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Single- and multiple-trait BLUP in genetic selection of parents and hybrids of grain sorghum

BLUP mono y múltiple característica en la selección genética de parentales e híbridos de sorgo

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Abstract

To increase yield, breeding programs must search for innovative and efficient methodologies for the development and selection of superior genotypes. Therefore, this study aimed to compare the single- and multiple-trait best linear unbiased prediction (BLUP) in genetic selection of parents and hybrids of grain sorghum. For this, an experiment conducted in alpha-lattice design with two replications was used. Flowering time (FT), plant height (PH), and grain yield (GY) were evaluated in 502 grain sorghum hybrids obtained by the cross of ten restorer lines and 54 male-sterile lines. Variance components were estimated via restricted maximum likelihood (REML). Significant effects of restorer lines, of male-sterile lines, and of specific combining ability were detected by the likelihood ratio test (LRT). The estimates of variance components, genetic parameters, and correlations were similar when obtained via single- and multiple trait-BLUP. Considering hybrids, the multiple-trait BLUP resulted in slightly higher predicted selection gain for the three evaluated traits and, therefore, can be efficiently applied in the genetic selection of grain sorghum.

Keywords
plant breeding • partial diallel • mixed model methodology • genetic correlation • heterosis

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Resumen

Para incrementar el rendimiento, los programas de mejoramiento deben buscar metodologías innovadoras y eficientes para el desarrollo y la selección de genotipos superiores. De esta forma, este estudio tuvo como objetivo comparar la mejor predicción lineal no viciada (BLUP) mono y múltiple característica, en la selección genética de parentales e híbridos de sorgo granelero. Para esto, fue usado un experimento con diseño alfa latice con dos repeticiones. Se evaluó el tiempo de floración (FT), la altura de planta (AP) y el rendimiento de granos (GY) en 502 híbridos de sorgo, obtenidos por el cruzamiento entre diez líneas restauradoras y 54 líneas macho estériles. Los componentes de varianza se estimaron mediante el método de máxima verosimilitud restringida (REML). Observamos que varias veces el BLUP múltiple característica da como resultado estimaciones de correlación genética más precisas. En este trabajo, se encontraron mayores correlaciones entre FT y GY a través de BLUP múltiple característica. Estos resultados llevaron a mayores ganancias genéticas para la selección de híbridos en comparación con BLUP mono característica.

Palabras clave
fitomejoramiento • dialelo parcial • modelos mixtos • correlación genética • heterosis

Introduction

Grain sorghum (*Sorghum bicolor* L. Moench) is among the five most widely cultivated species in the world. This crop stands out for its several uses, such as biomass, ethanol production, grains, fertilizers, and fiber (19). The high efficiency in solar energy conversion and water use are factors that favored the expansion of sorghum cultivation (16), enabling it develop in environments with low rainfall and high temperatures, such as the semiarid regions.

Aiming at increasing yield, grain sorghum has undergone modifications related to the genetic architecture, which has resulted in the development of early cultivars with high grain yield (4), and ideal plant height for harvest. Despite being less effective in autogamous than in allogamous species, the heterosis effect is widely explored in grain sorghum breeding programs. Thus, studies that address heterosis in sorghum have been reported in the literature, especially for traits related to grain yield (14, 24). Moreover, plant height and flowering time are target traits in grain sorghum breeding programs for they allow the early selection of more promising lines and with ideal height for mechanized harvesting. According to Silva *et al.* (2009), genotypes that present shorter height, associated with greater stem resistance, had less susceptibility to lodging or plant breakage. For grain sorghum, plant height should be between 100 and 150 cm (3), since sorghum harvesting uses adaptations of machines normally used for corn or soybeans, which operate in this height range. Improvement grain yield is associated with undesired increase in plant height and late flowering time (10).

Furthermore, in an effort to develop a suitable method for genetic evaluation,

Henderson (1975) proposed the best linear unbiased prediction (BLUP) to predict random effects, by adjusting the data to fixed effects. The empirical BLUP assumes that the values of the variance components estimated by the restricted maximum likelihood (REML) are true. Thus, the REML/BLUP is the standard procedure used to estimate variance components and to predict genetic values in plant breeding (21).

Currently, the single-trait BLUP has been widely used in genetic selection of grain sorghum. However, when the traits present genetic and residual correlations, the multiple-trait BLUP can be more accurate, as demonstrated by Viana *et al.* (2010). Therefore, the genetic evaluation via multiple-trait BLUP is considered as more efficient when traits are correlated, resulting in a lower mean bias than that of the single-trait BLUP (17, 18). This methodology is commonly used in animal breeding (9) and has been successfully applied to plant breeding (18, 29). Therefore, this study aimed to compare the single- and multiple-trait BLUP in genetic selection of parents and hybrids of grain sorghum.

Material and methods

Experimental data

The experiment consisted of a performance evaluation of 502 grain sorghum hybrids in the 2011/2012 season at Embrapa Agrosilvopastoral (lat. 55°36' W, long. 11°51' S, alt. 307 m a. s. l.), in Sinop/MT, Brazil. The hybrids were obtained by the cross of ten restorer lines and 53 male-sterile lines from the breeding program of Embrapa Maize and Sorghum. Due to the low pollen production of some restorer lines, there was imbalance in crosses, with loss of 38 hybrids (*).

Due to the large number of hybrids to be evaluated, the experimental design adopted was alpha-lattice design 42x12 with two replications. That is, each of the two replication of lattice were composed of 42 replicates and in each replicate 12 hybrids were evaluated, totalizing 504 plots in each of the two lattices. As their efficiency was low (<2%) for all traits, each lattice repetition was considered to a complete block (includes all treatments), and the usual model was used for analysis of complete randomized blocks, based in recommendations of Cochran and Cox (1957). Each plot consisted of two five-meter rows, spaced at of 0.50 m between rows. Planting fertilization and topdressing were carried out according to soil analyses. Cultural and phytosanitary treatments were performed based on the crop needs.

The evaluated traits were: flowering time (FT) (number of days from the emergence to when 50% of the plants in the plot reached the flowering stage); plant height (PH) (mean height of two plants of the useful area of the plot, measured from the soil surface to the apex of the panicle, at harvest time); and grain yield (GY) (corrected to 13% moisture and extrapolated to kg ha^{-1}).

* Identification of the two selected restorer lines (RL), of the eight selected male-sterile lines (ML), and the 25 hybrids selected for the traits flowering (FL), plant height (PH), and grain yield (GY), evaluated in 502 grain sorghum hybrids via single- and multiple-trait BLUP.

* Identificación de las dos líneas restauradoras selecionadas (RL), de las ocho líneas macho estériles (ML) seleccionadas y de los 25 híbridos seleccionados, para las características floración: (FL), altura de planta (PH) y rendimiento de grano (GY), evaluadas en 502 híbridos de sorgo vía BLUP mono y múltiple característica.

Statistical analyses

The REML/BLUP procedure was performed to estimate the variance components and to predict the genetic values, according to Resende *et al.* (2014). The statistical model associated with the evaluation of hybrids, obtained by partial diallel crosses, in a randomized block design, with one observation per plot, is given by:

$$\boldsymbol{y = Xr + Zm + Wf + T + e.} \quad (1)$$

where:

y = the observation vector

r = the vector of replication effects (assumed as fixed) summed to the general mean

m = the vector of the effects of the restorer lines (assumed as random)

f = the vector of the effects of male-sterile lines (assumed as random)

c = the vector of the effects of specific combining ability of restorer lines with the male-sterile lines (random)

e = the vector of residues (random)

X, Z, W, and T = the incidence matrices for the referred effects.

For the random effects of the model, significance for the likelihood ratio test (LRT) was tested using Chi-square statistics with one degree of freedom according to Resende (2014).

Accuracies were estimated using the single- and multiple-trait BLUP, based on the expression:

$$r^2_{\hat{u}a} = \sqrt{(\sigma^2_a - PEV)} / \sigma^2_a \quad (2)$$

where:

σ^2_a = estimator of the additive genetic variance

PEV = prediction error variance.

To estimate the genetic covariance via single-trait BLUP, individual analyses and the analysis of the sum of the values of traits were performed pair-to-pair so that the covariances were estimated based on the equation:

$$\sigma_{g(trait_i + trait_j)} = \frac{\sigma^2_{g(trait_i + trait_j)} + \sigma^2_{g(trait_i)} - \sigma^2_{g(trait_j)}}{2} \quad (3)$$

where:

$\sigma_{g(trait_i + trait_j)}$ = estimate of the genetic covariance between trait *i* and trait *j*

$\sigma^2_{g(trait_i)}$ = estimator of the genetic variance to trait *i* and trait *j*, respectively.

The genetic correlation coefficients (ρ) between the traits FT, PH, and GY for restorer lines, male-sterile lines, and hybrids, via single- and multiple-trait BLUP, were obtained based on the expression:

$$\rho = \frac{\sigma_{g(trait_i, trait_j)}}{\sqrt{\sigma^2_{g(trait_i)} \sigma^2_{g(trait_j)}}} \quad (4)$$

Selection gains (*SG*), considering each methodology, were predicted for the restorer lines, male-sterile lines, and hybrid, following the equation:

$$SG = \frac{\sum_{i=1}^{n} PGV_i}{n} \quad (5)$$

where:

PGV_i = predicted genetic value

n = quantity selected in function of the selection intensity of 2 (20%), 8 (15%), and 25 (5%) for restorer lines, male-sterile lines, and hybrids, respectively. These intensities of selection were established for purposes of comparison between single- and multiple-trait BLUP.

The selection coincidence between the individuals selected by single- and multiple-trait BLUP was verified for two restorer lines, eight male-sterile lines, and 25 hybrids. Statistical analyses were carried out in the software Selegen REML/BLUP (22), ASReml 4.1 (7) and R (27).

Results

Analysis of deviance

Significant effects of restorer lines (*g1*), of male-sterile lines (*g2*), and of specific combining ability of restorer lines with the male-sterile lines (*SCA*) ($P < 0.01$) were detected in the LRT for the traits FT, PH, and GY (table 1). Thus, according to the LRT, the full model was the most suitable to estimate the genetic parameters and to predict the genetic values. Consequently, the respective variance components are significantly different from zero, and so are the respective coefficients of determination.

Variance components

The estimates of genetic variance between restorer lines crossed with the male-sterile lines (σ^2_{g1}); male-sterile lines crossed with restorer lines (σ^2_{g2}); mean additive genetic variance (σ^2_a); residual variance (σ^2_e)and specific combining ability between two parents (σ^2_{sca}), via single and multiple-trait BLUP, for the traits FT, PH, and GY were similar (table 2, page 6).

Consequently, heritability values, mean accuracy of the restorer lines (Acc_{g1}), mean accuracy of the male-sterile lines (Acc_{g2}) and mean accuracy of hybrids (Acc_{hyb}) were also similar between the two models for all analyzed traits (table 2, page 6).

Genetic correlation

Table 3 (page 7) shows the genetic correlations between FT, PH, and GY estimated via single- and multiple-trait BLUP. The genetic correlations between the evaluated traits had similar results when comparing the two models, with no signal inversion and with low magnitude estimates.

Selection of parents

Selection coincidences and predicted selection gains of the male-sterile and restorer lines for FT, PH, and GY via single- and multiple-trait BLUP are presented in table 4 (page 7).

Table 1. Deviance and likelihood ratio test (LRT) for the traits: flowering time (FT), plant height (PH), and grain yield (GY), evaluated in 502 grain sorghum hybrids.

Tabla 1. Análisis de deviación y prueba de razón de verosimilitud (LRT) para los caracteres: tiempo de floración (FT), altura de planta (PH) y rendimiento de grano (GY), evaluados en 502 híbridos de sorgo.

	FT				GY	
Effect	**Deviance**	**LRT**	**Deviance**	**LRT**	**Deviance**	**LRT**
g1	3034.14	145.27**	-2644.27	185.97**	1224.23	334.30**
g2	3205.67	316.80**	-2607.62	222.62**	1007.38	117.45**
SCA	2987.82	98.95**	-2774.9	55.34**	930.87	40.94**
Full model	2888.87	-	-2830.24	-	889.93	-

g1: restorer lines; g2: male-sterile lines; SCA: specific combining ability; and **: significant at the 0.01 probability level according to the Chi-square test.

g1: líneas restauradoras; g2: líneas macho estériles; SCA: capacidad de combinación específica; y **: significativo en el nivel de probabilidad de 0,01 de acuerdo con la prueba de Chi-cuadrado.

Table 2. Estimates of variance components and genetic and non-genetic parameters for the traits: flowering time (FT), plant height (PH), and grain yield (GY), evaluated in 502 grain sorghum hybrids, via single- and multiple-trait BLUP.

Tabla 2. Estimación de componentes de varianza, parámetros genéticos y no genéticos para los caracteres: tiempo de floración (FT), altura de planta (PH) y rendimiento de grano (GY), evaluados en 502 híbridos de sorgo, a través de BLUP mono y múltiple característica.

Component	Single-trait BLUP			Multiple-trait BLUP		
	FT	PH	GY	FT	PH	GY
σ^2_{g1}	2.2067	0.0078	0.7065	2.2086	0.0078	0.7058
σ^2_{g2}	6.6537	0.0131	0.3031	6.6506	0.0131	0.3034
σ^2_a	8.8603	0.0209	1.0096	8.8591	0.0209	1.0092
σ^2_{sca}	2.6215	0.0065	0.2443	2.6217	0.0065	0.2465
σ^2_e	3.4353	0.0130	0.5774	3.4353	0.0130	0.5759
σ^2_f	14.9171	0.0404	1.8314	14.9161	0.0404	1.8317
h^2_{a1}	0.2959	0.3857	0.7716	0.2961	0.3867	0.7707
h^2_{a2}	0.8921	0.6503	0.3310	0.8917	0.6495	0.3313
c^2_{sca}	0.1757	0.1600	0.1334	0.1758	0.1599	0.1346
h^2_{dom}	0.1757	0.1600	0.1334	0.1758	0.1599	0.1346
h^2_a	0.5940	0.5180	0.5513	0.5939	0.5181	0.5510
h^2_g	0.7697	0.6779	0.6847	0.7697	0.6781	0.6856
Acc_{g1}	0.9303	0.9331	0.9414	0.9303	0.9334	0.9414
Acc_{g2}	0.9572	0.9413	0.9065	0.9573	0.9417	0.9069
Acc_{hyb}	0.9283	0.9095	0.9248	0.9284	0.9111	0.9262

σ^2_{g1}: genetic variance between restorer lines crossed with male-sterile lines; σ^2_{g2} : genetic variance between male-sterile lines crossed with restorer lines; σ^2_a : mean additive genetic variance; σ^2_{sca} : variance of specific combining ability between two parents;σ^2_e : residual variance; σ^2_f : individual phenotypic variance; h^2_{a1} : narrow-sense individual heritability in restorer lines; h^2_{a2}: narrow-sense individual heritability in male-sterile lines; c^2_{sca} : coefficient of determination of the effects of specific combining ability; h^2_{dom} : individual heritability of dominance effects; h^2_a: narrow-sense individual interpopulational heritability, mean for the two populations;h^2_g: broad-sense individual interpopulational heritability (total genotypic effects); Acc_{g1}: mean accuracy of restorer lines; Acc_{g2}: mean accuracy of male-sterile lines; and Acc_{hyb} : mean accuracy of hybrids.

σ^2_{g1}: varianza genética entre líneas restauradoras cruzadas con líneas macho estériles; σ^2_{g2} : varianza genética entre líneas macho estériles cruzadas con líneas restauradoras; σ^2: varianza genética aditiva media; σ^2_{sca}: varianza de la capacidad de combinación específica entre dos padres; σ^2_e : varianza residual; σ^2_f: varianza fenotípica individual; h^2_{a1}: heredabilidad individual de sentido estrecho en líneas restauradoras; h^2_{a2}: heredabilidad individual de sentido estrecho en líneas macho estériles; c^2_{sca}: coeficiente de determinación de los efectos de la capacidad de combinación específica; h^2_{dom}: heredabilidad individual de los efectos de dominio; h^2_a: heredabilidad interpoblacional individual en sentido estrecho, media para las dos poblaciones; h^2_g: heredabilidad interpoblacional individual de sentido amplio (efectos genotípicos totales); Acc_{g1}: precisión media de las líneas restauradoras; Acc_{g2}: precisión media de líneas macho estériles; y Acc_{hyb}: precisión media de los híbridos.

Table 3. Genetic correlations between flowering time (FT), plant height (PH), and grain yield (GY), evaluated in 502 grain sorghum hybrids, via single- (above diagonal) and multiple-trait BLUP (below diagonal).

Tabla 3. Correlaciones genéticas entre tiempo de floración (FT), altura de planta (PH) y rendimiento de grano (GY), evaluados en 502 híbridos de sorgo, a través de BLUP para mono (arriba de diagonal) y múltiples características (debajo de diagonal).

Traits	FT	PH	GY
FT	-	0.03[a] | -0.01[b] | 0.00[c]	0.28[a] | 0.04[b] | 0.08[c]
PH	0.04[a] | -0.03[b] | 0.00[c]	-	0.06[a] | 0.00[b] | 0.03[c]
GY	0.46[a] | 0.10[b] | 0.13[c]	0.05 [a] | 0.00[b] | 0.02[c]	-

[a]: restorer lines; [b]: male-sterile lines; and [c]: hybrids.

[a]: líneas restauradoras; [b]: líneas macho estériles; [c]: híbridos

Table 4. Selection coincidence and predicted selection gain for male-sterile and restorer lines for the traits: flowering time (FT), plant height (PH), and grain yield (GY), evaluated in 502 grain sorghum hybrids, via single- and multiple-trait BLUP.

Tabla 4. Coincidencia de selección y ganancia de selección predicha para líneas macho estériles y restauradoras, en las características: tiempo de floración (FT), altura de planta (PH) y rendimiento de grano (GY), evaluados en 502 híbridos de sorgo, por medio BLUP mono y múltiple característica.

Traits	Selection coincidence (%)	Predicted selection gain	
		Single-trait BLUP	Multiple-trait BLUP
FT	100[d] | 100[e]	-2.1479[d] | -4.3482[e]	-2.1479[d] | -4.3482[e]
PH	100[d] | 100[e]	-0.0789[d] | -0.1497[e]	-0.0789[d] | -0.1497[e]
GY	100[d] | 100[e]	1.2636[d] | 0.8578[e]	1.2636[d] | 0.8578[e]

[d]: restorer lines and e: male-sterile lines.

[d]: líneas restauradoras y e: líneas macho estériles.

For the evaluated traits, the predicted selection gains for restorer and male-sterile lines via single- and multiple-trait procedures showed no differences in values. Regardless of the trait evaluated, the models revealed selection coincidence of 100%, that is, the individuals selected by the single-trait model were the same as those selected by the multiple-trait model (table 4).

Selection of hybrids

Table 5 (page 8), presents the selection coincidences and predicted selection gains of hybrids for the traits FT, PH, and GY, via single- and multiple-trait BLUP. The multiple-trait BLUP resulted in slightly higher predicted selection gain for the three evaluated traits. Selection coincidences varied between traits (96% for FT, 88% for PH, and 84% for PY) and confirms the small difference between the hybrids selected by each methodology.

Table 5. Selection coincidence and predicted selection gain of hybrids for the traits flowering time (FT), plant height (PH), and grain yield (GY), evaluated in 502 grain sorghum hybrids, via single- and multiple-trait BLUP.

Tabla 5. Coincidencia de selección y ganancia de selección predicha para híbridos, en las características: tiempo de floración (FT), altura de planta (PH) y rendimiento de grano (GY), evaluados en 502 híbridos de sorgo, mediante BLUP mono y múltiple característica.

Traits	Selection coincidence (%)	Predicted selection gain	
		Single-trait BLUP	Multiple-trait BLUP
FT	96	-2.2273	-2.2280
PH	88	-0.1226	-0.1227
GY	84	0.6881	0.6921

Discussion

Variance components

Variance components is of great importance in plant breeding, since the population and the breeding method to be used depend on information that can be obtained from these components. The estimates of variance components, the prediction of genetic values, and the accuracies are crucial factors to a plant breeding program. A greater set of candidate genotypes must be used in selection for the development of new cultivars and the recommendation of newly-released varieties. The BLUP methodology is widely used to predict genetic values due to several benefits, such as unbiased prediction, and reduction of variances and errors when compared with other methods (31).

In this study, the estimates for variance components and genetic and non-genetic parameters obtained for the restorer and male-sterile lines and hybrids were the same for the traits analyzed for both single- and multiple-trait BLUP. This fact can be explained by the similarity in the deviations value between the estimation of the genetic and residual variance components considering the two procedures (29). Based on Schaeffer (23), when the heritability values of the traits are similar, and the genetic correlations between the traits are low, the use of the multiple-trait model may not increase the accuracy for the prediction of genetic values.

The estimates of the individual heritability of dominance effects and the narrow-sense individual interpopulational heritability mean for the two populations, indicate that most of the broad-sense individual interpopulational heritability is due additive effects. According to Viana (2005), regardless of the selection unit or type of epistasis, the bias in the estimate of the additive variance when assuming the additive-dominant model is considerable. This implies overestimation of the heritabilities at half sib family mean, plant within family, and plant levels; and underestimation if the selection units are full sib progenies. Thus, the predicted gains have a bias proportional to that of the heritability.

Genetic correlation

In plant breeding programs, the selection of superior genotypes based on several traits of agronomic interest is appropriate. However, when considering the existence of a correlation between traits, these correlations must be analyzed together to avoid selection bias (20). Viana *et al.* (2010) emphasized that the advantage of using the multiple-trait BLUP model is mainly dependent on heritability values and genetic correlations between the evaluated traits.

Piepho *et al.* (2008) stated that the multiple-trait model is more appropriate for highly correlated traits. However, when including information of traits of low magnitude correlation (as evaluated in this study) in multiple-trait BLUP analysis, no improvements in the estimate of genetic correlation were observed in relation to the single-trait BLUP (table 3, page 7).

Conversely, when considering the most correlated traits (GY and FT), multiple-trait BLUP correlations estimates were always higher. Persson and Andersson (2004) compared the genetic values predicted via single- and multiple-trait BLUP in *Pinus sylvestris* L. and reported that the multiple-trait model had a lower mean bias than the single-trait model. Moreover, the authors stated that the genetic correlations between traits by the single-trait BLUP were underestimated. Kadarmideen *et al.* (2003) observed that predicted genetic values and animal ranking were significantly different between single- and multiple-trait analyses in milk cattle. The same authors also reported that the single-trait analysis was shown to be biased, and thus recommended the use of multiple-trait BLUP. Kadarmideen *et al.* (2003), working with simulated low- and high-heritability trait data, found that the multiple-trait genomic model resulted in more accurate genomic predictions when compared with genomic single-trait model, especially for low heritability traits.

Parents selection

Grain sorghum genetic improvement must be performed individually in each group of lines (restorer or male-sterile). The improvement of male-sterile lines must be carried out in isogenic lines and, subsequently, introduced to cytoplasmic male sterility. Therefore, the identification of parents of each group that donate alleles with desirable effects is a promising strategy for the development of future hybrids with broad adaptability for these traits.

This work identified lines of each group that contribute to the desirable phenotype in the respective hybrids generated. The favorable alleles for each trait are found in different parents (* page 3). Finally, the two procedures evaluated led to the selection of the same parents from each line and, consequently, to the same estimates of selection gain (table 4, page 7).

The selection of relatives via single- and multiple-trait BLUP for annual crops such as sorghum is still scarce in the literature. The coincidence in the selection between the procedures is proportional to the absolute difference between the genetic and environmental correlations of the traits and the sample size (26). Thus, although there were small differences between the procedures for the genetic correlations of restorer lines and male-sterile lines (table 3, page 7), sample size did not allow detecting differences in selection coincidence and predicted selection gain for male-sterile and restorer lines (table 4, page 7).

In the breeding of perennial plants, the multiple-trait BLUP is a consolidated strategy. Several authors have detected superiority of this procedure in the selection of parents due to the great imbalance in the data that influences the residual correlation between the traits and consequently their heritability and genetic values. Imai *et al.* (2016), Costa *et al.* (2002), Kerr (1998), Alves *et al.* (2019) and Alves *et al.* (2018) applied multiple-trait BLUP in parent selection of perennial crops demonstrated the usefulness of the method in parent selection when the information of kinship among genotypes is known and in the presence of unbalanced data.

Hybrid selection

Currently, grain sorghum breeding programs seek to develop hybrids with high earliness, low plant height, and high grain yield (15). Thus, the selection of hybrids with the highest genetic value (* page 3) is fundamental to breeding programs. The differences in selection coincidence and predicted selection of hybrids for the traits can be explained mainly by the magnitude of the correlation obtained between GY and FL by single- (0.08) and multiple-trait BLUP (0.13), in addition to the larger sample size (504 hybrids).

In general, the gains obtained with the multiple-trait BLUP were slightly higher than the single-trait BLUP for all traits. Recently, Volpato *et al.* (2019) observed that multiple-trait BLUP is a suitable procedure for genetic selection of segregating soybean progeny. These results are important because they report the first application of this procedure for selection of hybrids in annual crops.

Conclusions

Significant effects of restorer lines, of male-sterile lines, and of specific combining ability were detected. The estimates of variance components, genetic parameters, and correlations were similar when obtained via single- and multiple trait-BLUP. Considering hybrids, the multiple-trait BLUP resulted in slightly higher predicted selection gain for the three evaluated traits and, therefore, can be efficiently applied in the genetic selection of grain sorghum.

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Effect of environmental factors on bee activity and onion (*Allium cepa* L.) seed yield

Efecto de factores ambientales en la actividad de la abeja y en el rendimiento de semilla de cebolla (*Allium cepa* L.)

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Abstract

Pollinators are required to produce onion seeds. This specie is one of the main vegetable crops. Two types of onion varieties are mainly grown worldwide: hybrids and open pollination (OP) cultivars. Although hybrids offer advantages to bulb growers, seed yields of hybrids are lower than OP cultivars and that is a significant problem. The influence of environmental factors (temperature, radiation, rainfall, relative humidity (RH) and wind speed) was determined, as well as bee attraction and seed production in three locations of the main onion seed production area in Argentina. Nine male sterile lines (MSL) and one OP were used. The results obtained showed a marked variability in the attraction of bees and seed production between the OP and MSL and within MLS. In addition, environmental factors such as minimun temperature or RH were determinant to modify bee foraging behavior, where values lower than 9°C and 22%, respectively, caused that bees stop their activity.

Keywords
Allium cepa L. • seed production • pollination • *Apis mellifera* L.

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Resumen

Los polinizadores son necesarios para producir semillas de cebolla. Esta especie es uno de los principales cultivos hortícolas. Dos tipos de variedades de cebolla se cultivan principalmente en todo el mundo: híbridos y cultivares de polinización abierta (PA). Aunque los híbridos ofrecen ventajas a los cultivadores de bulbos, los rendimientos de semillas de los híbridos son más bajos que los cultivares PA y eso es un problema importante. Se determinó la influencia de los factores ambientales (temperatura, radiación, precipitación, humedad relativa (HR) y velocidad del viento), así como la atracción de abejas y la producción de semillas en tres localidades, de principal zona productora de semillas de cebolla en Argentina. Se utilizaron nueve líneas androestériles (LAE) y una PA. Los resultados obtenidos mostraron una marcada variabilidad en la atracción de abejas y la producción de semillas entre la variedad PA y las LAE y dentro de las LAE. Además, los factores ambientales, como la temperatura mínima o HR, fueron determinantes para modificar el comportamiento de alimentación de las abejas, donde valores inferiores a 9°C y 22%, respectivamente, hicieron que las abejas detuvieran su actividad.

Palabras clave
Allium cepa L • producción de semilla • polinización • *Apis mellifera* L.

Introduction

Onion (*Allium cepa* L.) the most cultivated specie of the subgenus *Allium*, from the Alliaceae family (33). Onion seed production is very important in Argentina. Around 200 t per year are produced mainly in the provinces of Mendoza and San Juan. Half of the production is exported. Onion is an allogamous specie with protandria, anthers release pollen before the stigma is receptive, promoting the out-crossing among onion plants (12). In the world, two types of onion varieties are usually grown: open pollinated (OP) and hybrids (33). Hybrids offer greater vigor and uniformity than the OP materials (12, 14). However, reports from seed companies and previous studies have reported that hybrid seed yield is erratic and considerably lower compared to OP varieties (37).

For hybrid seeds production, systems based on cytoplasmic male sterility (CMS) are used throughout the world. Two main sources of CMS have been genetically characterized, identified as S and T. Type S is the most used due to the usual occurrence of the recessive allele in Ms (20). To obtain these seeds, it is necessary to cross a sterile male line (MSL) with a fertile one. Almost two thirds of the onion cultivars of the most important seed companies' catalogs belong to the hybrid category (12). Very variable seed yield has been observed among the different onion hybrids. Poor seed yields are often due to poor pollination (19, 24, 34, 35, 36). Therefore, increasing seed yield depends on increasing the activity of the cross-pollinating insects (3).

There are at least 276 species of insects identified as pollinators of onion flowers, however bees (*Apis mellifera* L.), are the most efficient and handling them in the crops is easier, compared to other

insects used, as flies (8). A distinctive characteristic of bees with respect to other insects is that bees visit only one type of flower at a time, this fact that some authors call floral constancy, makes the transfer of pollen from flower to flower more efficient, making possible the pollination (22).

Onion seed yield of hybrid varieties mainly depends on bee activity (25), the frequency at which they move and carry the pollen from the fertile plants to the sterile ones, as well as the proportion of fertile plants and their distribution in the crop (13, 28, 41). A poor pollination is one of the main causes of low yield; therefore, bees are necessary for a good seed set. However, the widespread use of bees as a pollinator does not always bring expected results because of the onion nectar is not particularly attractive to bees (7, 19, 40).

Around 10 beehives per hectare are used for onion pollination. Beehives usually are not introduced at the same time, starting when a 10% of fertile flowers are opened (40) until 50% of flowering (10). The flowers open irregularly during a period that lasts between two and four weeks (32).

Another important factor to consider in hybrid materials is the synchronization in flowering between the parental lines "Nicking", *i.e.* the coincidence between the period in which the pollen is viable and the stigma is receptive (28).

Environmental factors influence both plant and pollinators. An onion bulb can have more than one floral scape, each scape can have an umbel, and therefore, a plant that produces several inflorescences can open its flowers for a month or more. Each umbel has hundreds of perfect flowers (18). The production of onion flowers is induced by environmental factors. The abundance and viability of pollen is low at 14°C, but it is higher at 23°C. Pollen tube growth and seed formation are negatively affected by temperatures above 40°C (10).

On the other hand, bee behavior is controlled by both environmental and genetic factors (38). The influence of climatic conditions on bee flight activity has been reported (29, 31). Radiation and temperature are the most important factors on both, flight and communication between bees (1, 23, 31). Although, other studies have shown that with humidity higher than 75%, or precipitations, bee foraging activity is almost null, as well as when the wind speed exceeds 40 $km.h^{-1}$ (30). The aim of this work is to determine the influence of environmental factors (temperature, humidity, rainfall, wind and cloudiness) on the attraction of bees and the production of onion seed in the Cuyo region.

Materials and methods

Plant material

Ten onion lines, 9 male sterile lines (LAE) and one fertile line (OP) were selected, looking for materials that differed in their genetic characteristics and harvest yields. On April 8, 2015 bulbs produced in San Juan from each of these lines were planted in identical conditions in three locations. The management of the crop was the same for the three farms, no pesticides were used throughout the experiment, and the plants were drip irrigated. A number from 1 to 10 was assigned to each line, being number 4 the OP variety (table 1, page 16). All the lines were provided by Bayer Argentina.

Locations where trials and experimental design were implemented

Three locations were selected in different environments within the onion seed productive area.

Table 1. Characteristics of the lines and seed yield for the three studied areas (g. umbel^{-1}).

Tabla 1. Características de las líneas y rendimientos de semilla para las tres localidades (g.umbela^{-1}).

LINE	Bulb color	Bolting	Bulb shape	Luján de Cuyo	Zonda	Media Agua
♀ 1	Red	Early	Globe	4.03 ab	3.86 b	0.72 ab
♀ 2	Yellow	Medium	Flat	3.96 ab	3.76 b	1.45 acd
♀ 3	Yellow	Late	Flat	4.06 ab	3.89 b	5.43 d
♂ 4	White	Late	Globe	5.09 c	5.39 c	3.23 dc
♀ 5	White	Late	Globe	3.79 ab	3.70 ab	2.85 dc
♀ 6	Yellow	Early	Globe	4.06 ab	3.87 b	0.53 a
♀ 7	Yellow	Medium	Globe	4.08 ab	3.79 b	0.92 ab
♀ 8	Yellow	Early	Globe	4.22 b	3.97 b	1.64 abc
♀ 9	White	Late	Globe	3.54 a	3.53 ab	2.58 bcd
♀ 10	Yellow	Late	Globe	3.62 a	3.04 a	3.27 dc
			Average:	4.04	3.88	2.26

Values represent mean of three determinations. Values in the same column with different letters present significant differences P ≤ 0.05.

Los valores representan la media de tres repeticiones. Los valores en la misma columna con diferente letra representan diferencias significativas *P* ≤ 0,05.

One of the trials was located at INTA Mendoza Agricultural Experimental Station in Luján de Cuyo, Province of Mendoza (33°00'22 "S- 68°51'35,15" W), the other two were located in the province of San Juan, one of them in the department of Sarmiento (32°00'23" S- 68°21'38" W) and another in a farm located towards the northwest of San Juan in the Valle de Zonda (31°32'11" S- 68°42'19" W).

A randomized complete block design was used, with three repetitions and 150 bulbs in each of them. The bulbs were distributed in 3 rows of 5 meters long and a density of 10 bulbs per meter. Rows were about one meter apart. The ratio between sterile and fertile plants was 3: 1, which is the usual ratio used in commercial crops. Among all fertile and sterile lines there was 1 meter of distance.

Bee foraging behavior evaluation

Two bee hives (*Apis mellifera* L.) were placed in each experimental plot. Other insects also visited the flowers, but their work on the umbels was almost insignificant, with bees mainly found on the umbels during the trial period. At 10% of the anthesis of the fertile flowers (Line 4) the hives were introduced in the experimental parcels. Each hive had 4 open breeding frames and 2 frames with operculated brood. The queens came from an apiculture located in the Valle de Uco that has the Proapi certification. These bees had as remarkable characteristics, meekness and very good foraging ability.

An observation area was delimited, taking into account that there was the greatest homogeneity possible among them; each area had a length of 1 m. Visits were count visually; the observation was made alongside the group of umbels, during a period of two minutes per repetition, following the methodology proposed by Maldonado (2014). A visit was recorded when a bee stayed more than 3 seconds on the umbel. The number of bee visits was registered during seven days from 50% of flowering, defining as reading schedules from 12:00 a.m. to 2:00 p.m. The only days that no readings were taken were the days with strong wind or rain.

Flowering period

To evaluate the flowering percentage, the number of bloomed flowers per umbel inside the marked area was recorded daily, considering as an open umbel the one that had at least one flower with anthesis between the months of October and December. Then an average was calculated between the three repetitions of each of the experimental plots.

Environmental parameters

Air temperature and humidity were recorded in the 3 locations with temperature and humidity sensors (Onset® Mark, Model Pro v2). This instrument took readings every 15 minutes through the entire crop cycle, storing more than 9000 readings in each experimental plot. Sensors were place on a wooden stake at umbels height. Precipitation, wind and cloud data were extracted from nearby meteorological stations.

Seed harvest

The umbels were harvested manually between 15th and 20th January of 2016, taking as an optimum point of maturity when black seeds were shown in the umbels, then they were placed in mesh bags to allow drying. The bags were placed in dryers that allowed air circulation and exposure to the Sun. The moisture loss was controlled every two days with a John Deer moisture sampler calibrated for onion, when the average humidity reached 14%, the samples were milled, approximately 15 days after harvesting. The seed was processed in a sieve machine and then by hand the remaining impurities were extracted. The seeds obtained in each of the repetitions were weighed separately.

Statistical analysis

The statistical analysis of data was carried out using the analysis of variance (ANOVA) and means were compared using the Tukey significance test. The results were considered significant at $P \leq 0.05$ unless specified otherwise. Principal component analysis was applied to highlight the data structure and to find the overall relationships between environmental parameters and the effectiveness of MSL pollination for the production of hybrid onion seeds. Basic statistics and multivariate analysis were performed with the statistical package InfoStat2016 for Windows (Córdoba, Argentina). All data were reported as the mean ± standard deviation (SD) of three replicates.

Results

Seed production

Seed production was variable, there were significant differences among lines and locations, and there was interaction between lines and location. The line * location interaction observed was due to the fact that Media Agua suffered a special situation due to the fact that bees stopped visiting the crop after the third week of pollination, yields of Media Agua lines were very erratic, therefore, to determine the source of the variability associated with the production, this locality was removed from the analysis. When Media Agua was eliminated, we observed that there was no significant interaction between line * locality. The major source of variability was explained (81.42%) by the difference among the studied lines. In Luján de Cuyo were obtained the highest yields for all the lines compared to the yields obtained by the same lines in San Juan. The line with the highest yield per umbel was line N°4 (OP) in both Luján de Cuyo and Zonda (table 1, page 16). In both locations, the OP line (L4) differed significantly from the MSL. Among the MSL, differences were also observed, line 10 had the lowest seed production in the two locations and line 8 was the most productive.

Relationship between onion lines and bee activity

The blooming period in the onion lines cultivated in Luján de Cuyo was about 10 and 15 days later than the lines that were cultivated in the Zonda and Media Agua localities. The most precocious variety was MSL N° 10 while the latest one was MSL N° 6, this pattern was repeated in the three locations, while the open pollination line N° 4 had an intermediate behavior (figure 1).

At 50% of flowering the bee activity in Luján de Cuyo, was always higher than the observed in Zonda. This behavior was observed through all the flowering period and was related to seed yields. The OP line (L4) was the most visited in both locations.

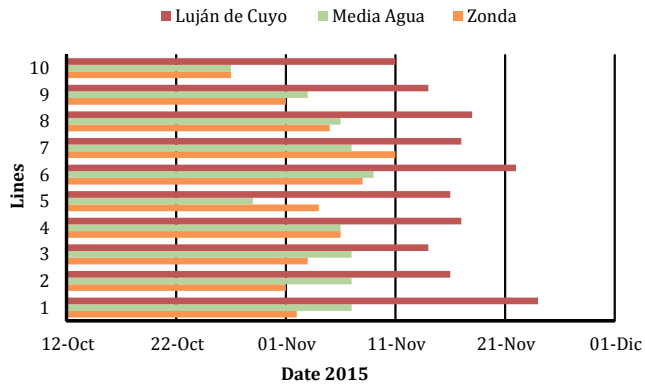


Figure 1. Date when the different onion lines reached 50% of flowering in the three studied locations.

Figura 1. Fecha en la que diferentes líneas de cebolla alcanzaron el 50% de floración en las tres localidades de estudio.

Within the MSL, L8 was the most visited and L10 the least visited in Luján de Cuyo whereas in Zonda the least visited for that percentage of flowering was L6. On the other hand, in Media Agua, it was observed that those lines that had more visits did not have the highest seed yield, for the reasons previously mentioned (figure 2). When we analyzed the source of variation, it was observed that these differences were explained by the difference between the locations in 11.43% and by the line factor in 47.40%. Bee visits at 50% flowering in all the onion lines in Luján de Cuyo and Zonda showed a high correlation with seed yields (r = 0.69).

Bee activity varied through the flowering period as well as along the day. In those days when the weather conditions were not favorable for bees at noon, no insects were observed on the umbels and bee increased during the afternoon. This behavior was similar for all the onion lines. For line 4 (OP) from Luján de Cuyo, during the first four days of readings, more bee visits were counted in the afternoon hours (5:00 p.m. to 6:00 p.m.) than during midday (12:00 p.m. 2:00 p.m.). The highest number of bees for this line was registered at 90% of flowering. The same was observed in male sterile lines. Bee activity at 50% of flowering was low and increased until it reached 85%, remaining constant until the end of flowering (figure 3, page 20).

Effect of environmental factors on bee visits

The temperature values registered in Luján de Cuyo, during the observation days, reached a maximum of 38.5°C and a minimum of 7.3°C and the values of relative humidity varied from 10% to 100% on days when rainfall was recorded; while in Zonda, San Juan the minimum and maximum temperatures were 16 and 38.8°C respectively and the recorded humidity varied between 18% and 100%. The maximum humidity values, as well as the precipitations were the environmental factors that had more influence on the bee activity. Although the most extreme values were observed in Media Agua (Max Temp 50°C and Min Temp -6,5°C), if we consider the mean values between the three locations there were no significant differences.

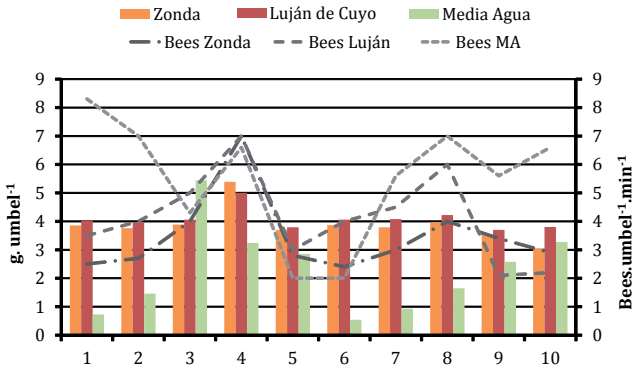
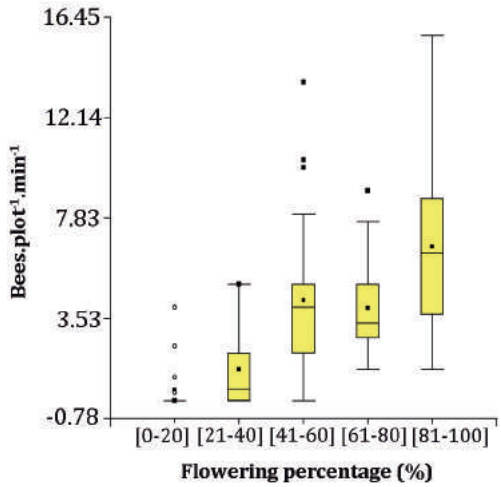


Figure 2. Seed yield per umbel and bee activity at 50% of flowering.
Figura 2. Rendimiento por umbela y actividad de abeja al 50% de floración.



Horizontal bars represent medians, box plots represent interquartiles and bars represent minimum and maximum values.

Las barras horizontales representan las medianas, los diagramas de caja representan intercuartiles y las barras representan valores mínimos y máximos.

Figure 3. Bee activity according to the flowering percentage in Zonda and Luján de Cuyo.

Figura 3. Actividad de la abeja de acuerdo con el porcentaje de floración en la localidad de Zonda y Luján de Cuyo.

The flowering percentage as well as the temperature influenced positively on bee activity under open field conditions; on the other hand, RH (Max and Min), cloudiness and precipitation are the parameters that most is negatively influenced bees (figure 4, page 21).

On cloudy days (6-8 oktas) or when the wind speed was higher than 50 $km.h^{-1}$, bees did not visit onion flowers. In addition, in the presence of precipitations bees reduced the frequency of visits or it became null. If rainy days are not taken into account, between 50% and 100% of flowering, the factor that has greatest influence in each locality was the minimum RH. In days where the minimum percentage of HR was low (less than 20%) or it was very high (above 55%), bees did not fly. Analyzing those days, with an optimal range of humidity, radiation and without wind, the factor that influenced the most, is temperature; the activity of bees decreased below 9°C and the highest frequency of visits was recorded between 22°C and 33°C. As an example, in figure 5 (page 21), Luján de Cuyo and Zonda locations are represented and it shows how the environmental factors influenced in the total number of visits of bees per plot. In addition, it was observed that not only the climatic conditions were very variable during the flowering period, but also through the day (figure 6, page 21).

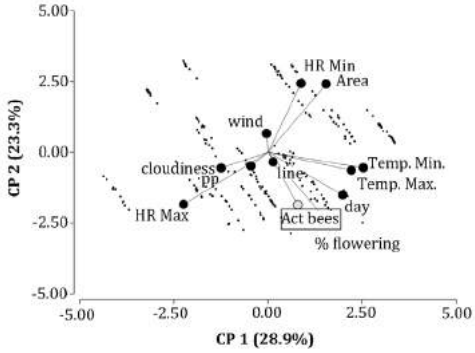


Figure 4. Principal components analysis graph: interaction between climatic factors with the bee activity, lines and the percentage of flowering for the three studied areas.

Figura 4. Gráfico de componentes principales: interacción entre todos los factores climáticos, la actividad de la abeja y las líneas y el porcentaje de floración para las tres localidades estudiadas.

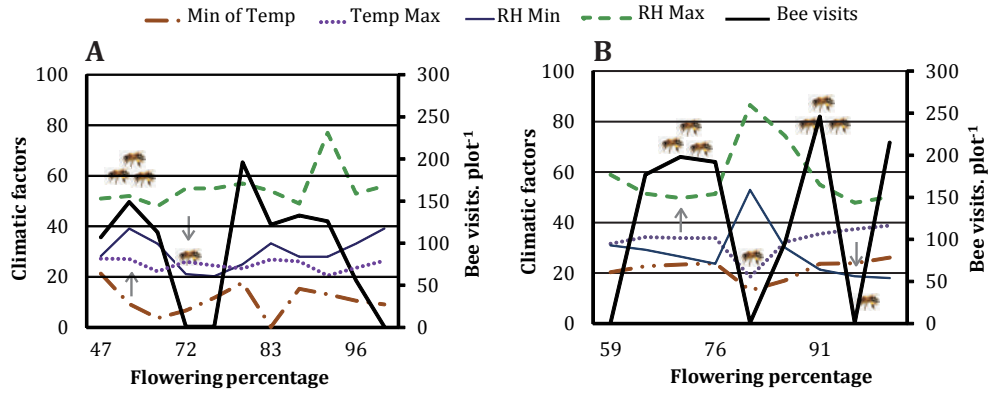


Figure 5. Influence of climatic factors on bee activity between 50 y 100% of blooming. A: Luján de Cuyo; B: Zonda.

Figura 5. Influencia de los factores climáticos en la actividad de las abejas entre el 50 y 100% de floración. A: Luján de Cuyo; B: Zonda.

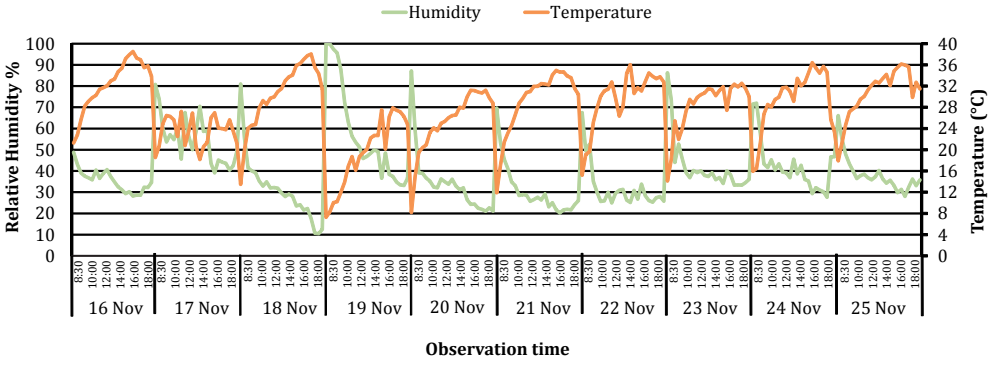


Figure 6. Temperature and humidity record during 10 days in Luján de Cuyo.

Figura 6. Registro de temperatura y humedad durante 10 días en la localidad de Luján de Cuyo.

Discussion

Seed yield differed between the OP variety and the studied hybrids, in addition differences were observed among the MSL. Although an interaction between location and seed yield was observed, the differences were maintained in the locations of Luján de Cuyo and Zonda. Media Agua was affected by the natural flora that attracted more the bees than onion flowers. The OP variety produced 1.4 folds more seeds than the N° 10 line, which had the lowest seed yield among all male sterile lines. Silva and Dean (2000) and Ahmad *et al.* (2003) reported differences in the seed production per umbel among the different hybrids. These results are in concordance with those reported by Maldonado (2014) who observed lower yield in male sterile lines than the OP in cage conditions.

The seed production of the OP line as well as the MSL was correlated with bee visits. These results are in agreement with other studies where 2 fold more bees on the OP lines than in MSL were observed (9, 34), which is expected since in pollination open lines also have pollen (4, 10). Other authors consider that this difference is due to a greater amount of nectar and concentration of sugars in the fertile lines (9, 26, 41). Silva and Dean (2000) reported that the average amount of nectar produced in onion flowers has a significant positive correlation with the number of bee visits.

In an average umbel that can have up to 600 flowers, 20 to 30% of the flowers have receptive stigmas. Benedek (1977) suggested that there should be between 5 to 8 bee visits on an umbel to have a satisfactory setting through the flowering season. When the lines were close to 50% flowering bee activity was register and the recording time was past noon between 2:00 p.m. and 4:00 p.m. Maldonado (2014) observed 1.5 times more bees at 50% of flowering than at 30% or 70% of flowering, furthermore reported that the highest foraging activity occurred at 3:00 pm, finding up to 1.5 times more bees at that time than at 9:30 am. It should be noted that these results were obtained under experimental conditions in a cage. However, several studies report that the highest activity of bees occurs from late morning to early afternoon, both in the field (16) and cage (42). Our results showed that the highest bee activity occurs when the climatic conditions were ideal for the pollinator. According to reports by Silva and Dean (2004), the highest bee activity is observed in the afternoon, which is negative for the pollination of onion flowers because they produce more nectar in the middle and late morning. However, other authors say that the lowest forage occurs from 10:00 a.m. to 5:00 p.m. (6).

Bee activity was very erratic through the flowering period even when the climatic conditions were favorable for foraging, sometimes no bees were seen visiting the flowers. Some days, the weather conditions at the recording time were not ideal for bees, therefore the activity was almost null, but this situation was solve in the course of the afternoon when conditions improved, increasing the bee activity on the umbels. Neupane *et al.* (2006), reported that there are several factors involved in the preference of bees and that the frequency of their visits varies through the day, as well as with the flowering stages. The seed yields found in the different lines indicate that the climatic conditions may have improved at certain times of the day, making it possible for the bee to efficiently perform its work.

The bee activity increased when the temperature was between 22 and 33°C and the day was sunny, conditions that only occurred in the afternoon some days. The differences in the attraction according to the time of the day could be attributed to variations in temperature and cloudiness, which are the most influential parameters in the flight of bees, when rain and strong winds (greater than 80 $km.h^{-1}$) are not consider since under this situation bees are tent to stop all activity outside the hive. Our results are in agreement with observations previously made by other authors, if the temperature is below 9°C, bees do not fly (21, 23). The flight and temperature are linearly correlated in the range of 14-22°C under field conditions, but the residence time per onion umbel is not influenced by the air temperature (7). Tan *et al.* (2012) observed that at 20°C the bee activity was the highest, while at 43°C the activity was reduced almost completely, the same as at temperatures below 10°C. Although, the most precocious lines yield better in warmer locations, like San Juan, onion seed production for all lines was higher in Mendoza. Temperature had the same effect in the attraction of bees for the different lines; therefore, the differences of attraction of bees would be explained by the differences among the genetic materials used.

In Media Agua it was observed that during the days when the temperature exceeded 38°C the bee stopped visiting the umbels regardless of the line observed, nor were observed foraging flowers of other species, but they could be grouped in large numbers on the puddles of water that remained after irrigation. Some studies have reported that a high temperature (higher than 40°C) increases bees water collection to the detriment of foraging or pollen collection (15, 17) and that foraging activity in the field correlates negatively with the increase in temperature (6). Voss *et al.* (1999) also determined that with temperature increases the onion nectar becomes more viscous and therefore less attractive for the bee.

Humidity and cloudiness influenced negatively in bee activity. Even when there are adequate temperatures, bees do not fly if there is not enough light, on cloudy days they stay near the hive. According to the results obtained, the minimum RH had a great influence on the behavior of the bees. At values lower than 20% or higher than 55% bees drop their activity. These results are in agreetment with Puškadija *et al.* (2007), who reported that the highest bee activity for sunflower pollination is between 40 and 50% of humidity, but they are lower than those reported by other authors (2, 3). Burill and Dietz (1981) found that, bee foraging increased when air temperature rises, but was not correlate to changes in atmospheric pressure and relative humidity. So far in most of the studies reported, it was found that the combination of several meteorological parameters control the foraging pattern of pollinators and that the cessation of activity seems to be governed by the rapid decrease in the intensity of light. In our work we observed that climatic factors affected the frequency of visits for all lines in the same way, therefore, the differences between the visits are explained by the variability between the genetic materials used and not by the meteorological parameters.

Conclusions

Onion seed production for all lines was higher in Mendoza than in San Juan. It would not be convenient to carry out an onion seed production in areas where the temperatures are very high during the month of November, month where the highest frequency of bee visits occurs between 50 and 85% of flowering.

The results showed a marked variability in the attraction of bees and seed production between the OP and MSL and within the MSL. In addition, it was shown that environmental factors such as temperature and humidity modify the rate of bee visits on the flowers.

This study contributes to the understanding of the factors that affect the bees foraging behaviour for the production of onion seeds. According to the results obtained, it is presume that the variability in the bee attraction by the studied lines would be influenced by the climatic factors; however, these would not explain the total variability in the performance of the different studied lines. Therefore, the study of other factors such as the floral traits of OP and MSL and the composition of the nectar, which directly or indirectly affect the bee attraction, would be necessary.

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Artificial seed viability of sugarcane (*Saccharum officinarum* L. cv. Mex 69-290) under conditions of Huimanguillo-Tabasco, Mexico

Viabilidad de la semilla artificial de caña de azúcar (*Saccharum officinarum* L. cv. Mex 69-290) bajo condiciones de Huimanguillo-Tabasco, México

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Abstract

To develop an artificial seed of sugar cane using sodium alginate and starch, it was possible to bring shoots resistance and protection in addition to a germination of 100 and 84%, respectively. In order to improve the technology of the artificial seed of sugarcane, two experiments were carried out to evaluate different polymer concentrations (sodium alginate and starch) and determine the maximum storage time for the artificial seed, using a completely random design, and a factorial 5x5 completely random design. The study variables were as follows: physical condition, rheological, mechanical test and seedling emergence. The results obtained have allowed to us to conclude that the best physical condition, resistance and seedling emergence, were obtained with 2% (w/v) sodium alginate and 15% (w/v) starch, corroborating that the initially proposed encapsulation is reliable for the artificial seed elaboration. The seed viability at the fifth day of elaboration was the best choice with a seedling emergence of 100% at the 30 days of planting. Therefore, artificial seed can only be stored for five days to ensure a 100% seed germination.

Keywords
artificial seed • sugar cane • viability • encapsulated • germination

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Resumen

Al desarrollar la semilla artificial de caña de azúcar, utilizando alginato de sodio y almidón, fue posible brindar resistencia y protección a las yemas, además de una germinación de 100 y 84% respectivamente. Con la finalidad de mejorar la tecnología de la semilla artificial de caña de azúcar, se realizaron dos experimentos para evaluar diferentes concentraciones de polímeros (alginato de sodio y almidón) y determinar el tiempo máximo de almacenamiento de la semilla artificial, para ello, se utilizó un diseño completamente al azar y un diseño factorial 5x5 completamente al azar. Las variables de estudio fueron: estado físico, prueba reológica, prueba mecánica y emergencia de plántulas. Los resultados obtenidos permiten concluir que, el mejor estado físico, resistencia y emergencia de plántulas, se obtuvieron con alginato de sodio al 2% (p/v) y almidón al 15% (p/v), corroborando que el encapsulado inicialmente propuesto es confiable para elaborar la semilla artificial. La viabilidad de la semilla a los cinco días de elaborada fue la mejor obteniendo una emergencia de plántulas de 100% a los 30 días de siembra. Por lo tanto, la semilla artificial solo puede almacenarse cinco días para asegurar una emergencia de plántulas del 100%.

Palabras clave
semilla artificial • caña de azúcar • viabilidad • encapsulados • germinación

Introduction

In Mexico, the sugarcane industry is an activity of high social impact, due to jobs generation and to be deeply rooted in the economy and culture of the country (1, 14). The sugarcane plantation in Mexico is a semi-mechanical activity by combining manual and mechanized operations (11, 14), however, the labor used in this system is increasingly expensive and difficult to achieve, which indicates the need for a fully mechanical operation, whose main advantage is the reduction of labor and operating costs (12, 13).

Even when using the planters' technology employing whole stems or sugarcane pieces, the efficiency of precision mechanized planting had not been achieved (12). During the process of obtaining sugarcane pieces with harvesters and in the transshipment to the seeders, the buds are mechanically damaged, this reduces the seed germination percentage, and after almost three decades of experience, it is still estimated that only 70% of commercially planted shoots manage to germinate (17).

Due to this problem, the technology of artificial seeds arises, which are vegetal structures of normally asexual origin, encapsulated with a gelling substance that protects it and that have the capacity to regenerate a plant completely identical to its parent. This technology includes two parts: a system of micro propagation through somatic embryos and another through buds (6, 7, 8, 9).

The technique of somatic embryogenesis has been the most widely used to obtain sugar cane seedlings *in vitro*, with the objective of seeding them semi-mechanically. The technique involves encapsulating a somatic embryo in a

gelling matrix, which must protect it and allow it to be manipulated during storage, transport and planting, as well as to facilitate germination (5, 9).

However, obtaining sugarcane seedlings through this technique is highly costly, due to the need for reagents, materials, technicians and a specialized laboratory, to obtain it *in vitro*, to this is added, greenhouse acclimatization and the subsequent transplant to the field (6).

On the other hand, encapsulated buds have the same advantages as embryogenesis, in addition to being able to take them directly to the soil for their germination and to mechanize the sowing process. The axillary buds can be isolated from a portion of the stem and mixed with a biodegradable polymer. This can be achieved under non-sterile conditions by adding fungicides to avoid contamination (7).

Given these concerns, the artificial sugarcane seed was developed, which consists of a piece of sugarcane stem of 35 mm in length with a single shoot, disinfected and encapsulated with a mixture of ground sugar cane straw and a biodegradable polymer.

In fact, sodium alginate and starch bring them shoots resistance and protection (2), in addition, had achieved a seed germination of 100% using alginate of sodium and 84% using starch (3). Bearing this in mind, the objective of the present investigation was to evaluate the viability of artificial sugarcane seeds by comparing different concentrations of polymers for their elaboration and to determine the maximum storage time. In order to have a better management of the planting material and obtain higher percentages of germination.

Materials and methods

The experiments were carried out in the plant physiology laboratory of the Colegio de Postgraduados Campus Tabasco, Mexico. Sugarcane samples were taken from eight-month-old sugarcane plantations of the cultivar Mex 69-290, in the village C-34 from the municipality of Huimanguillo-Tabasco, Mexico whose geographic coordinates are as follows: 17°58'16'' N, -93°37'30'' W. This variety has a good index of nitrogen efficiency (Salgado *et al.*, 2017).

With the use of a machete, the sugar cane stems were cut, later, with the use of a hacksaw, pieces of stems with shoots of 35 mm in length were cut (20 mm of reserve from the foliar scar towards above and 15 mm at the bottom) of the middle section of the stem. Shoots were disinfected for 10 minutes in a solution of Malathion 50EC (Agroquímica Tridente) to 0,2% and Carbendazim (Prozycar® 500) to 0,1%, subsequently, have allowed to dry for 10 minutes.

Encapsulation improvement of the sugarcane artificial seed

The experiment was carried out in October 2016. A completely randomized design was used with nine treatments (table 1, page 30) and 20 replicates.

Encapsulation of the sugarcane shoots using sodium alginate + calcium chloride (T1, T2, T3 and T4)

Sodium alginate was mixed with 1 L of water in a beaker, stirring to avoid lumps formation. In the same way, calcium chloride was mixed in 1 L of water in a beaker. To the sodium alginate mixture, 300 g of dried ground sugarcane milled straw was added to form a paste, with which the shoots were manually covered.

Table 1. Encapsulation treatments used.
Tabla 1. Tratamientos de encapsulación utilizados.

Treatments (T)	Description
T1	Sodium alginate to 2% + calcium chloride to 10%
T2	Sodium alginate to 4% + calcium chloride to 10%
T3	Sodium alginate to 6% + calcium chloride to 10%
T4	Sodium alginate to 4% + calcium chloride to 13%
T5	Sodium alginate to 4% + calcium hydroxide to 10%
T6	Starch to 10%
T7	Starch to 12.5%
T8	Starch to 15%
T9	Starch to 17.5%

The encapsulated shoots were immersed into a calcium chloride solution for five minutes, and subsequently, placed in a plastic tray to dry for 72 hours in the shade (26°C approximately). The encapsulation thickness was approximately 5 mm.

Encapsulation of the sugarcane shoots using sodium alginate + calcium hydroxide (T5)

Sodium alginate was mixed with 1 L of water in a beaker, stirring to avoid lumps formation. In the same way, calcium hydroxide in 1 L of water was mixed in a beaker. To the sodium alginate mixture was added 300 g of dried ground sugarcane milled straw to form a paste, with which the shoots were manually covered. The encapsulated shoots were immersed into a calcium hydroxide solution for five minutes and placed into a plastic tray to dry them for 72 hours in the shade (26°C approximately). The encapsulation thickness was approximately 5 mm.

Encapsulation of sugar cane shoots using starch (T6, T7, T8 and T9)

The starch was dissolved into 250 mL of water in a beaker, subsequently 750 mL of hot water was added, was stirred until homogenization. To the starch mixture was added 300g of dried ground sugarcane milled straw to form a paste, with which the shoots were manually covered and placed into a plastic tray to let them dry for 72 hours in the shade (26°C approximately). The encapsulation thickness was approximately 5 mm.

Study variables

Physical state of the artificial seed

A visual analysis of the encapsulation was performed after 72 h of storage. An encapsulation in good physical condition should not present detachments or germination.

Mechanical strength test

To observe the strength or fragility of the encapsulated with good physical condition, they were placed in a plastic tray, which stirred from one place to another on 10 occasions, simulating the movement that takes place during the planting process.

Rheological test

With a pocket penetrometer (model E-280, AMS®, USA), a strength was exerted on the center of the encapsulates that passed the mechanical strength

test and necessary effort was recorded (kg/cm^2) to break down or deform them.

Seedling emergence

Ten replicates of each treatment in good physical condition after the previous tests were selected and planted in a completely random plastic trays containing river sand. Irrigation was applied every second day to field capacity. 30 days after planting, a count of the seedling emerged was carried out per treatment.

Seedling vigor

30 days after panting, all the seedling emerged were extracted, in order to measure the roots length and stem height.

Statistical analysis

An analysis of variance was performed with a completely randomized design, and Tukey's multiple means comparison test, using the SAS version 9.3 package. To obtain an approximate normal distribution of the data, a transformation was performed by means of the arcsine function of the square root [$\sqrt{X}$ / (100)], for the variables evaluated in percentage (physical state, mechanical resistance and emergence of seedlings), for the rest of the variables (rheological test and seedling vigor) a logarithmic transformation was carried out.

Storage time of the artificial seed of sugarcane

The experiment was carried out in November 2016. A factorial 5x5 completely random design was used; 5 treatments (table 2) and 5 storage time with 25 replicates. The storage times were: 5, 8, 11, 14 and 17 days in the shade at room temperature (24°C approximately).

Encapsulation of sugarcane shoots using starch and sodium alginate + calcium chloride

For the encapsulation process of the sugar cane shoots, the same procedure described in the previous experiment was carried out for each corresponding polymer.

Study variables

Physical state of the artificial seed

For its evaluation, a visual analysis of the encapsulation was performed after 5, 8, 11, 14 and 17 storage days.

Seedling emergence

At each date of assessment of physical condition, were planted in a completely random design 5 replicates per treatment on plastic trays containing river sand. Irrigation was applied every second day at field capacity. 30 days after planting, in the phenological stage of germination, a count of the seedlings emerged was carried out per treatment.

Table 2. Encapsulation treatments used.

Tabla 2. Tratamientos de encapsulación utilizados.

Treatments (T)	Description
T1	Sodium alginate to 2% + calcium chloride to 7%
T2	Sodium alginate to 2% + calcium chloride to 10%
T3	Sodium alginate to 2% + calcium chloride to 13%
T4	Starch to 10%
T5	Starch to 15%

Seedling vigor

30 days after planting, all the seedlings that emerged were extracted, in order to measure the roots length and stem height.

Statistical analysis

An analysis of variance was performed with a factorial 5x5 completely randomized design, and Tukey's multiple means comparison test, using the SAS version 9.3 package. To obtain an approximate normal distribution of the data, a transformation was performed by means of the arcsine function of the square root [$\sqrt{X}$ / (100)], for the variables evaluated in percentage (physical state and emergence of seedlings), for the rest of the variables (seedling vigor) a logarithmic transformation was carried out.

Results and discussion

Encapsulation improvement of the sugarcane artificial seed

Physical state of the artificial seed

The analysis of variance indicates highly significant differences between treatments. According to Tukey test, the best physical state of the artificial seed (100%) was presented in the T8 with 15% starch (table 3), this value exceeds 95% reported by Alvarez *et al.* (2016), when using 10% starch to encapsulate sugarcane buds; the mixture of 15% starch with ground cane straw, produces a polymer that allows better encapsulation of the buds, although its concentration is increased by 5%, this is cheaper than alginate and calcium chloride, which places it as the best polymer to encapsulate sugarcane buds.

Table 3. Physical tests of the encapsulated after 72 hours of storage.

Tabla 3. Pruebas físicas de los encapsulados después de 72 horas de almacenamiento.

Encapsulation treatments (T)	Physical state (%)	Mechanical strength (%)	Rheological test (kg/cm^2)
T1 Sodium alginate to 2 % + calcium chloride to 10%	95 b	100 a	4.5 a
T2 Sodium alginate to 4 % + calcium chloride to 10%	80.4 d	80 b	4.5 a
T3 Sodium alginate to 6 % + calcium chloride to 10%	70 e	40 d	4.5 a
T4 Sodium alginate to 4 % + calcium chloride to 13%	95 b	80 b	4.5 a
T5 Sodium alginate to 4 % + calcium hydroxide to 10%	85 d	80 b	3.6 b
T6 Starch to 10%	60 f	66.7 c	4.5 a
T7 Starch to 12.5%	93.3 bc	87.5 b	4.5 a
T8 Starch to 15%	100 a	100 a	4.5 a
T9 Starch to 17.5%	90.4 c	100 a	4.5 a
Average (%) CV (%) F probability of T DMS	85.4 2.6 0.0001** 0.06	81.5 4.2 0.0001** 0.1	4.4 1.9 0.0001** 0.2

† Means with the same letter in the column are statistically equal. Tukey test (P≤ 0.05).

** Highly significant difference.

† Medias con la misma letra en la columna son iguales estadísticamente. Prueba de Tukey (P≤ 0,05).

** Diferencia altamente significativa.

The T1, T4 and T7 presented physical state greater than 93%. These results coincide with that reported by Arias *et al.* (2016), that the polymer 2% sodium alginate plus 10% calcium chloride, allow to obtain encapsulates of the artificial seed with physical states higher than 94%. The rest of the treatments are discarded since they present physical states of less than 90%.

Mechanical strength test

In the results of the analysis of variance can be observed that there are highly significant differences among encapsulation treatments. The Tukey test indicates that the treatments that presented 100% of their replicates in good condition after performing the test were as follows: T1, T8 and T9, respectively (table 3, page 32), corroborating what is reported by Álvarez *et al.* (2016) for T1, and surpassing 84% of physical state after carrying out the test with encapsulated of starch (at 10%), compared to 100% of the T8 and T9. In the case of T2 and T3, it is observed that, increasing the concentration of sodium alginate and maintaining the concentration of calcium chloride, does not favor the physical condition or the mechanical resistance of the encapsulates, indicating that the concentrations of the Polymers should be increased equally, as observed in T4, which obtained similar results to T1. On the other hand, substitution of calcium chloride by calcium hydroxide (T5), in an effort to reduce the processing costs, does not favor good physical condition or mechanical resistance to obtain only 85 and 80% of encapsulated in good physical state in each test respectively.

Rheological test

In the rheological test, the analysis of variance indicates highly significant differences among encapsulation treatments. The Tukey test indicates that the lowest resistance was obtained by T5 (3.6 kg/cm^2) because this treatment does not solidify properly. The rest of the treatments are statistically equal to each other, with an average resistance of 4.5 kg cm^{-2} (table 3, page 32). These results are similar to the 4.2 and 4.3 kg cm^{-2} reported by Álvarez *et al.* (2016) for 10% starch encapsulates and sodium alginate at 2% plus calcium chloride at 10% respectively. These results corroborate that the polymers T8 and T1, allow to elaborate an excellent artificial seed of sugarcane.

Seedling emergence (%)

The analysis of variance indicates that there are highly significant differences between the encapsulation treatments. The Tukey test indicates that the T1 and T8 treatments presented a 100% emergence of seedlings, followed by T2, T4 and T5, all of them with a 90% emergence of seedlings (table 4, page 34). These results are comparable and even superior in terms of the variability of the report by Muralles *et al.* (2009), who reported that, in a study under controlled conditions in North Florida, they obtained germination percentages of 73% using cuttings from the upper stem of sugarcane, while Galal (2016) reported a percentage of germination of 92.66% at 35 days using shoots planted in trays, surpassing the germination of traditional sowing in 8.43%. Of these results, T1 and T8 stand out, which obtained the best results in the physical state and the mechanical and rheological tests, which indicates that despite the hardness of the encapsulation, this does not prevent the development and seedling emergence of the sugarcane buds.

Table 4. Emergence and seedlings vigor resistance after 30 days of planting.
Tabla 4. Emergencia y vigor de plántulas a los 30 días después de la siembra.

Encapsulation treatments (T)	Seedling emergence (%)	Root length (cm)	Stem height (cm)
T1 Sodium alginate to 2% + calcium chloride to 10%	100 a	11.4 d	41.3 d
T2 Sodium alginate to 4% + calcium chloride to 10%	90.4 b	14.9 bc	33.3 d
T3 Sodium alginate to 6% + calcium chloride to 10%	80.4 c	14.3 c	40.7 cd
T4 Sodium alginate to 4% + calcium chloride to 13%	90.4 b	19.6 a	44.4 bcd
T5 Sodium alginate to 4% + calcium hydroxide to 10%	90.4 b	16.8 b	43.3 bcd
T6 Starch to 10%	71.2 d	13.8 c	44.6 bcd
T7 Starch to 12,5%	80.4 c	14.3 c	76.7 a
T8 Starch to 15%	100 a	10.2 d	64.6 abc
T9 Starch to 17,5%	80.4 c	14.6 bc	68.8 ab
Mean (%) CV (%) F Probability of T DMS	87 1.6 0.0001** 0.04	14.7 2.6 0.0001** 0.1	45.7 8.1 0.0001** 0.5

† Means with the same letter in the column are equal statistically. Tukey test (P≤ 0.05)
** Highly significant difference.
† Medias con la misma letra en la columna son iguales estadísticamente. Prueba de Tukey (P≤ 0,05).
** Diferencia altamente significativa.

Seedling vigor

For the length of the root, the analysis of variance indicates highly significant differences between the encapsulation treatments. The Tukey test indicates that T4 obtained the highest root length (19.6 cm), while the lowest treatments were T1 and T8 (11.4 and 10.2 cm respectively), being statistically equal to each other. In this case it is observed that there is no relation between the length of roots and the emergence of the buds, and that perhaps the length of roots is influenced by the quantity of reserves of the sugarcane bud, which has not been possible control, despite collecting stems of similar diameter to extract the buds. The variety Mex 60-290 has an intermediate nitrogen consumption, requires 1.9 kg of N per t of stems (Salgado *et al.*, 2017).

The length of roots of T1 and T8 are less than those reported by Arias de la Cruz (2015), who reported 13.8 cm of root length for encapsulation with 10% starch and 14.0 cm root length with 2% sodium alginate plus chloride of calcium at 10%, at 45 days after planting.

With respect to, the stem height in the analysis of variance, can be observed that there are highly significant differences among encapsulation treatments. The Tukey test indicates that the highest stem height was obtained with the T7 (77.6 cm), followed by the T8 and T9 (64.6 and 68.8 cm respectively), being statistically equal. While the lowest stem height was obtained with T1 and T2 (41.3 and 33.3 cm respectively), being statistically equal to each other (table 4).

The differences between stem height could be explained, as mentioned above by the reserve of the buds, the polymer in this case only protects the buds from desiccation and possible physical damage during handling for planting.

The short observation period did not allow observing the relationship between root length and stem height, reported by Budi *et al.* (2016), who mention that the root system affects the growth of seeds and seedlings to reach the optimum height. The stem height of T1 and T8, could be considered adequate, if compared to the height reported by Arias de la Cruz (2015) 45 days after the development of the sugarcane plant, who found 59.5 cm of stem height for the starch encapsulation 10% and 58.8 cm of stem height with al 2% sodium alginate plus 10% calcium chloride.

Storage time of the sugarcane artificial seed

Physical state of the artificial seed

According to the results of the analysis of variance, highly significant differences were observed for the encapsulation treatment (T), storage days (SD) and their interaction T * SD (table 5).

The results of the Tukey test, for the encapsulation treatment, indicate that T2 and T3, presented 100% of the artificial seeds in good physical condition until 17 SD (table 5). These results corroborate that the encapsulation with 2% sodium alginate plus 10% calcium chloride reported by Alvarez *et al.* (2016), is sufficient to maintain a good physical condition up to 17 SD, and increase the concentration of calcium chloride, would be an unnecessary expense of the encapsulation material, on the contrary, by reducing the concentration of calcium chloride (T1), the percentage of encapsulates in good physical condition is reduced.

According to the results of the analysis of variance, highly significant differences were obtained for the storage days' factor (SD), where the highest percentage of artificial seed in good physical condition (98.4%) was obtained at five SD.

Table 5. Physical state (%) of the encapsulated at different Storage Days (SD).
Tabla 5. Estado físico (%) de los encapsulados a diferentes días de almacenamiento.

Encapsulation treatments (T)	Storage Days (SD)					T mean
	5	8	11	14	17	
T1 Sodium alginate to 2% + calcium chloride to 7%	96 b	95 b	93.3 b	90 b	80 b	90.8 c
T2 Sodium alginate to 2% + calcium chloride to 10%	100 a	100 a	100 a	100 a	100 a	100 a
T3 Sodium alginate to 2% + calcium chloride to 13%	100 a	100 a	100 a	100 a	100 a	100 a
T4 Starch to 10%	96 b	90 c	86.5 c	80 c	60 c	82.5 d
T5 Starch to 15%	100 a	95 b	93.3 b	90 b	80 b	91.6 b
Mean of Days of storage	98.4 a	96 b	94.6 b	92 c	84 d	
CV (%)	2.9					
F probability						
Encapsulation treatment (T)	0.001**					
Days of storage (SD)	0.001**					
Interaction (T*SD)	0.001**					
DMS (T)	0.03					
DMS (SD)	0.03					

† Means with the same letter in the column are equal statistically. Tukey test (P≤ 0.05)

** Highly significant difference.

† Medias con la misma letra en la columna son iguales estadísticamente. Prueba de Tukey (P≤ 0,05).

** Diferencia altamente significativa.

In fact, it was observed that the three best encapsulation treatments (T2, T3 and T5) achieved a better interaction at five days, with 100% of the artificial seeds in good physical condition, followed by the interaction at eight days where said treatments, obtained 100, 100 and 95% of the artificial seeds in good physical condition respectively (table 5, page 35).

The above indicates that the shelf life of the artificial seed can be from 5 to 8 days, depending on the treatment used. Sufficient time to carry out the mechanized planting, without damaging the artificial seed. On the contrary, T1 and T4, presented the lowest percentages of encapsulated in good physical condition, this can be attributed to the fact that, having a lower concentration of polymer, the humidity content could be higher, which would delay the drying process and would cause the sprouting of the yolk, in comparison with the rest of the treatments with higher concentration of polymers.

Seedling emergence (%)

According to the results of the analysis of variance, highly significant differences were observed for the encapsulation treatment (T), Storage days (SD) and their interaction T * SD (table 6).

The results of the Tukey test, for the encapsulation factor, indicate that the worst treatment was T3, which had no seedling emergence, attributed to the hardness of the encapsulation.

Table 6. Seedling emergence of artificial seeds planted after 5, 8, 11, 14 and 17 Storage days (SD).

Tabla 6. Emergencia de plántulas a partir de semillas artificiales sembradas después de 5, 8, 11, 14 y 17 días de almacenamiento.

Encapsulation treatments (T)	Seedling emergence (%)					T mean
	5 SD	8 SD	11 SD	14 SD	17 SD	
T1 Sodium alginate to 2 % + calcium chloride to 7%	80 b	60 c	60 b	0 a	0 a	40 b
T2 Sodium alginate to 2 % + calcium chloride to 10%	100 a	80 b	0 d	0 a	0 a	36 c
T3 Sodium alginate to 2 % + calcium chloride to 13%	0 c	0 d	0 d	0 a	0 a	0 d
T4 Starch to 10%	100 a	80 b	80 a	0 a	0 a	52 a
T5 Starch to 15%	100 a	100 a	40 c	0 a	0 a	48 a
Mean of Days of storage	76 a	64 b	38 c	0 d	0 d	
CV (%)	3.1					
F probability						
Encapsulation treatment (T)	0.001**					
Days of storage (SD)	0.001**					
Interaction (T*SD)	0.001**					
DMS (T)	0.01					
DMS (SD)	0.01					

† Means with the same letter in the column are equal statistically. Tukey test (P≤ 0.05).

** Highly significant difference.

† Medias con la misma letra en la columna son iguales estadísticamente. Prueba de Tukey (P≤ 0,05).

** Diferencia altamente significativa.

The decrease in the emergence of the seedlings after eight days can be attributed to the dehydration and loss of the nutrient reserve due to the period of inactivity of the shoots, in this respect Carneiro *et al.* (1995), mention that the organic reserve of the buds, it influences the time of germination and development of the seedlings.

For the Storage days' factor, it was observed that, with the artificial seeds with five SD before planting, the highest percentage of seedling emergence are obtained (76 %) (table 6, page 36). Which is very favorable, since Shrivastava *et al.* (2008), indicate that, in order to achieve a good germination, sugarcane should not be allowed to remain unplanted more than two days after cutting, this demonstrates the favorable effect of the encapsulation by providing an adequate means for the shoots to maintain its viability.

T * SD interaction, it is observed that the highest percentage of seedling emergence (100%) was obtained with T2, T4, and T5 at the five SD, and even at 8 SD for T5 (table 6, page 36).

In general, a significant decreasing in seedling emergence was observed after 11 SD, similar results reported by Shrivastava *et al.* (2008), where, after a period of inactivity of 10 days, the shoots of the upper and lower part of the stem did not germinate and only a few of the middle section manage to germinate.

Therefore, the best viability of the artificial seed is observed at five SD where is possible to observe a 100% seedling emergence, being higher than that reported by Galal (2016), who showed that the shoots planted in plastic trays can reach a viability percentage of 95%. These results corroborate that the polymers T2 and T5, allow to elaborate an excellent artificial seed of sugarcane.

Seedling vigor

According to the results of variance analysis for root length, highly significant differences were observed for the treatment of encapsulation (T), storage days (SD) and interaction T * SD (table 7, page 38).

The results of the Tukey test for the encapsulation factor indicate that the largest root length was obtained with T4 (10.6 cm), with T3 being the worst (table 7, page 38), as it did not show root growth due attributed to the encapsulation hardness by increasing the concentration of calcium chloride.

The artificial seed of T2 and T5, allow a good growth of roots, as already discussed previously in the previous stage. What corroborates the viability of this technology. For the Days of Storage factor, it was observed that, with the artificial seeds with five SD before planting, the highest root length was obtained (9 cm), as the SD increases, the root length is reduced, this is attributed to the loss of nutrient reserve of the buds by dehydration due to days of inactivity after the cut of the stem. T * SD interaction, is observed that in general with the artificial seeds seeded after five SD, a greater root length is obtained, and when increasing the SD before planting, the length of the root system decreases (table 7, page 38).

With respect to the height of the stem, the results of the analysis of variance showed highly significant differences for treatment of encapsulation (T), storage days (SD) and interaction T * SD (table 8, page 38).

The results of the Tukey test for the encapsulation treatment factor, indicates that the highest height of the stem was obtained in T4 with an average of 41.9 cm, in contrast, T3 presented the worst performance when not developing the stem (table 8, page 38), attributed to the hardness of the encapsulation due to the increase in chloride concentration of calcium (13%) that prevented the softening of the cover during irrigation and the hydration of the buds.

Table 7. Root length of artificial seeds planted after 5, 8 and 11 Storage days (SD).
Tabla 7. Longitud de raíz de semillas artificiales sembradas después de 5, 8 y 11 días de almacenamiento.

Encapsulation treatment (T)	Root length (cm)			T mean
	5 SD	8 SD	11 SD	
T1 Sodium alginate to 2% + calcium chloride to 7%	11.3 ab	9 a	2.4 c	7.5 c
T2 Sodium alginate to 2% + calcium chloride to 10%	10 b	7.8 a	2 c	6.6 d
T3 Sodium alginate to 2% + calcium chloride to 13%	0 c	0 b	0 d	0 e
T4 Starch to 10%	12.3 a	8.4 a	11.2 a	10.6 a
T5 Starch to 15%	11.3 ab	8 a	5.4 b	8.2 b
Mean of Days of storage	9 a	6.6 b	4.2 c	
CV (%)	2.7			
F probability				
Encapsulation treatment (T)	0.001**			
Days of storage (SD)	0.001**			
Interaction (T*SD)	0.001**			
DMS (T)	0.1			
DMS (SD)	0.07			

† Means with the same letter in the column are equal statistically. Tukey test (P≤ 0.05)
** Highly significant difference.
† Medias con la misma letra en la columna son iguales estadísticamente. Prueba de Tukey (P≤ 0,05).
** Diferencia altamente significativa.

Table 8. Stem height of seedlings planted after 5, 8 and 11 Storage days (SD).
Tabla 8. Altura de tallos de plántulas sembradas después de 5, 8 y 11 días de almacenamiento.

Encapsulation treatments (T)	Stem height (cm)			t mean
	5 SD	8 SD	11 SD	
T1 Sodium alginate to 2% + calcium chloride to 7%	35 c	41 ab	32.6 a	36.2 b
T2 Sodium alginate to 2% + calcium chloride to 10%	30.5 d	37.6 bc	17.6 c	28.5 c
T3 Sodium alginate to 2% + calcium chloride to 13%	0 e	0 d	0 d	0 d
T4 Starch to 10%	63 a	34.8 c	28.1 b	41.9 a
T5 Starch to 15%	50 b	43.2 a	18.2 c	37.1 b
Mean of Days of storage	35.7 a	31.3 b	19.3 c	
CV (%)	2.7			
F probability				
Encapsulation treatment (T)	0.001**			
Days of storage (SD)	0.001**			
Interaction (T*SD)	0.001**			
DMS (T)	0.07			
DMS (SD)	0.05			

† Means with the same letter in the column are equal statistically. Tukey test (P≤ 0.05).
** Highly significant difference.
† Medias con la misma letra en la columna son iguales estadísticamente. Prueba de Tukey (P≤ 0,05).
** Diferencia altamente significativa.

Figure 1. Details of the encapsulated after 72 hours of storage, rheological test and seedling. **a).** Encapsulated of Sodium alginate to 2 % + calcium chloride to 10 %. **b).** Encapsulated of starch to 15%. **c).** Encapsulated subjected to rheological test. **d).** Seedling with root system and stem with good plant development.

Figura 1. Detalle de los encapsulados después de 72 horas de almacenamiento, prueba reológica y plántula, **a).** Encapsulado de alginato de sodio al 2 % + cloruro de calcio al 10 %. **b).** Encapsulado de almidón al 15 %. **c).** Encapsulado sujeto a la prueba reológica. **d).** Plántula con raíces y tallo en buen desarrollo.

For the Storage days' factor (SD), it was observed that, with the artificial seeds with 5 SD before planting, the highest height of the stem (35.7 cm) was obtained. Subsequently, interaction T * SD, it was observed that the highest stem height, was obtained with the T4 in the five SD (table 8, page 38). As previously mentioned, the encapsulation can protect or in any case retard the emergence of buds, but does not influence its development, since this to be conditioned by the amount of reserves in the bud. This coincides with that reported by Nieves *et al.* (2003), who observed that differences in height and diameter of the stem of plants derived from *in vitro* cultures and plants derived from cuttings, planted in field conditions diminish with time and even disappear at 12 months of age.

In figure 1 (page 39), the detail of the encapsulated in good physical condition, the performance of the rheological test and a seedling with a portion of the encapsulation can be shown, demonstrating that the encapsulation does not impede the seedling germination and development.

Conclusions

The experiment focused on the improvement of the encapsulation of the artificial seed, using 2% sodium alginate + 10% calcium chloride and 15% starch, the best encapsulation hardness is obtained for the protection of the shoots without affecting the germination and development process of the seedlings. As for the experiment regarding the storage time of the artificial seed, 2% sodium alginate + 10% calcium chloride, maintains 100% of its artificial seed in good physical condition until 17 storage days. However, the optimum emergence of the seedlings (100%) was obtained with the seeds planted after five storage days.

For the 15% starch, the best physical condition (100%) was obtained after five storage days, while the optimum emergence of the seedling (100%) was obtained with the seeds planted to five and eight storage days. Therefore, the viability of the seed made with 2% sodium alginate + 10% calcium chloride and 15% starch, was better after five storage days, obtaining 100% germination at 30 days of planting. Given these concerns, it is concluded that artificial seeds can only remain at storage for five days to ensure a good physical condition, germination and emergence of 100% seedlings.

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Effects of nitrogen fertilization and seasons on the morphogenetic and structural characteristics of Piatã (*Brachiaria brizantha*) grass

Efectos de la fertilización nitrogenada y las estaciones sobre las características morfogenéticas y estructurales del pasto Piatã (*Brachiaria brizantha*)

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Abstract

The objective of this study was to evaluate the morphogenetic and structural characteristics of Piatã grass under rotational stocking and nitrogen fertilization during the seasons. A randomized complete block design in a split-plot arrangement with three replications was used. The main plots were the applications of 0, 150, 300 and 450 kg per ha of N in the form of urea, and the subplots were seasons of the year: late summer/fall, winter, spring and summer. No interaction was detected between nitrogen fertilization and season for leaf appearance rate, leaf lifespan, number of live leaves and final length leaves. However, an interaction ($P<0.05$) of nitrogen fertilization and season influenced leaf elongation rate, phyllochron, leaf senescence rate and stem elongation rate. The leaf elongation rate and leaf appearance rate were linearly affected ($P<0.05$) by nitrogen fertilization. The seasons affected ($P<0.05$) the leaf lifespan and number of life leaves. The leaf lifespan decreased by 0.06 days for each kg of N applied. On the other hand, the number of live leaves increased by 0.0026 leaves/tiller for each kg of N. Fertilization with nitrogen positively affects morphogenetic and structural characteristics of Piatã grass under rotational stocking. This effect can be optimized during rainy periods in spring and late summer/autumn.

Keywords

Brachiaria brizantha • grazing management • leaf appearance rate • leaf elongation rate • leaf lifespan • leaf senescence rate

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Resumen

El objetivo de este estudio fue evaluar las características morfogenéticas y estructurales del pasto Piatã bajo pastoreo rotacional y fertilización nitrogenada durante las diferentes estaciones del año. Se utilizó un diseño de bloques completos al azar con un arreglo de parcelas divididas con tres repeticiones. Las parcelas principales fueron las aplicaciones de 0, 150, 300 y 450 kg/ha de N en forma de urea, y las subparcelas fueron las estaciones del año: finales de verano/otoño, invierno, primavera y verano. No se detectó interacción entre la fertilización nitrogenada y la estación para la tasa de aparición de hojas, la esperanza de vida de la hoja, el número de hojas vivas y longitud final de las hojas. Sin embargo, una interacción ($P<0,05$) de la fertilización nitrogenada y la temporada influyeron en la tasa de elongación de la hoja, filocrono, tasa de senescencia foliar y tasa de elongación del tallo. La tasa de elongación de la hoja y la tasa de aparición de la hoja se vieron afectadas linealmente ($P<0,05$) por la fertilización nitrogenada. Se observó un efecto ($P<0,05$) de las estaciones sobre la vida media foliar y el número de hojas vivas. La vida útil de la hoja disminuyó en 0,06 días por cada kg de N aplicado. Por otro lado, el número de hojas vivas aumentó en 0,0026 hoja/macollo por cada kg de N. La fertilización con nitrógeno influye positivamente en las características morfogenéticas y estructurales del pasto Piatã bajo pastoreo rotativo. Este efecto se puede optimizar durante los períodos de lluvia, en la primavera y al final del verano/otoño.

Palabras claves

Brachiaria brizantha • manejo del pastoreo • tasa de aparición de hojas • tasa de elongación de hojas • vida media foliar • tasa de senscencia de las hojas

Introduction

Brazil has an area of more than 160 Mha of pastures, and at least 100 Mha of these are sown pastures (7). *Brachiaria* spp. are the most commonly used grasses in Brazilian pastures. The *Brachiaria brizantha* cv. Piatã is one of the new recently cultivar introduced in Brazilian pasture. This cultivar has advantage of promoting slightly higher animal performance in the dry season (19).

The efficiency of forage use can be defined as the proportion of tissues that are removed by animals before forage entering the senescent state (8).

Therefore, to explore the maximum grass potential, it is necessary to know, understand and control its morphological characteristics, which is possible through morphogenetic studies associated with strict control of the height of the forage canopy (6, 19).

Tillers are considered the growth units of forage grasses, and the pasture is a population of tillers. For pasture to become perennial and persistent, there must be a balance between the appearance and death of tillers throughout the year, which allows grazing to adapt to different management conditions (15).

Leaf tissue production is regulated by environmental factors and influenced by the population density of tillers, and the interaction between these factors determines the morphogenetic rhythm of the plants (14). Thus, at the individual plant level, morphogenesis can be described by three basic characteristics: appearance, elongation and lifespan of the leaf (1).

The combination of these basic morphogenetic variables is responsible for the main structural characteristics of the pasture: leaf blade size, population density of tillers and number of live leaves per tiller (16). Thus, the morphogenetic rhythm determines the speed of recovery of leaf area after defoliation or its ability to maintain equilibrium in the case of pastures managed in rotational and continuous stocking, respectively (1).

Among the management practices that determine morphogenetic responses and structural characteristics, nitrogen fertilization is one of the most important. Therefore, due to the association of nitrogen fertilization and its role in several morphogenetic characteristics, involving the dynamics of leaves and tillers, it is necessary to evaluate the effects of this nutrient on grasses (5, 14) under environmental conditions of the southern region of the state of Mato Grosso.

Hypothesis

The hypothesis tested in this study was that nitrogen fertilization and seasons of the year affect morphogenetic and structural characteristics of Piatã grass.

Objetive

Evaluate the morphogenetic and structural characteristics of Piatã grass under rotational stocking and nitrogen fertilization during the seasons.

Materials and methods

Location of the experiment

The study was conducted at the Experimental Farm of the Universidade Federal de Mato Grosso from February 2014 to March 2015. This research station is located at 15°04'36" S, 56°04'36" W and is 141 m above sea level.

Climatic conditions

According to Koppen's classification, the climate is Aw, with a tropical megathermal climate, characterized by two well-defined dry (May to September) and rainy (October to April) seasons. The mean annual rainfall is 1,500 mm, with maximum intensity during January, February and March. The mean rainfall, insolation and temperature during the experimental period are shown in figures 1, 2 and 3 (page 45).

Treatments and experimental design

An established *Brachiaria brizantha* cv. piatã pasture was used. The experimental area was subdivided into 12 paddocks of 6 × 6 m, separated from each other by electrified fences and screens. A randomized complete block design in a split-plot arrangement with three replications was used. The main plots were the applications of 0, 150, 300 and 450 kg per ha of N in the form of urea, and the subplots were seasons of the year: late summer/fall, winter, spring and summer.

At the end of November of 2013, the pasture was cut at a height of 5 cm from the soil. Based on the soil analysis results, the soil was limed with dolomitic limestone, with 80% of lime's total relative neutralization (LTRN) carried out on the surface, aiming to raise the base saturation to 50%.

The fertilizer doses used were close to the recommendations of Souza and Lobato (2004), according to the requirement of the grass. After regrowth, the average height of 20 cm was maintained in all paddocks. At the end of December 2013, 120 kg ha^{-1} of P_2O_5 and 80 kg ha of K_2O using single superphosphate and potassium chloride, respectively, was applied. The source of phosphorus was applied in a single dose and the potassium in two doses. Nitrogen fertilization was divided into four doses according to the number of grazing cycles (table 1, page 46).

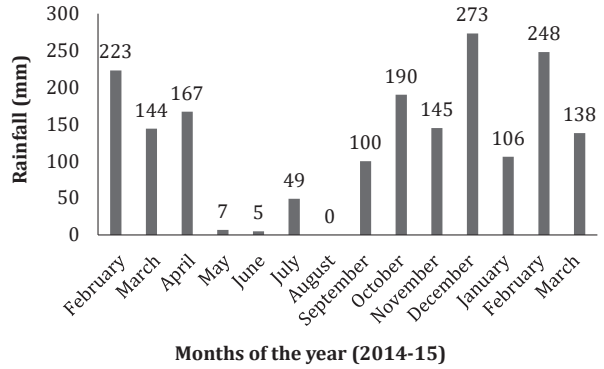

Figure 1. Monthly rainfall during the experiment.
Figura 1. Precipitación mensual durante el experimento.

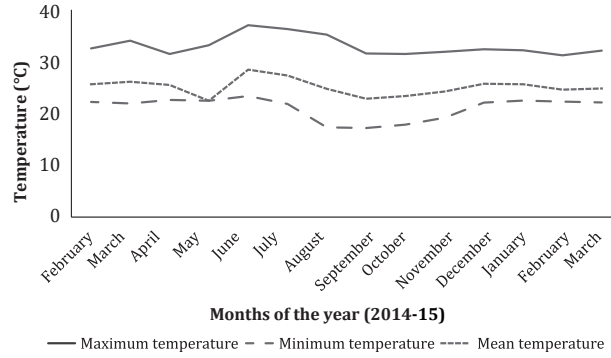

Figure 2. Mean, maximum and minimum temperatures during the experiment.
Figura 2. Temperaturas medias, máximas y mínimas durante el experimento.

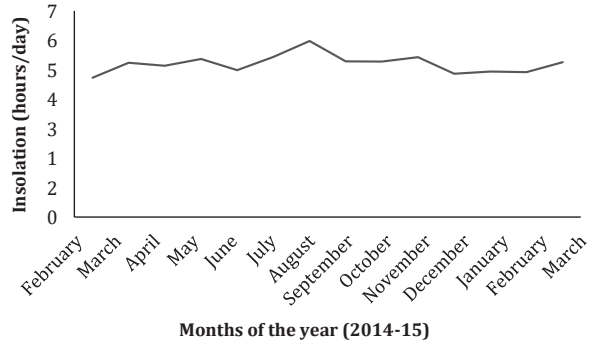

Figure 3. Insolation (hours/day) during the experiment.
Figura 3. Heliofanía (horas/día) durante el experimento.

Table 1. Nitrogen fertilization (kg/ha of N) applied to each paddock and its respective date of application during the experimental period.

Tabla 1. Fertilización con nitrógeno (kg/ha de N) aplicada a cada parcela y su respectiva fecha de aplicación durante el período experimental.

Level*	block	Date of application	1st application	Date of application	2nd application	Date of application	3rd application	Date of pplication	4th application	Total
0	I	-	-	-	-	-	-	-	-	0
0	II	-	-	-	-	-	-	-	-	0
0	III	-	-	-	-	-	-	-	-	0
150	I	2/8/14*	37.5	3/26/14**	37.5	5/8/14	37.5	11/2/14	37.5	150
150	II	2/8/14*	37.5	3/26/14**	37.5	5/8/14	37.5	11/2/14	37.5	150
150	III	2/8/14*	37.5	3/26/14**	37.5	5/8/14	37.5	11/2/14	37.5	150
300	I	2/8/14*	75	3/19/14**	75	10/6/14	75	11/9/14	75	300
300	II	2/8/14*	75	3/19/14**	75	10/6/14	75	11/9/14	75	300
300	III	2/8/14*	75	3/19/14**	75	10/6/14	75	11/9/14	75	300
450	I	2/8/14*	112.5	3/12/14**	112.5	10/19/14	112.5	11/17/14	112.5	450
450	II	2/8/14*	112.5	3/12/14**	112.5	10/19/14	112.5	11/17/14	112.5	450
450	III	2/8/14*	112.5	3/12/14**	112.5	10/19/14	112.5	11/17/14	112.5	450

*: First application of K_2O; **: Second application of K_2O.

*: Primera aplicación de K_2O; **: Segunda aplicación de K_2O.

The grazing management with rotational stocking strategy was characterized by the animal entry into and exit from the paddocks when the pasture showed 95% of light interception ($LI_{95\%}$) (pre-grazing) and 20 cm of height (post-grazing), respectively.

The pasture was grazed by sheep with average body weights of 50 kg using the mob-grazing technique (11), and the number of animals was dimensioned so that the lowering time of pastures would not exceed a daytime. Thus, a variable number of sheep was used per treatment, but maintained the same number of animals per repetition of treatment.

Grazing occurred on the same day for all replicates of the evaluated treatments, *i.e.* LI was considered as the average of the three replicates under the same treatment. After grazing, when the pasture showed 20 cm of height, the sheep were removed from the paddocks, placed in a one-hectare reserve pasture, and were returned when the pasture of Piatã grass reached the established pre-grazing target.

The monitoring of light interception was done using the sward analyzer AccuPAR Linear PAR/LAI ceptometer, Model PAR 80 (DECAGON Devices) in 10 places per paddock, in W-shaped trajectories (representative locations of the average conditions of the pasture during the sampling). Two readings were taken at each point: one above the sward and another at the soil surface (below the sward). These measurements were performed every six days until the approximation of the pre-established grazing target and every 2 days thereafter. The grazing intensity of Piatã grass was obtained from the results presented by Prado *et al.* (2014), who used LI as a parameter of grazing frequency.

Evaluation of the morphogenetic and structural characteristics

The evaluation of the morphogenetic and structural characteristics was performed in seven tillers per experimental unit. After each grazing cycle, tillers were randomly tagged on representative spots of the average sward condition (by visual evaluation of height and forage mass).

Every three tillers were assessed and measurements were taken of the leaf blade and stem (stem + leaf sheaths) lengths, leaf appearance, leaf expansion and senescence. These evaluations enabled the calculation of the leaf appearance rate (LAR, number of leaves appearing per tiller divided by the number of days of the evaluation period-leaf/tiller/day), the elongation rate (LER, sum of all the leaf blade elongations per tiller divided by the number of days under evaluation-mm/tiller/day) and the leaf senescence rate (LSR, sum of the senesced lengths of leaf blades present on the tiller divided by the number of days of the evaluation period - mm/tiller/day), the stem elongation rate (SER, sum of all the elongations of stems (stems + leaf sheaths) per tiller divided by the number of days of the evaluation period–mm/tiller/day), the number of live leaves per tiller (average number of expanding leaves and leaves expanded per tiller, not considering the leaves with more than 50% of this length senesced-leaves/tiller), phyllochron (which is the opposite of LAR-days/leaf), leaf lifespan (number of live leaves × phyllochron) and final leaf length (FLL, mean length of all expanded leaf blades–mm/tiller) (8).

Statistical analysis

To analyze the data, it was weighed the values of the variables that were grouped according to season. This is because some of the grazing cycles took place over two seasons. Thus, the value obtained for each variable was proportional to the number of days within each season of the year.

Therefore, data were grouped into four seasons: late summer/fall (from February 8 to June 20 of 2014); winter (from June 21 to September 21 of 2014); spring (from September 22 to December 20 of 2014); and summer (from December 21 of 2014 to March 19 of 2015).

The data were submitted to analysis of variance (ANOVA) and regression analysis using the MIXED procedure of SAS version 9.2 (21). When there was an interaction, data were unfolded to evaluate the effect of season in each nitrogen dose and the effect of dose in each season.

All statistical procedures were conducted by using 0.05 as the critical probability level for a type I error. Regression equations were chosen based on the determination coefficient and on the significance of the regression coefficients using the t test, adopting $\alpha = 0.05$.

Results

No interaction was detected between nitrogen fertilization and season for LAR, leaf lifespan, number of live leaves and FLL. However, an interaction ($P<0.05$) of nitrogen fertilization and season influenced LER, phyllochron, LSR and SER. The LER was linearly affected ($P<0.05$) in all seasons. The highest values of LER were verified in spring, late summer/autumn, summer and winter, respectively (table 2).

The LAR was linearly affected ($P<0.05$) by nitrogen fertilization. An increase of 48.96% was observed for LAR when compared to the absence of nitrogen fertilization with the highest dose. The highest values of LAR were seen in spring and late summer/autumn (table 3, page 49).

The phyllochron was linearly affected ($P<0.05$) by nitrogen fertilization in all seasons of the year (table 4, page 49). The highest phyllochron was found during winter of 44.06 days/leaf, which increased by 0.03 day/leaf for each kg of N applied. This value was higher than 50% of its value in spring (17.10 days/leaf), which only increased by 0.01 day/leaf for each kg of N.

The highest and lowest values of phyllochron were found during winter and spring, respectively, except in the dose of 450 kg of N/ha, which did not differ ($P>0.05$) among seasons.

Table 2. Leaf elongation rate (mm/tiller/day) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 2. Tasa de elongación foliar (mm/macollo/día) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotativo y fertilización nitrogenada durante las estaciones del año.

Seasons	Nitrogen fertilization (kg/ha)				Regression equations	r^2
	0	150	300	450		
Late Summer/Fall	3.32b	8.81b	13.38b	22.13b	$\hat{Y} = 2.7636 + 0.0406\ N$	0.9792
Winter	1.77c	2.17d	3.07d	3.24d	$\hat{Y} = 1.7693 + .0035\ N$	0.9416
Spring	7.65a	15.59a	20.13a	30.60a	$\hat{Y} = 7.4866 + .0489\ N$	0.9784
Summer	4.09b	4.47c	7.51c	11.50c	$\hat{Y} = 3.1046 + .0168\ N$	0.9036
CV (%)	10.40					

Different letters within a column indicate significant differences ($P \leq 0.05$); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas ($P \leq 0,05$); CV = coeficiente de variación.

Table 3. Leaf appearance rate (leaf/tiller/day) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 3. Tasa de aparición de hojas (hoja/macollos/día) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones.

Nitrogen fertilization (kg/ha)					
0	**150**	**300**	**450**	**Regression equation**	**r^2**
0.0465	0.0511	0.0792	0.0838	$\hat{Y}$ = 0.0441 + 0.000094 N	0.8994
Seasons					
Late summer/fall	Winter	Spring	Summer	CV (%)	
0.072ab	0.043c	0.091a	0.052bc	31.74	

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

Table 4. Phyllochron (days/leaf) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 4. Filocrono (días/hoja) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones.

Seasons	**Nitrogen fertilization (kg/ha)**				**Regression equations**	**r^2**
	0	**150**	**300**	**450**		
Late Summer/Fall	20.43b	11.71b	9.83ab	6.61a	$\hat{Y}$= 18.6496 - 0.0288 N	0.8959
Winter	27.38a	20.57a	15.68a	10.00a	$\hat{Y}$= 26.9656 - 0.0380 N	0.9958
Spring	10.83c	10.24b	8.73b	5.11a	$\hat{Y}$= 11.5293 - 0.0124 N	0.8805
Summer	26.12ab	15.77ab	10.28ab	9.38a	$\hat{Y}$= 23.7483 - 0.0371 N	0.8741
CV (%)	23.00					

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

The LSR and SER were linearly affected (P<0.05) by nitrogen fertilization in all seasons (tables 5 and 6, page 50). The highest values of LSR and SER were verified in spring, late summer/autumn, summer and winter, respectively.

The seasons affected (P<0.05) the leaf lifespan and number of life leaves (table 7, page 50 and table 8, page 51). The leaf lifespan decreased by 0.06 days for each kg of N applied. On the other hand, the number of live leaves increased by 0.0026 leaves/tiller for each kg of N. The FLL was linearly affected (P<0.05) by nitrogen fertilization (table 9, page 51) and increased by 0.063 mm/tiller for each kg of N applied, but it was not affected (P>0.05) by season.

Discussion

The highest LER values in spring and late summer/autumn were caused by favorable climatic conditions, such as light, temperature, nutrient availability and mainly, water availability, as the greatest rainfall was recorded during this period (figures 1, 2 and 3, page 45).

Table 5. Life lifespan (days) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 5. Vida media foliar (días) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones del año.

Nitrogen fertilization (kg/ha)					
0	**150**	**300**	**450**	**Regression equation**	**r^2**
65.75	54.50	45.75	39.00	Ŷ= 64.6000 - 0.0593 N	0.9873
Seasons					
Late Summer/Fall	Winter	Spring	Summer	CV (%)	
52.33ab	58.83a	47.25b	46.58b	19.01	

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

Table 6. Number of live leaves (leaves/tiller) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 6. Número de hojas vivas (hojas/macollo) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones.

Nitrogen fertilization (kg/ha)					
0	**150**	**300**	**450**	**Regression equation**	**r^2**
3.18	3.48	3.73	4.44	Ŷ= 3.1056 + 0.0026 N	0.9355
Seasons					
Late Summer/Fall	Winter	Spring	Summer	CV (%)	
3.69b	3.40b	4.74a	3.00b	20.70	

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

Table 7. Leaf senescence rate (mm/tiller/day) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 7. Tasa de senescencia de la hoja (mm/macollo/día) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones**.**

Seasons	**Nitrogen fertilization (kg/ha)**				**Regression equations**	**r^2**
	0	**150**	**300**	**450**		
Late Summer/Fall	1.82b	7.31b	11.88b	20.62b	Ŷ= 1.2636 + 0.0406 N	0.9792
Winter	0.57c	0.67d	1.57d	1.74d	Ŷ= 0.4793 + 0.0029 N	0.8911
Spring	6.15a	14.09a	18.63a	29.10a	Ŷ= 5.9866 + 0.0489 N	0.9784
Summer	2.59b	2.97c	6.01c	10.00c	Ŷ= 1.6046 + 0.0168 N	0.9036
CV (%)	6.20					

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

Table 8. Final leaf length (mm/tiller) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 8. Longitud final de la hoja (mm/macollo) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones.

Nitrogen fertilization (kg/ha)					
0	**150**	**300**	**450**	**Regression equation**	**r^2**
147.58	163.89	156.83	176.56	Ŷ= 147.11 + 0.0626 N	0.9867
Seasons					
Late Summer/Fall	Winter	Spring	Summer	CV (%)	
163.85a	157.16a	166.15a	157.69a	15.84	

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

Table 9. Stem elongation rate (mm/tiller/day) of *Brachiaria brizantha* cv. Piatã subjected to rotational stocking and nitrogen fertilization during the seasons.

Tabla 9. Tasa de elongación del tallo (mm/macollo/día) de *Brachiaria brizantha* cv. Piatã sometida a pastoreo rotacional y fertilización nitrogenada durante las estaciones.

Seasons	**Nitrogen fertilization (kg/ha)**				**Regression equations**	**r^2**
	0	**150**	**300**	**450**		
Late Summer/Fall	0.83b	2.20b	3.34b	5.53b	Ŷ= 0.6923 + 0.0101 N	0.9794
Winter	0.44c	0.54d	0.76d	0.81d	Ŷ= 0.4440 = 0.0000 N	0.9437
Spring	1.91a	3.90a	5.03ª	7.65a	Ŷ= 1.8746 + 0.0122 N	0.9784
Summer	1.02b	1.12c	1.88c	2.88c	Ŷ= 0.7766 + 0.0042 N	0.9044
CV (%)	5.54					

Different letters within a column indicate significant differences (P≤0.05); CV = coefficient of variation.
Letras diferentes dentro de una columna indican diferencias significativas (P≤0,05); CV = coeficiente de variación.

Leaf expansion is one of the most sensitive physiological processes to drought, as it interrupts leaf and root elongation long before photosynthetic processes and cell division are affected (12, 24). The LER is directly related to the recovery speed of the leaf area index of the pasture after grazing (2). Thus, the increase of nitrogen fertilization promotes a faster recovery of the remaining leaf area index, reducing the grazing interval and consequently promoting an increase in the number of grazing cycles, ensuring a perennial pasture.

According to Duru and Ducrock (2000) the influence of nitrogen on LAR can be viewed as the result of a combination of several factors such as sheath height, leaf elongation and temperature. Thus, nitrogen stimulates the growth of the plant, with consequent elongation of the internodes. It can be inferred that the increase in nitrogen fertilization associated with grazing management based on light interception (LI 95%) and variable stocking rate allowed greater control of stem elongation. This management allows the new leaf to be pushed out of the leaf sheath, promoting the increase of LAR (13).

The highest LAR values in spring and late summer/fall can be explained by the occurrence of better climatic conditions observed in spring and late summer/ autumn (figures 1 and 2, page 45) and the time of nitrogen fertilization (20). The results of phyllochron are in agreement with LAR because the larger the phyllochron the longer the time necessary for the expansion of a new leaf blade (25).

The development of stems increases the forage mass with a negative influence on pasture structure and light competition, compromising the grazing efficiency due to the decrease in the leaf:stem ratio (4, 18). The highest LSR during spring likely resulted from the higher rainfall (figure 1, page 45) associated with nitrogen fertilization, causing the plant begins senescence due to nutrient translocation for the expansion of new leaf blades (10). Thus, there was an acceleration of the biomass flow providing high LSR during this season.

The LSR is a very important morphogenetic characteristic in pasture management under rotational stocking especially when submitted to high nitrogen doses (22). The control of this characteristic by means of adjustment in the stocking rate, duration of grazing period and resting the pastures allows the loss of leaf tissue due to the senescence process being minimized (9). Thus, it can be inferred that grazing management using ecophysiological concepts (LI95%) allows the ideal grazing interval to occur when the greatest accumulation of leaves happens, but before the beginning of an accentuated accumulation of stem and dead material (2) promoting greater forage harvest efficiency.

A reduction of 59% in the leaf lifespan was found when comparing pastures without N fertilization and with 450 kg of N/ha. These results show that grasses without N fertilization use the maintenance of live leaves longer in detriment of the expansion of new leaves, as a survival strategy (table 2, page 48). On the other hand, the reverse occurs with high doses of N due to intense leaf renewal (9).

Nitrogen activity in FLL can be explained by the increase in the number of dividing cells, stimulating the production of new cells and providing an increase in LER (table 2, page 48), which contributed to changes in FLL (4) and the maintenance of a perennial pasture.

Conclusions

Fertilization with nitrogen positively affects morphogenetic and structural characteristics of Piatã grass under rotational stocking. This effect can be optimized during rainy periods in spring and late summer/autumn.

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Phenology, pollen synchronization and fruit characteristics of european hazelnut (*Corylus avellana* L.) cv. " Tonda de Giffoni" in three sites of central Chile

Fenología, sincronización polínica y características frutales de avellano europeo (*Corylus avellana* L.) cv. "Tonda de gifoni" en tres localidades de Chile central

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Abstract

Phenology, pollen synchronization and fruit characteristics were studied during the season 2011-2012 for European hazelnut (*Corylus avellana* L.) cv. "Tonda di Giffoni" and three of its pollinizers ("Tonda Romana", "Tonda Gentile delle Langhe" and "Barcelona") in three agro-ecological conditions of central Chile. Male and female blooms occurred from June through August, with a flowering span ranging between two to two and a half months depending on the cultivar, pollinizer and study sites. "Tonda di Giffoni" male flowering onset occurred during the first week of June, up to two weeks earlier than female flowers (271 to 417 chilling hours) showing a marked protandrus dichogamy. "Tonda Gentile delle Langhe" and "Barcelona" pollinizers completely covered the female flowering period of "Tonda di Giffoni", while "Tonda Romana" fail to cover the first flowering week. In general, starting dates for the different phenological stages were directly and significantly ($P<0.05$) correlated with chilling hour accumulation and growing degree days. Fruit set (34.1%) and maximum fruit diameter (16.6 mm) were significantly lower in the case of "Tonda Gentile delle Langhe" compared to "Tonda Romana" (82.2%, 17.3 mm) and "Barcelona" (74.7%, 17.4 mm).

Keywords
filbert • chilling hours • growing degree days •pollen compatibility

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Resumen

Se estudió la fenología, sincronización polínica y características frutales de avellano europeo (*Corylus avellana* L.) cv. "Tonda di Giffoni" y tres polinizantes ("Tonda Romana", "Tonda Gentile delle Langhe" y "Barcelona") bajo tres condiciones agroecológicas de Chile central durante la temporada 2011-2012. Las floraciones masculina y femenina ocurrieron entre junio a agosto y la floración varió entre dos y dos meses y medio dependiendo del cultivar, polinizante y sitios de estudio. El inicio de la floración masculina de "Tonda di Giffoni" ocurrió durante la primera semana de junio, hasta dos semanas antes que las flores femeninas (271 y 417 horas frío), manifestando una marcada dicogamía de tipo protándrica. "Tonda Gentile delle Langhe" y "Barcelona" cubrieron por completo el período de floración femenina de "Tonda di Giffoni", mientras que "Tonda Romana" no cubrió su primera semana de floración. En promedio, las fechas de inicio para las diferentes etapas fenológicas se correlacionaron directa y significativamente ($P<0,05$) con la acumulación de horas frío y días grado. El porcentaje de cuaja de frutos (34,1%) y el diámetro máximo de frutos (16,6 mm) fueron significativamente menores cuando se comparó el polinizante "Tonda Gentile delle Langhe" con "Tonda Romana" (82,2%, 17,3 mm) y "Barcelona" (74,7%, 17,4 mm).

Palabras clave
avellano • horas frío • días grado • compatibilidad polínica

Introduction

Hazelnut (*Corylus avellana* L.) is one of the oldest and most important dried fruit bushes (3, 8), holding the second place in the nut global market, with productions exceeding 800,000 metric t per year (24). This fruit has been labeled as a functional food and its consumption has been associated to several human health benefits due to the high concentration of bioactive compounds, mainly tocopherol and phenols (9, 11, 13). In recent years the demand for this species has risen significantly, mostly driven by a fastly growing functional food, chocolate and confectionery industries. The market is currently dominated by Turkey (75% of world production), followed by Italy and the United States (10). Chile, with an approximated cropping area of 18,000 ha, is the main Southern hemisphere supplier, and it is expected that by 2025 the country will become the third world producer, with a cultivated area of ~30,000 ha. Regarding its floral biology, it is a monoecious plant with female (glomeruli) and male flowers (catkins), although most varieties and cultivars are self-incompatible (19, 30). This sporophytic incompatibility is controlled by a single S locus with multiple alleles (31). The pollination is anemophilic, and in many cases there is dichogamy, where there is a lack in synchronization between pollen release and stigma receptivity from the female flower. Pollination and fruit setting are strongly conditioned by ecological and environmental factors, such as climate and soil type where the species develops (6, 27). Pollination occurs during winter, when female flowers emerge for several

weeks and pistils can be receptive in alternating periods of time (32). Factors such as average temperature, chilling hours, degree days, solar radiation, rainfall, wind speed and relative humidity, among others, strongly affect the occurrence of different phenological stages, as well as pollination and fruit set success (18). Thus, having a compatible and synchronized pollinizer and main variety combination becomes a requirement to achieve sufficient viable pollen production during the long flowering period (16). Several authors (11, 23) have studied the effects of different pollen origins on fruit set, yield and various quality parameters (weight of the nut, proportion of skin / fruit, proportion of fruit, etc.).

All have concluded that selecting the right pollinizer and main variety combination adapted to a specific area is a requirement to achieve sustainable and economic productions (11, 23). Despite a wide agroclimatic cultivation area, there are no systematic studies in Chile concerning the effect of agroclimatic conditions on phenological development, pollinizer compatibility and synchronization, and yield and fruit quality.

In addition, climate change adds an extra layer of complexity to hazelnut cropping, given its direct effect on pollen synchronization; thus, making floral phenology monitoring a permanent task to achieve high fruit sett, yield and quality. "Tonda di Giffoni" is the second main cultivar for the Chilean hazelnut industry, and is characterized by showing medium vigor and yields ranging between 2,000 and 3,000 kg/ha. In addition, its medium fruit size (14mm) and excellent organoleptic characteristics (10) contribute to a high demand from the chocolate industry.

The main goal of this study was to characterize the phenology, pollen synchronization, and fruit characteristics for European hazelnut (*Corylus avellana* L.) cv. "Tonda di Giffoni" and three pollinizers: "Tonda Romana", "Tonda Gentile delle Langhe" and "Barcelona" in three agro-ecological conditions of central Chile.

Materials and methods

Study site characterization

The study was carried out during the years 2011 and 2012, at three different sites located in the Maule Region, central zone of Chile (table 1, page 58).

Plant material

Six-year-old hazelnut trees of cv. "Tonda di Giffoni" and three pollenizers (11%) were selected: "Tonda Romana" (TR), "Tonda Gentile delle Langhe" and "Barcelona" (table 1, page 58).

In each study site the plantation framework was 5 x 4 m in north-south oriented rows. Trees were trained to a multi-axis system and watered weekly to water balance from November to the end of March with a micro-sprinkler system (40 L/h). Conventional cultivation practices (fertilization, phytosanitary management, weed control and pruning) were carried out, avoiding the use of growth regulators.

Floral phenology

A phenological monitoring was carried out weekly during the reproductive stage (From May 1, 2011 to the end of March 2012). In each study site (table 1, page 58) five uniform trees corresponding to "Tonda di Giffoni" and five nearby pollinating trees were selected.

Table 1. Study site and orchard specifications for Hazelnut (*Corylus avellana* L.) cv. "Tonda di Giffoni" and its pollinizers (11%).

Tabla 1. Sitios de studio y características de los huertos de avellano europeo (*Corylus avellana* L.) cv. "Tonda di Giffoni" y sus polinizantes (11%).

Study site	Coordinates	Soil type (5)	Climate (29)	Pollinizers (11%)
Los Niches	35°3'522" S; 71°7'3127" W	Huecán soil series. Sandy clay loam.	Average maximum temperature during January: 27.5°C. Average minimum temperature during July: 4.0°C, 1380 degree days (base 10°C) and 1472 chilling hours (base 7°C).	"Tonda Romana"
Camarico	35°16'16 82" S; 71°22'13 82" W	San Rafael soil series. Silt loam (FL). Soil phase SRF-6.	Average maximum temperature during January: 30.1°C. Average minimum temperature during July: 4.0°C, 1788 degree days (base 10°C) and 1283 chilling hours (base 7°C).	"Tonda Gentile delle Langhe"
San Rafael	35°15'34 70" S; 71°33'24 65" W	San Rafael soil series. Silt loam (FL). Soil phase SRF-7.	Average maximum temperature during January: 31.4°C. Average minimum temperature during July: 5.5°C, 2228 degree days (base 10°C) and 536 chilling hours (base 7°C).	"Barcelona"

In each tree, 50 cm long segments located in the middle third of five homogeneous branches, with current season's shoots, were selected and marked. In these segments the phenology of catkins and glomeruli ("Tonda di Giffoni"), or only catkins in the case of pollinizers, was studied (2).

The occurrence of phenological stages was compared with accumulated growing degree days (GDD) and chilling hours (CH) records. Climatic records were obtained from nearby meteorological stations belonging to the Chilean national agroclimatic network (www.agromet.cl).

For the calculation of GDD, the thermal average method (average daily temperature-base temperature) was utilized.

$$GGD = \sum_{i=1}^{n} (Ta - Tb) \qquad (1)$$

where:

n = number of days from May 1 (biofix) to the date of occurrence of each phenological stage.

Ta = average daily temperature and *Tb* is the base temperature (10°C). The Weinberger method (14, 32), still in frequent use (17), was used to estimate the accumulation of CH.

$$CH = \sum_{i=1}^{n} H;\ if\ 0°C\langle T\langle 7°c, 1\ is\ added, ifnot, 0 \qquad (2)$$

where:

H = number of hours in which temperature (*T*) is between 0°C and 7°C, from May 1 (biofix) and the date of occurrence of each phenological stage.

Fruit characteristics

The following evaluations were carried out in the same "Tonda di Giffoni" marked trees and shoot segments utilized in the phenological monitoring.

Fruit setting

Fruit setting percentage was calculated based on the total number of fruits related to the previously counted female flowers for each shoot segment.

Fruit weight and diameter and seed yield

Seed yield (%) = (Seed weight/fruit weight)*100 (3)

Data analysis

Floral phenograms and graphs comparing accumulated GDD and CH among localities were built to describe phenological stages and its relationships with these temperature parameters. Pearson correlations and linear regression analyses were conducted for thermal accumulation and the occurrence of phenological events. Analyses of variances (ANOVA) were conducted to examine variation among study sites for all productive parameters (percentage of fruit set, diameter and weight of fruits and seeds). Means were compared using Tukey's multiple comparison test ($P<0.05$). All statistical analyses were carried out using the statistical software program SPSS 15.0© Inc 2006.

Results

Floral phenology

Differences in phenological stages for hazelnut cv. "Tonda di Giffoni" across study sites are shown in figures 1 and 2 (page 60) to figures 3 and 4 (page 61).

During the seson 2011-2012, male and female bloom occurred from June to August, with a span varying between two and two and a half months according to the main cultivar, pollinizers and study sites (figure 1, page 60). "Tonda di Giffoni" presented a marked protandrous dichogamy in the three study sites. Male flowering began during the first days of June and lasted on average 64 days and male full blooming (more than 50% of the catkins releasing pollen), lasted 43 days in Los Niches and San Rafael and 49 days in Camarico. Female flowering (pistillated flowers) began the second and third week of June (up to two weeks later than the staminate) and lasted for 59 days. "Tonda Romana", "Tonda Gentile delle Langhe" and "Barcelona", flowering lasted on average 71 days, with "Tonda Romana" being 10 days shorter than "Tonda Gentile delle Langhe" and "Barcelona". Pollen release occurred at the three sites while "Tonda di Giffoni" stigmas were receptive (pollen synchrony), although there were differences for full female flower coverage. "Tonda Gentile delle Langhe" and "Barcelona" completely covered "Tonda di Giffoni", however, "Tonda Romana" did not release pollen the first week of "Tonda di Giffoni" full blooming. The evolution of the phenological stages varied across study sites (figures 1 and 2, page 60; to figures 3 and 4, page 61).

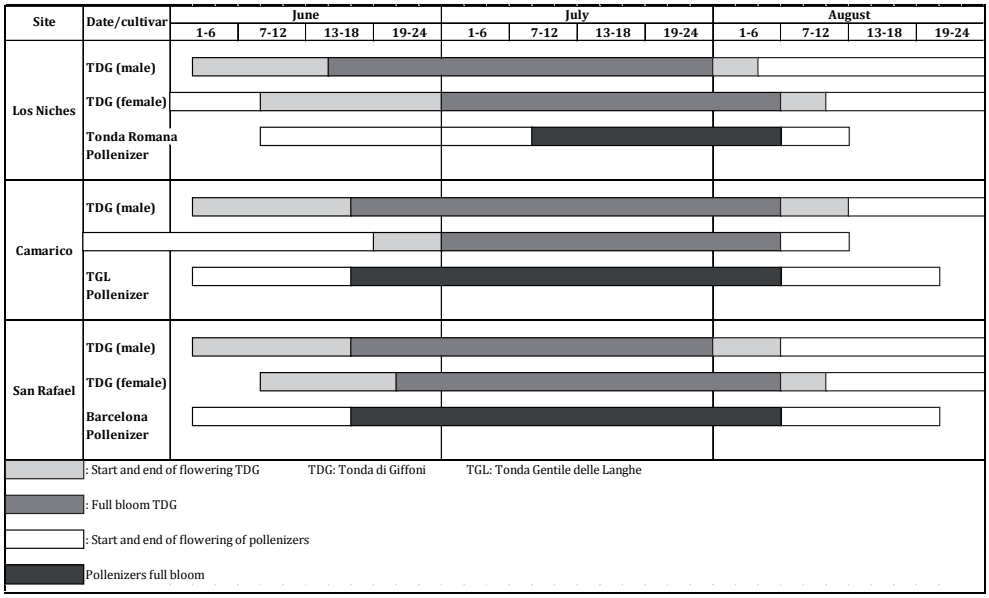


Figure 1. Floral phenology for hazelnut (*Corylus avellana* L.) cv. "Tonda di Giffoni" and their pollenizers at study sites.

Figura 1. Fenología floral para avellano europeo (*Corylus avellana* L.) cv. "Tonda di Giffoni" y sus polinizantes en los sitios de estudio.

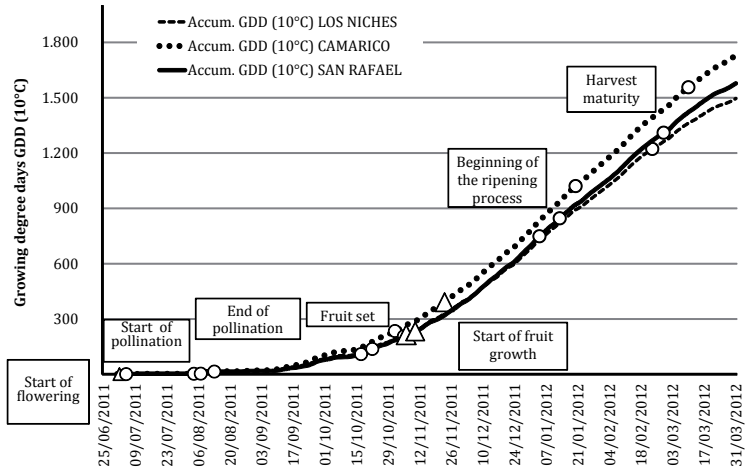


Figure 2. Floral phenology of Hazelnut (*Corylus avellana* L.) cv. "Tonda di Giffoni" according to accumulated growing degree days (GDD) at study sites.

Figura 2. Fenología floral de avellano europeo (*Corylus avellana* L.) cv. "Tonda di Giffoni" de acuerdo con la acumulación de días grado (DG) en los sitios de estudio.

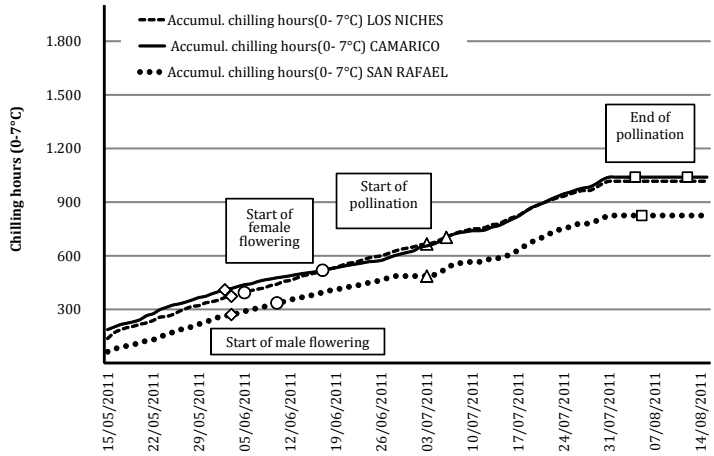


Figure 3. Floral phenology of hazelnut (*Corylus avellana* L.) cv. "Tonda di Giffoni" at study sites according to accumulated chilling hours.

Figura 3. Fenología floral de avellano europeo (*Corylus avellana* L.) cv. "Tonda di Giffoni" de acuerdo con la acumulación de horas frío en los sitios de estudio.

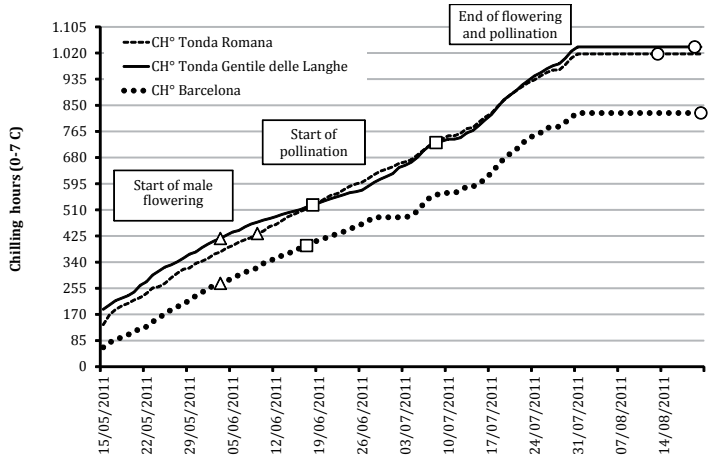


Figure 4. Floral phenology of hazelnut (*Corylus avellana* L.) pollinizers at study sites

Figura 4. Fenología floral de los polinizantes de avellano europeo (*Corylus avellana* L.) en los sitios de estudio.

Los Niches proved to be the earliest site, ahead for most phenological stages harvesting when compared to other productive areas. This site was followed very closely by San Rafael and finally by Camarico, which showed the latest harvest.

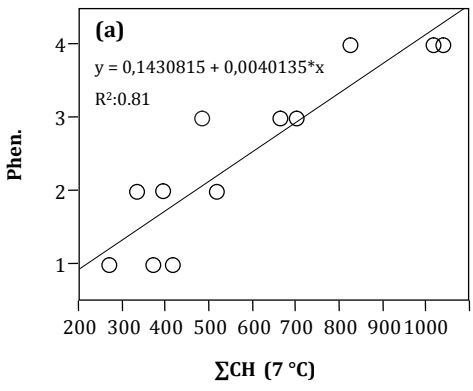
Correlation of phenological stages and accumulation of growing degree days and chilling hours

The occurrence of phenological stages during the season 2011-2012 was positively and significantly correlated ($P<0.05$) with CH and GDD accumulation, with coefficients of determination (R^2) of 0.81 and 0.82, respectively (figure 5).

However, important differences between study sites were observed. This is especially evident in the case of Camarico, where in spite of a greater accumulation of CH and GDD, the occurrence of phenological stages was clearly delayed when compared to Los Niches and San Rafael (figures 1 and 2, page 60; to figure 3 and 4, page 61).

Chilling hours

"Tonda Di Giffoni" male bloom begun at same time (3.6.11) at the three study sites, up to two weeks earlier than female flowers (protandrous-type dichogamy), with an accumulation of chilling hours that varied between 271 and 417 CH.



1:Start of male flowering, 2: Start of female flowering, 3: Start of pollination, 4: End of pollination

1: Inicio de floración masculina, 2: Inicio de floración femenina, 3: Inicio de polinización, 4: Fin de polinización

3: Start of pollination, 4: End of pollination, 5: Fruit set, 6: Start of fruit growth, 7: Beginning of the ripening process, 8: Harvest maturity

3: Inicio de la polinización, 4: Fin de la polinización, 5: Cuaja de frutos, 6: Inicio del crecimiento de frutos, 7: Inicio del proceson de maduración, 8: Madurez de cosecha

Figure 5. Linear adjustment for the relationship between phenological development (Phen.), accumulated degree days (∑DG) (a) and chilling hours (∑HF) (b) for hazelnut cv. "Tonda di Giffoni" and their pollinizers at study sites (2011-2012).

Figura 5. Ajuste lineal para la relación entre desarrollo fenológico, acumulación de días grado (∑DG) (a) y horas frío (∑HF) (b) para avellano europeo cv. "Tonda di Giffoni" y sus polinizantes en los sitios de estudio (2011-2012).

The earliest onset of female flowering occurred in Los Niches (5.6.11), with an accumulation of 394 CH and the latest in Camarico (17.6.11), with an accumulation of 519 CH. When "Tonda Di Giffoni" floral phenological stages are compared with the accumulation of chilling hours for each locality (figure 3, page 61), it can be observed that in Los Niches, "Tonda Di Giffoni" accumulated 1,017 CH, in Camarico, 1,040 CH, and in San Rafael, 825 CH. In the case of the pollinizers (figure 4, page 61), "Tonda Romana" (Los Niches) accumulated 433 CH at the beginning of male bloom, 733 CH at the beginning of pollination, and 1,017 CH at the end of flowering and pollination. "Tonda Gentile delle Langhe" (Camarico) accumulated 417 CH at the beginning of male bloom, 526 CH at the beginning of pollination and 1,040 CH at the end of flowering. "Barcelona" (San Rafael) accumulated 271 CH at the beginning of flowering, 394 CH at the beginning of pollination and 825 CH at the end of flowering. Thus, the highest accumulation of CH occurred for "Tonda Romana" and "Tonda Gentile delle Langhe", while "Barcelona" showed the lowest.

Growing degree days

Camarico produced the highest values for each phenological stage, followed by San Rafael and then Los Niches. The accumulation of growing degree days was 1556.9 GDD in Camarico, 1310.5 GDD in San Rafael and 1229.2 GDD for Los Niches. In Camarico, phenological stages showed, in general, two weeks of delay compared to Los Niches and San Rafael. Fruit set occurred with an accumulation range of 115 and 239 GDD. The beginning of fruit growth ranged between 205.7 and 374.1 GDD, the beginning of maturity between 740.6 and 1028.8 GDD, and harvest maturity between 1229.2 and 1556.9 GDD.

Productive parameters

From all the evaluated parameters, only fruit setting and fruit equatorial diameter were significantly different among studies sites (table 2).

Los Niches and San Rafael showed statistically similar percentages of fruit setting (82.2% *vs.* 74.7%, respectively). In contrast, Camarico, produced the lowest fruit setting (34.1%) and equatorial diameter (16.6 mm), compared to Los Niches (82.2%, 7.3 mm) and San Rafael (74.7%, 17.4 mm).

Table2. Productive parameters for hazelnut *cv.* "Tonda di Giffoni" at study sites.

Tabla 2. Parámetros productivos para avellano europeo *cv.* "Tonda di Giffoni" en los sitios de estudio.

Location	Fruit setting (%)	Fruit ecuatorial diameter (mm)	Fruit weigh (with peel) (g)	Seed weight (g)	Seed yield (%)
Los Niches	82.2 b	17.3 b	3.1 a	1.4 a	44.3 a
Camarico	34.1 a	16.6 a	2.9 a	1.3 a	45.0 a
San Rafael	74.7 b	17.4 b	3.1 a	1.4 a	43.8 a

* Different letters for each column indicate statistical differences according to Tukey ($p < 0.05$).

* Diferencias en las letras para cada columna indicant diferencias significativas según Tukey ($p < 0,05$).

Discussion

The strong protandric dichogamy showed by "Tonda di Giffoni" at the three study locations coincides with previous reports concerning this cultivar, which generally displays varying degrees of protandric dichogamy across seasons, mostly as a response to fall and winter temperatures (27, 29). Capik and Molnar (2014) found that European hazelnuts behave as protandrous or protogynous depending on their genetic makeup and the regional climate conditions where they are grown (15, 17). In regions with Mediterranean-type climates, such as those like the present study, the protandric dichogamy tends to be more common, while protogynous tends to predominate in areas with cold and long winters (20, 21, 26).

Grau (2014) in a study conducted in the province of Ñuble (Chile), observed that only 60% of hazelnut varieties were protandrous, while the rest was protogynous. In addition, "Tonda di Giffoni" evaluations conducted in the Chilean Bio-Bio region reported that male flowering occurred between the end of May and July, while female flowering was observed between the middle of June 15 the end of and July (1). Thus, pollen availability to fully cover "Tonda di Giffoni" female flowering becomes a limiting factor, especially when using a single pollinator, a practice commonly used in Chile (ranging between 5% and 11%).

The risk of limited pollen synchrony even increases when considering seasonal climate variations, given the direct effect of climatic variables, such as temperature, humidity, rainfall, and wind, on floral staminated and glomeruli behavior. Undoubtedly, an inefficient coverage of feminine flowers will affect fruit setting and therefore fruit yields. Thus, two or more pollinators are recommended per main cultivar, and has become and standard practice among the main hazelnut-producing countries (16). Hazelnut trees tolerate extreme cold, including temperatures as low as -15°C, being CH requirements an adaptive condition for preventing development when flowers or leaves are likely to be damaged by frost (7). Estimated CH accumulations observed in this study (between 825 and 1040 CH) is in agreement with previously reported values for "Tonda di Giffoni" (7). Differences observed for male and female flowers CH accumulation were also described in previous studies (4), with staminated flowers typically showing lower CH requirements compared to glomeruli flowers to break fall dormancy. Positive and significant correlations ($P < 0.05$) between phenological stage dates and CH ($R^2=0.81$) and GDD ($R^2=0.82$) accumulations points out a strong relationship between these variables, in agreement with Črepinšek *et al.* (2012). Even though temperature accumulation (CH and GDD)/phenological evolution trend was similar in all study sites, Camarico showed higher CH and GDD accumulations, but not an earlier phenological development compared to the other two study sites. This behavior has been observed in previous studies (18, 33). Differences among sites, in terms of phenological evolution and CH and GDD accumulations, have been explained by factors, such as relative humidity, photoperiod, solar radiation, cultivar genetics, cropping and soil types (18, 33).

Pollinizers are essential to ensure pollen availability, fruit setting and yield. However, there must exist synchrony between

pollinizers and main cultivar flowering windows. "Tonda Gentile delle Langhe" showed the earliest flowering and pollen release when compared to the rest of the evaluated pollinizers. This fast flowering behavior has been reported previously (12), and it has been explained by a lower CH requirement. Phenological variation for "Tonda di Giffoni" and its pollinizers at different study sites agrees with previous findings, where synchronization between glomeruli opening and pollinizer's pollen release has been associated to genetic and cultural factors, photoperiod and solar radiation, among others (18, 22, 28, 33).

Differences for fruit setting and diameter depended on the pollen source and agrees with descriptions by various authors (11, 25), who found that several fruit quality and yield traits, such as nut weight, husk/fruit ratio, fruit diameter, etc., were affected by the pollen source. "Tonda Gentile delle Langhe" showed the earliest flowering and was the least CH demanding pollinizer, in agreement with previous reports (12). The phenological variation observed for "Tonda di Giffoni" and its pollinizers at the three study sites is in accordance with similar studies (18, 22, 28, 33), which also reported that the synchronization between glomeruli aperture and pollen release depended on genetic and cropping factors, photoperiod, and solar radiation among others.

Conclusions

During the season 2011-2012 "Tonda di Giffoni" male and female flowering occured during June through August, with flowering spans ranging from two to two and a half months, depending on the specific location and pollinizers, showing a clear dichogamous behavior (protandric).

The studied pollinizers displayed differences on pollen synchrony. In general, the beginning of phenological stages were significant and positively correlated ($P<0.05$) with CH and GDD accumulation. Differences in CH and GDD accumulations were detected across study sites. Camarico showed the greater CH and GDD accumulations but the most delayed phenology compared to Los Niches and San Rafael. Fruit setting and diameter were significantly influenced by the pollinizer. Results generated by this study may aid the decision-making when selecting cropping areas for hazelnut "Tonda di Giffoni" in similar climates. An adequate selection of type, number and proportion of pollinizers under specific CH and GDD accumulation conditions may significantly increase yields and fruit quality.

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Seed oil content and composition of *Jatropha curcas* (L.) and grafted *Jatropha curcas* (L.) on *Jatropha cinerea* (Ortega) Muell. Arg. rootstock

Composición y contenido de aceite en semillas de *Jatropha curcas* (L.) y *Jatropha curcas* (L.) injertada en porta injertos de *Jatropha cinerea* (Ortega) Muell. Arg.

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Abstract

Jatropha curcas has been investigated for its high content of oil, its moderate salinity and drought tolerance, and *Jatropha cinerea* is a species that can withstand long drought periods and tolerate salinity up to 100 mM of NaCl. The aim of this study was to graft *J. curcas* plants on *J. cinerea* and grow them in experimental semiarid conditions, different soil and climate conditions from those of *J. curcas* native area to analyze their effects on oil seed composition and content. The survival of grafted *J. curcas* on *J. cinerea* rootstock was 95%. Seeds from grafted and non-grafted plants were analyzed to determine their oil content. The grafted plants showed greater height (150.7 cm) and oil content (51.3%) than the non-grafted plants (123.5 cm and 49.2%, respectively) without affecting their fatty acid composition. The meteorological information of the experimental plot (Baja California Sur, Mexico) showed values below those necessary for good phenological development; nonetheless, the graft improved its characteristics. Therefore, the use of grafted plants is an option for the establishment of *J. curcas* plantations in other parts of the world with different soil and climate conditions than those where they grow in the wild.

Keywords
Jatropha • fatty acids • grafts • rootstock • climate

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Resumen

Jatropha curcas ha sido investigada como fuente de aceite y es moderadamente tolerante a la sequía y salinidad; *Jatropha cinerea* es una especie que puede resistir largos periodos de sequía y tolerar salinidades hasta 100 mM de NaCl. El objetivo de este estudio fue injertar plantas de *J. curcas* sobre *J. cinerea* y cultivarlas en una parcela experimental en condiciones semi-áridas, condiciones de suelo y clima diferentes del área nativa de *J. curcas*, para analizar los efectos en la composición y contenido de aceite de las semillas. La sobrevivencia de *J. curcas* injertadas sobre el porta injerto de *J. cinerea* fue de 95%. Las plantas injertadas presentaron mayor altura (150,7 cm) y contenido de aceite (51,3%) que las plantas no injertadas (123,5 cm altura y 49,2% aceite) sin afectar la composición de los ácidos grasos. La información meteorológica de la parcela experimental (Baja California Sur, México) mostró valores por debajo de los necesarios para un buen desarrollo fenológico de *J. curcas*; sin embargo, el injerto mejoró las características de la planta. Por lo tanto, el uso de plantas injertadas es una opción efectiva para el establecimiento de plantaciones de *J. curcas* en condiciones de suelo y clima diferentes a aquellos donde se desarrollan en condiciones silvestres.

Palabras clave
Jatropha • ácidos grasos • injertos • portainjerto • clima

Introduction

The genus *Jatropha* comprises approximately 170 to 175 known species, of which 45 are found in Mexico (18), including *J. curcas* that has been investigated in depth as source of raw material for biodiesel production since it produces oil-rich seeds (27 to 40%) (6, 10); its fatty acid composition is mainly linoleic (28.9-47.5%) and oleic (31.7-47.1%) acids (22). *Jatropha curcas* grows in a wide range of climate conditions (annual precipitation from 300 to 3000 mm) with tolerance to high temperatures and a preference for deep and well-drained soils (21) with moderate tolerance to salinity up to 50 mM NaCl (9).

Jatropha cinerea is distributed in wild populations of northwestern Mexico; it is a shrub from 1-3 m in height that can withstand long periods of drought and tolerate up to 100 mM of NaCl (11). Studies have reported medicinal and industrial applications (16, 22, 23). Its latex is useful for healing all types of wounds and burns; it is used as a mordant (color fixative) and also as astringent and a remedy to remove warts (12).

The biological distribution of both species is found in distinct ecological zones. Behera *et al.* (2010) indicated that it was important to know the biological distribution of the species because of its wide variation in oil content. Zamarripa and Díaz (2008) reported an area in Mexico with high and medium potential for the establishment of *J. curcas* in the state of Sinaloa, which had the largest area (557641 ha) for this crop in contrast to the state of Baja California Sur where areas with productive potential had not been detected.

Jatropha curcas and *J. cinerea* are mainly propagated by seeds, cuttings, *in vitro* culture and grafting (7, 8). Soto-Landeros *et al.* (2016) mentioned that the use of rootstocks could be used as

an alternative to reduce water and saline stress of the plants cultivated in semi-arid zones and low precipitation. Both the climate of a site where *J. curcas* develops in the wild and that of the experimental field of our research facilities were compared to be able to have a reference of the development of the plants in semi-arid conditions. Therefore, the objective of this study was to graft *J. curcas* plants on *J. cinerea* and grow them in experimental semiarid conditions, different soil and climate conditions from those of *J. curcas* native area to analyze their effects on oil seed composition and content.

Materials and methods

Plant material

Seeds of *J. curcas* from Sinaloa, Mexico were collected from an experimental cultivation field in Estación Dimas (23°46' 35.4" N, 106°46'48.3" W; 42 m a. s. l.) in October 2013 and *Jatropha cinerea* seeds from wild plants located in the town of San Antonio de la Sierra, B. C. S., Mexico (23°45'06.2" N, 110°07'03.5" W; 452 m a. s. l.) in November 2013.

Seed germination

The experiment was performed in the Laboratory of Plant Biotechnology at Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, BCS, Mexico. The seeds of both species were placed in sterile paper wetted with sterile water for their germination and incubated in a growth chamber at 25 ± 2°C under dark conditions in March 2014. Once the seeds germinated, they were sowed in 250 mL polyethylene cups, perforated on the base and filled with plant transplant growing mix Sphagnum moss Sogemix® (Quakertown Canada).

When seedlings developed, they were placed in a greenhouse under sunlight and temperature of 28 ± 7°C.

Grafting method

Thirty-day old seedlings of both species with similar diameter and stem height were selected; *J. cinerea* seedlings were cut in a "V" shape under the cotyledons to be used as rootstocks, discarding the aerial part to avoid growth of the axillary buds found in the knot of the cotyledon leaves. On the other hand, *J. curcas* (graft) plants were cut in an arrow shape above the cotyledons avoiding the bud union, in such a way that it joined the rootstock, discarding the root (7). The grafting parts were joint perfectly and covered with Parafilm® (American National Can, CT, U.S.A.) tape to hold them together and prevent air entrance.

The seedlings recently grafted (50 plants) were placed in a greenhouse at a temperature of 28 ± 7°C, relative humidity from 70 to 80% and sunlight. Three-month grafted seedlings were transferred to 5 L pots with substrate (Sogemix®, Quakertown, CAN) and perlite (Perlita Group, Torreón, Coahuila, MX) (75% and 25%, V / V, respectively).

Growing conditions

After three months, grafted and non-grafted plants were sown on an experimental plot (CIBNOR) (24°08' 05.0" N, 110°25' 31.0" W; 6 m a. s. l.) at a distance of 2 m between rows and 2 m between plants (12 plants, 8 grafted and 4 non-grafted). Observations were made one month later; the experimental parameters were plant growth, height and stem diameter above the graft union. After four months, the plants were pruned at 1 m in height to homogenize them. Later, the relationships of soil characteristics and seed fatty acid content were evaluated.

The soil analyses consisted on evaluating pH, electrical conductivity, organic matter and texture. Each plant was fertilized with 50 g NPK formula (17-17-17) and watered once a week.

Seed characteristics and oil content

Seeds were harvested from grafted and non-grafted plants three months after planting. Their characteristics, such as number of seeds per plant, mass, length and diameter were assessed. Then, they were sent to Centro de Investigación en Alimentación y Desarrollo (CIAD), Culiacán, Sinaloa, Mexico for oil content and composition analyses. Each of the 25 seeds per plant of each type were measured in length and diameter with a Truper® digital Vernier (Mexico City, MX). Weight was determined using an analytical balance with an accuracy of ± 0.2 mg. The seeds were cracked open using tweezers; the seed coat was carefully removed, and the kernels were stored in a desiccator prior to sample preparation. Kernels, subsequently tarred and milled, were weighed (5 g) in a porcelain crucible. Moisture content of the samples was determined by oven drying to a constant weight. A 2 g sample was placed in a pre-weighed extraction thimble and put in the Soxhlet system for 16 h using hexane. The solvent was distilled under vacuum rotary evaporation (30°C) (BÜCHI, 816HE, BÜCHI Labortechnik AG, Flawil, CH).

The thimble was placed in an oven (Yamato DKN602C, Tokyo, JP) at 103 ± 2°C for 24 h, using method 920.39, A.O.A.C. (1) to remove residual solvent and then weighed on an analytical balance (Sartorius AX124, Göttingen, DE). The result was expressed as the percentage of oil in dry matter. The oil was stored at 5°C in amber bottles for further analysis of fatty acid composition.

Oil composition

Fatty acid composition was determined by gas chromatography (Agilent, 7820, Santa Clara, CA). Oil methylation was performed using method 920.33 A.O.A.C. (1). The oil samples were analyzed using a 30 m x 0.32 mm x 0.2 µm capillary column (Omega wax 320, Sigma-Aldrich Corp., St. Louis MO, U.S.A). The injector temperature was 260°C. The carrier gas was helium, which was maintained at a constant flow of 1 mL/min for 40 min. The analysis was performed in triplicate using a commercial sample (Supelco-Sigma-Aldrich, Bellefonte, PA, U.S.A.) with 37 fatty acids (19). The percentage of individual fatty acids was calculated by comparing the peak areas with the commercial standard and expressed as the total proportion of fatty acids in each lipid fraction (2). The ANOVA statistical test and Tukey's mean comparison test were performed with MINITAB 17 software.

Weather information

Daily environmental conditions (precipitation, relative humidity and temperature) were recorded in an automated Davis Vantage Pro 2 Plus (Davis Instruments, CA, U.S.A.) located at CIBNOR experimental plot, CIBNOR, La Paz, BCS, Mexico (24°08'08.6" N, 110°25'40.1" W 6 m a. s. l.). The meteorological data of the cultivation site located in Estación Dimas, Sinaloa, Mexico were obtained from an automated climate station Adcon Telemetry® (Vienna, AT), located 5 km from where wild *J. curcas* grows (23°44' 01" N, 106°49'11" W; 4 m a. s. l.).

Results

Seed germination

At third day, germination of *J. curcas* seeds were 40% and *J. cinerea* seeds were 10%. The rest of the germination for both species happened heterogeneously, obtaining 60% for *J. curcas* with a total of 120 plants and 25% for *J. cinerea* with a total of 50 plants in a period of 12 days. Seedling emergence was 7 to 10 days after sowing.

Grafts

A survival of 95% of grafted plants was obtained after 25 days. Callus formation was observed in the graft union area when the parafilm (Sigma-Aldrich, St. Louis, MO, U.S.A.) was removed increasing stem diameter. The stems of the grafted plants showed the integration of *J. curcas* tissues with *J. cinerea* rootstock and developed as a single plant.

Crop development

Table 1 shows the parameters recorded in non-grafted and grafted *J. curcas* plants after a 10-month period, displaying significant differences ($p > 0.05$) between the height of the two types of plants, 26 cm in average; in terms of stem diameter, no significant difference ($p \leq 0.05$) was found between grafting and non-grafting. The yield of the number of seeds was significantly different ($p \leq 0.05$), which was higher in grafted plants with an average increase of 36 seeds per plant. Seeds harvested from grafted and non-grafted plants showed significant differences among weights. Comparing the weight of the seeds developed in Estación Dimas (0.63 ± 0.3) (3) and those obtained in this study, the grafted plants had a similar weight (0.56 ± 0.13). However, in terms of length and diameter, no statistical difference was observed. The differences between plant height, weight and number of seeds were due to the fact that *J. cinerea* rootstock benefited from *J. curcas* grafting because it is not a wild plant that grows in BCS, Mexico. In contrast, *J. cinerea* is adapted to semi-arid conditions and distributed naturally throughout northwestern Mexico.

Table 1. Results of *Jatropha curcas* grafted and non-grafted characteristics of plant and seeds.

Tabla 1. Resultados de las características de plantas y semillas de *Jatropha curcas* injertada y no injertada.

Parameters	*Jatropha* Non-grafted	*Jatropha* grafted
	Plants	
Height (cm)	123.5±8.96 [b]	150.7±18.63 [a]
Diameters (mm)	60.7±2.76 [a]	58.2±3.23 [a]
	Seeds	
Seeds for plant	142±19.2 [b]	178±23.5 [a]
Mass (g)	0.49±0.11 [b]	0.56±0.13 [a]
Length (mm)	16.7±0.93 [a]	18.3±0.73 [a]
Diameter (mm)	9.5±0.48 [a]	10.2±0.39 [a]

Value of the mean is ± SD. Different superscripts in the same line indicate significant differences among plant types (Test Tukey, $p < 0.05$).

El valor de la media es ± SD. Diferentes superíndices en la misma línea indican diferencias significativas entre los tipos de plantas (Prueba de Tukey, $p < 0,05$).

Soil analysis in BCS showed pH 7.5, alkaline soil, sandy-clay texture, organic matter of 0.4% and with respect to electrical conductivity; it is classified as slightly saline soil (14).

Weather information

Annual precipitation was 223 mm in the experimental plot (CIBNOR), distributed mainly from July to October whereas for the field at Estación Dimas where *J. curcas* grows in wild conditions, it occurred from June to October with an annual precipitation average of 700 mm. In the experimental plot (CIBNOR), relative humidity data showed an annual average of 62 ± 1.4% while at the field Estación Dimas it showed an annual average of 81 ± 2%. The average monthly maximum and minimum temperatures ranged from 25.5± 1.1°C to 38.95 ± 1.6°C and from 10.2 ± 1.6°C to 25 ± 1.4°C, in the experimental plot (CIBNOR) respectively, and average monthly maximum and minimum temperatures ranged from 26.4 ± 0.4°C to 33.2 ± 0.3°C and from 13.7 ± 0.7°C to 25.3°C, respectively in Estación Dimas. CIBNOR experimental field had lower precipitation, higher temperature and lower percentage of humidity than the field at Estación Dimas.

Oil content and composition

The oil content of grafted and non-grafted *J. curcas* plants was 51.3% and 49.2%, respectively with significant differences ($p \leq 0.05$). The highest amounts of fatty acids observed in grafted and non-grafted plants were linoleic and oleic acids with an average of 45.83% and 40.30%, respectively, and lower proportions of palmitic (12.20%) palmitoleic (0.56%), linolenic (0.34%) arachidonic (0.40%) and myristic (0.20%) acids. The saturated fatty acids (myristic, palmitic and arachidonic) represented approximately 12.70% of the total content. Significant differences ($p \leq 0.05$) were only observed in palmitic acid content with respect to non-grafted plants. Unsaturated fatty acids, (palmitoleic, oleic, and linolenic) constituted 87.29%. (table 2).

Table 2. Composition of fatty acids in oil obtained from seeds of *Jatropha curcas* harvested in an experimental plot (CIBNOR) in La Paz, Baja California Sur, Mexico.

Tabla 2. Composición de ácidos grasos en aceite obtenido de semillas de *Jatropha curcas* cosechadas en una parcela experimental (CIBNOR) en La Paz, Baja California Sur, México.

Fatty acids	Treatments	
	Non-grafted plants (%)	Grafted plants (%)
Myristic (C14:0)	0.21±0.05 [a]	0.20±0.04 [a]
Palmitic (C16:0)	12.72±0.44 [a]	11.49±0.36 [b]
Palmitoleic (C16:1n7c)	0.58±0.05 [a]	0.54±0.11 [a]
Oleic (C18:1n9c)	40.30±2.67 [a]	42.08±0.68 [a]
Linoleic (C18:2n6c)	45.83±2.36 [a]	44.67±0.40 [a]
Linolenic (C18:3n3a)	0.33±0.05 [a]	0.34±0.04 [a]
Arachidonic (C20:0)	0.32±0.04 [a]	0.49±0.45 [a]

Value of the mean is ± SD. Different superscripts in the same line indicate significant differences among plant types (Test Tukey, $p < 0.05$).

El valor de la media es ± SD. Diferentes superíndices en la misma línea indican diferencias significativas entre los tipos de plantas (Prueba de Tukey, $p < 0,05$).

Discussion

Hishida *et al.* (2013) observed that the beginning of *J. curcas* germination occurred on day three, reaching its maximum rate on day six compared with *J. cinerea* that began at day four with a maximum rate at day 10. Two factors could have affected this difference; the first one might have been due to the seed coat of *J. curcas*, which was thinner than that of *J. cinerea*; a large number of forest species seeds do not germinate because the hard seed coat prevents water ingression (physical latency), and the seed does not germinate unless the seed coat is scarified (15). Another factor that could have been affecting germination percentage was seed quality since the seeds of *J. curcas* were from cultivated plants obtaining a better physiological maturation. As mentioned by Budi *et al.* (2012), who studied the viability of *J. curcas* seeds in different maturity stages of plants grown in an experimental field, they found that the best stage for seed germination was physiological maturity (yellow fruit). For the same reason, because *J. cinerea* seeds came from wild plants, heterogeneous fruits and seeds were obtained.

About graft, Cholid *et al.* (2014) assessed grafting compatibility on *J. curcas* rootstock, following two methods, using lateral plating, joining a diagonal cut and slit-grafting cutting the rootstock and scion in "V" shape with a survival rate of 89.5 and 93.8% after two to three months, respectively, similar as the results. Soto-Landeros *et al*. (2016) have reported that *J. cinerea* rootstock accumulated a greater number of starch granules in its cells, which functioned as osmotic regulators preventing the plant from water deficit. In the results, this characteristic lead it to more biomass production reflected in plant height and seed production.

The seeds produced by grafted plants showed greater weight, which mean higher oil content in their germ. The rootstock favored development and prevented the plant from water deficit, affected by weather conditions (solar radiation, temperature, relative humidity, precipitation and wind speed). Araiza-Lizarde *et al.* (2015) mentioned that environmental conditions (temperature and wind speed) influenced *J. curcas* seed oil content but not its physic-chemical properties.

The fatty acid results obtained in this study were consistent with those reported by Araiza-Lizarde *et al*. (2015) where they recorded 44.1 ± 0.09% of oleic acid and 42.63 ± 1.06% of linoleic acid at Estación Dimas. They also mentioned that high temperatures influenced oil content. Sosa-Segura *et al.* (2014) evaluated oil yield and germ fatty acid composition of three species of *Jatropha* (*J. curcas*, *J. platyphylla* and *J. cinerea*). The results obtained for *J. curcas* showed higher oil content (61.5%) compared to those reported in this study, probably because climate conditions were more favorable since they were developed in a climate with heavier rainfall and higher relative humidity.

However, in the fatty acid profile analysis, the results were similar, mainly in palmitic, linoleic and oleic acids. The fatty acid profile showed that the oil from grafted and non-grafted *Jatropha* was dominated by unsaturated fatty acids (oleic, linoleic and linolenic acids) with a significant amount ($p \leq 0.05$) in palmitic and oleic acids. These data were consistent with those reported by Mazumdar *et al.* (2013), who studied the production of biodiesel from *J. curcas* seed oil and found that vegetable oil with a high content of unsaturated fatty acids was an alternative to replace fossil fuels since it met the requirements of ASTM (American Society for Testing and Materials) international standards.

Environmental conditions were important; *J. curcas* could survive with only 250 to 300 mm of annual rainfall; however, 600 mm were needed for flowering and fruit production (5). In this study, although weather conditions were not favorable, it was observed that grafted plants produced more seeds because *J. cinerea* rootstocks helped for seed production.

Climate factors (temperature, precipitation, relative humidity, etc.) had a significant effect ($p \leq 0.05$) on plant growth, distribution, productivity, seed yield and oil content. Rodrigues *et al*. (2016) evaluated the ability of high relative humidity, associated with the supply of K^+ to mitigate the harmful effects caused by saline stress on the physiological parameters of *J. curcas* plants and two different levels of relative humidity, low (40%) and high (80%); they concluded that the combined effects of high relative humidity and a supply of K^+ were able to improve growth, leaf gas exchange and ionic homeostasis of *J. curcas* plants. It is important to consider relative humidity because the experimental plot in this study had a lower humidity percentage than that in the plot where wild *J. curcas* grows.

The optimum temperature range is from 18 to 28°C. Higher temperatures can reduce yields. Some plants change sex in flowering (protandria) with few immature seeds at conditions of 40 ± 2°C during summer, so it is important to investigate in future studies if it happens in *J. curcas* (12). Wassner *et al.* (2016) studied the quality and composition of *J. curcas* oil under subtropical conditions and found that environmental conditions (precipitation, temperature and relative humidity) modified seed quality and oil composition during grain filling while its concentration was not affected.

Conclusion

The compatibility of *J. curcas* grafts on *J. cinerea* rootstocks was 95% of the total grafted plant survival, which ensured plant breeding and the possibility of establishing commercial plots. The grafted plants showed greater height (150.7 cm) and more oil content (51.3%) than the non-grafted plants, 123.5 cm and 49.2%, respectively, without affecting the composition of fatty acids in both cases. The grafting method was beneficial because increased plant height and seed weight, without affecting the fatty acid profile of the seed germ. Therefore, the use of grafted plants is a good option for the establishment of commercial plantations in low quality soils (from moderate to high salinity).

According to the climate data shown for the development of *J. curcas* under wild conditions, the use of grafting with *J. cinerea* rootstock improved the production and development of *J. curcas* under unfavorable conditions as less rainfall, relative humidity, and rainy season with higher temperatures. On the other hand, sowing grafted plants in Baja California Sur, Mexico or in semi-arid areas in other parts of the world with similar weather conditions offers great advantages especially because they are pest-free and despite the extreme dry summer conditions, the rest of the year is a temperate climate with the possibility of rain.

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The edaphic macrofauna in three components of the coffee plant arrangement associated with different management typologies, Antioquia, Colombia

La macrofauna edáfica en tres componentes del arreglo vegetal cafetero asociada con diferentes tipologías de manejo, Antioquia, Colombia

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Abstract

The balance and sustainability of coffee agroecosystems in southwestern Antioquia depend on the interactions and synergisms that take place above and below the ground. Within these, the functional groups of edaphic macrofauna constitute bio-indicators of soil quality. The present research on coffee systems evaluates the edaphic macrofauna in three components of the plant arrangement, under different management typologies. The study was carried out in the San Gregorio, La Soledad, La Clara and Egypt townships of Santa Rita in the municipality of Andes, Antioquia. The assessment of the edaphic macrofauna was carried out by random stratified sampling under coffee canopy, under banana canopy and in the furrow, in three zones of the slope of each productive system. A general linear model, multivariate techniques of Manovas and Biplot were used as statistical methods. The greatest interaction of the macrofauna groups was presented in the order of the systems: Transition II (Use of organic inputs)>Transition I (Rationalization of synthetic inputs)>Conventional (Use of chemical inputs), and by plant arrangement components in the order Banana>Coffee>Furrow.

Keywords
Agroecology • agroecosystems • biota • conventional • transition

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Resumen

El equilibrio y la sostenibilidad de los agroecosistemas cafeteros del suroeste antioqueño, dependen de las interacciones y sinergismos que se dan lugar por encima y debajo del suelo. Dentro de estas, los grupos funcionales de la macrofauna edáfica constituyen bioindicadores de la calidad del suelo. La presente investigación de los sistemas cafeteros evalúa la macrofauna edáfica en tres componentes del arreglo vegetal, bajo diferentes tipologías de manejo. El estudio se realizó en las veredas San Gregorio, La Soledad, La Clara y Egipto del corregimiento de Santa Rita en el municipio de Andes, Antioquia. Se realizó la evaluación de la macrofauna edáfica mediante un muestreo aleatorio estratificado debajo del dosel del café, debajo del dosel del plátano y en el surco, en tres zonas de la pendiente de cada sistema productivo. Se emplearon como métodos estadísticos un modelo lineal general, técnicas multivariadas de Manovas y Biplot. La mayor interacción de los grupos de la macrofauna se presentó en el orden de los sistemas: Transición II (Utilización de insumos orgánicos)>Transición I (Racionalización de insumos sintéticos)>Convencionales (Utilización de insumos químicos), y por componentes del arreglo vegetal en el orden Plátano>Café>Surco.

Palabras claves
Agroecología • agroecosistemas • biota • convencional • transición

Introduction

Today, the advance of modern agriculture has transformed the natural ecosystems of plants into simplified crop systems, characterized by a high degree of imbalance in the components of the agroecosystems (21).

In Colombia's coffee growing systems, the intensification of coffee production from a social, environmental, economic and technological point of view have a negative impact on the landscape, which in turn leads to the loss of biodiversity of microorganisms, plants and animals native to different regions of the country (20, 29).

In the municipality of Andes, Southwestern Antioquia, coffee growers and organizations in the sector use conventional systems as their main productive model. These systems use monocultures, heavy machinery, fertilizers and chemical pesticides. This model threatens the biodiversity of the territory and causes serious impacts such as; contamination of water sources (19), progressive degradation of the physical and chemical properties of the soil (27), exploitation of energy sources (24), breaking of complex biological trophic networks of the soil (7, 20, 28), resistance to pests, invasive diseases and weeds (5, 16, 25), intensification in the emission of greenhouse gases and greater vulnerability to climate change (13, 21, 29).

According to Altieri and Niholls (2008); Armbretch (2016) and Vázquez and Matienzo (2010) in order to curb the ecological deterioration that has been occurring in coffee systems, the solution is to increase interactions and positive ecological synergisms between the biotic functional groups above and below the ground.

The interactions of functional groups, ensure the integrity of the agroecosystems through various synergies and ecosystem services that occur between plant diversity

and the presence of multifunctional biological organisms. Services include: nutrient recycling, increased organic matter, stability of the physical and chemical properties of soil, biological control of pests and diseases. All these services together guarantee productive, sustainable, biodiverse and resilient systems (1, 14, 21).

In direct relation with the management of soil fertility, the trophic groups of the edaphic macrofauna are manifested. These are considered soil engineers (6, 16, 18, 21) as they modify agroecosystems through their action in the decomposition of organic matter and its influence on the cycle of carbon and other nutrients. They also improve porosity, permeability, infiltration and structural stability of soil aggregates (7, 8, 26).

Therefore, this research will make it possible to evaluate the functional groups of edaphic macrofauna in three components of the plant arrangement under different management systems for coffee production.

Materials and Methods

The study area was located in the Santa Rita district of Andes municipality, Antioquia. It is located southwest of the municipality, about 12.1km. It is located at 5°39'13" N and 75°3'37" W. It presents a humid tropical temperate climate and corresponds to the life zones of premontane humid forest. In general, the climatic conditions show an average temperature between 18°C and 24°C and an average annual rainfall between 1000 and 2000 mm (19).

The investigation was carried out in 13 coffee farms of the village, located on the path lane: San Gregorio, La Soledad, La Clara and Egypt, on an altitudinal strip between 1700 and 2000 meters above sea level. The soils of the study systems have been developed from metamorphic, igneous and sedimentary rocks with volcanic ash deposits (30). They have a texture of sandy and clayey loamy soils, in some cases with high mineral content and a strongly acidic pH. They are very deep, with an organic surface horizon-mineral of dark color and beneath it, another yellow or reddish brown color, good drainage, low moisture retention and a jagged appearance is manifested. Organic matter and nutrient content vary depending on the type of management. The types of soil present in the village are Typic Dystrudepts, Typic Fulvudands and Humic Dystrudepts. Also the inclusions Lithic Dystrudepts, Oxic Dystrudepts and Typic Eutrudepts are present (15).

The study systems belong to coffee producers with more than 20 years of experience under sun exposure or diversified shade, with three and four hectare farms and different management types (27) (table 1, page 81).

The experimental design of the research started with stratified random sampling of different functional groups of the edaphic macrofauna in three components of the plant arrangement, under three management typologies.

The sampling sites analyzed during the investigation were: I (Under coffee canopy), the coffee crops presents the same height and vegetative state and with the same topographical inclination; II (Under banana canopy), the banana crops are in the fruiting stage and are associated with coffee at a distance of one meter with 50 centimeters; III (Furrow between coffee and banana), the furrow has a width of 70 centimeters, the tillage is zero and no weeds were observed. The sampling was collected in the middle of the furrow.

Table 1. Characterization of management typologies coffee-growing systems.
Tabla 1. Caracterización de las tipologías de manejo de los sistemas cafeteros.

Characteristics	Management typologies		
	Conventional	Transition I	Transition II
Coffee farms	1, 2, 3, 4, 5 and 6	9, 10, 11, 12 and 13	7 and 8
Production systems	Farms of intensive production based on mono-culture and in excessive use high toxicity chemical products.	Farms in an initial phase of the conversion process toward agro-ecological systems. These systems assure the progressive elimination of chemical products through rationalization and with organic fertilizers.	Farms in a second phase of the conversion process toward agro-ecological systems. They only use organic products prepared with internal products of the system such as: vermin-culture, rustic compost, bocashi, mineral soups, among others, bio-fermented products based on manure and minerals.
Vegetal strate	The low stratum presents coffee plantation and invading weeds such as (*Cynodon dactylon* L.), (*Triumfetta semitriloba* L.), (*Cyperus rotundus* L.), (*Portulaca oleracea* L.), (*Rumex obtusifolius* L.), (*Digitaria sanguinalis* L.). In medium stratum, (*Manihot esculenta* Crantz.) and (*Musa* sp. L.) were crops observed in a dispersed way and without organization in the field as a basic food of the family.	The low stratum presents coffee plantation, invading weeds such as (*Cynodon dactylon* L.) and (*Eleusine indica* L.). In medium stratum (*Manihot esculenta* Crantz.), (*Zea mays* L.), and (*Musa* sp. L.) were crops found in association with the main crop. High stratum there were found fruit trees such as: (*Persea americana* Mill.) and (*Mangifera indica* L.).	The low stratum presents coffee plantation, not invading weeds, medicinal plants such as (*Cymbopogon citratus* Stapf.), (*Aloe vera* L.), (*Rosmarinus officinalis* L.), (*Salvia officinalis* L.), (*Ocimum basilicum* L.), and (*Capsicum annuum* L.). In medium stratum, there were crops observed such as (*Manihot esculenta* Crantz.), (*Zea mays* L.), (*Phaseolus vulgaris* L.), and (*Musa* sp. L.*).* In high stratum, the system showed fruit and wood trees, such as (*Persea Americana* Mill.), (*Mangifera indica* L.), (*Cordia alliodora* Oken.), (*Cedrela odorata* L.), and *(Inga edulis* Mart.).

The study of the edaphic macrofauna was carried out by coffee systems, through nine soil monoliths 25 x 25 x 30 cm deep, 20 meters apart, and distributed in three monoliths by components of the plant arrangement. In each system, the macroinvertebrates present were collected following the Methodology of the International Program "Biology and Fertility of Tropical Soil" or TSBF. Collected samples were kept in jars with 70% ethanol and 30% formaldehyde which were used to preserve the worms. Subsequently, the counting and separation of samples was carried out using a stereoscopic microscope, according to specifications of different extraction methods and taxonomic and functional identification of the macroinvertebrates (6, 7, 16).

For the interactions assessment, the general linear model (GLM) was used, where the control factors were the systems and the plant arrangements, the response variables were the detritivores, omnivores, herbivores and predators of the edaphic macrofauna. The variables were transformed based on the Box-Cox family, with the aim of establishing the optimal lambda, which would allow validating the statistical assumptions associated with the classification model. The Manova multivariate technique was also implemented, with canonical contrast of orthogonal type, in order to simultaneously evaluate all the variables under study. In addition, the Biplot technique and the descriptive process were applied in order to detect the joint relationships between all the variables studied simultaneously. The statistical packages used were SAS University Edition version 3.0.1, R and SPAD version 3.5.

Results and Discussion

Taxonomic and functional composition of the edaphic macrofauna.

The taxonomic composition of macrofauna present in the three sampling sites, showed that the macroinvertebrates are represented by three Phyla (Annelida, Mollusca and Arthropoda), seven classes (Clitellata, Insecta, Diplopoda, Chilopoda, Gastropoda, Malacostraca, Arachnida), six orders (Haplotaxida, Hymenoptera, Coleoptera, Isopoda, Dermaptera and Araneae) and seven families of different orders. The functional composition of the edaphic macrofauna by components of the plant arrangement produced four trophic groups: Detritivores, Predators, Omnivores and Herbivores (table 2).

Table 2. Taxonomic and functional composition of the edaphic macrofauna.
Tabla 2. Composición taxonómica y functional de la macrofauna edáfica.

Phyla	Common Name	Classes	Orders	Families	Functional Group
Annelida	Earthworms	Clitellata	Haplotaxida	Megascolecidae Glossoscolecidae	Detritivores
Mollusca	Slugs and Snails	Gastropoda	-	-	Detritivores
Arthropoda	Millipede	Diplopoda	-	-	Detritivores
	Pillbugs	Malacostraca	Isopoda	-	Detritivores
	Beetles	Insecta	Coleoptera	Carabidae Tenebrionidae Scarabaeidae Elateridae	Detritivores, Herbivores and Predators
	Earwigs	Insecta	Dermaptera	-	Detritivores
	Ants	Insecta	Hymenoptera	Formicidae Subfamilia: Myrmicinae	Omnivores
	Centipede	Chilopoda	-	-	Predators
	Spiders	Arachnida	Araneae	-	Predators

Statistical analysis of functional groups of edaphic macrofauna by sampling sites and management typologies

Statistical difference was detected for the furrow zone between Transition II system, compared to the Conventional and Transition I systems, for the amount of detritivores and omnivores present ($p<0.05$). For the other combinations of management systems and sampling sites, no significant differences were detected ($p>0.05$) (table 3, page 84).

The multivariate analysis of the Manova variance, which takes into account all the variables related to macrofauna in its comparison, made it possible to detect a statistical difference ($p<0.05$) between Transition II and Conventional systems. For coffee and banana crops, no difference was found between systems in the amount of detritivores, omnivores, herbivores and predators ($p>0.05$) (table 3, page 84).

The largest number of detritivores and omnivores in the furrow of the transition II system with respect to the other systems are due to the fact that these sites have an organic management of the plantations and present a high biodiversity of microorganisms, plants and animals that cover the entire surface of the soil. Functional edaphic groups under this typology find characteristics similar to a natural ecosystem, endowed with quantity, variety and quality of plant and animal food, as well as microhabitats and resources to establish nesting sites (5, 11, 12).

Overall results in conventional systems indicate high levels of disturbance. The simplicity of these systems affects the decomposition of organic matter and nutrient recycling, as well as increasing negative impacts such as soil erosion, gradual loss of organic matter and plant cover, generate high temperatures that alter the local microclimate.

Consequently, these characteristics diminish the sustainability of coffee and lower soil quality (9).

Omnivorous populations were represented by ants, a very diverse group of food habits that find favorable conditions for reproduction and development in open coffee systems, disturbed, with few plant species, as occurs in conventional systems, where only predominantly banana cultivation. These conditions stimulate the presence of larger generalist ant species and soldiers made up of large heads and thickened bodies (3).

Omnivores participate in many key ecosystem processes, such as soil improvement, nutrient circulation and regulation of populations of herbivorous insects and predators, which explains the low populations of these groups in all systems (25).

In Transition I and II systems, these insects had greater resources available for different microhabitats and nutrients, which explains why high omnivorous populations are represented equally in these systems (4), as well as detritivores in turn, find a greater source of food to carry out physiological processes in order to improve soil properties and guarantee the fertility of the soil (5, 17, 28).

Significant differences observed in the furrow zone between systems are due to the fact that the coffee systems in Transition II, present a greater vegetable cover made up of leguminous, medicinal and weed species, which maintains a favorable microclimate in the soil, with abundant food and shelter, for the establishment of populations of detritivores and omnivores, which decreases in the Transition I and Conventional systems (11).

Table 3. Analysis Anovas and Manovas of the edaphic macrofauna by sampling sites and management typologies.

Tabla 3. Análisis Anovas y Manovas de la macrofauna edáfica por sitios de muestreo y tipologías de manejo.

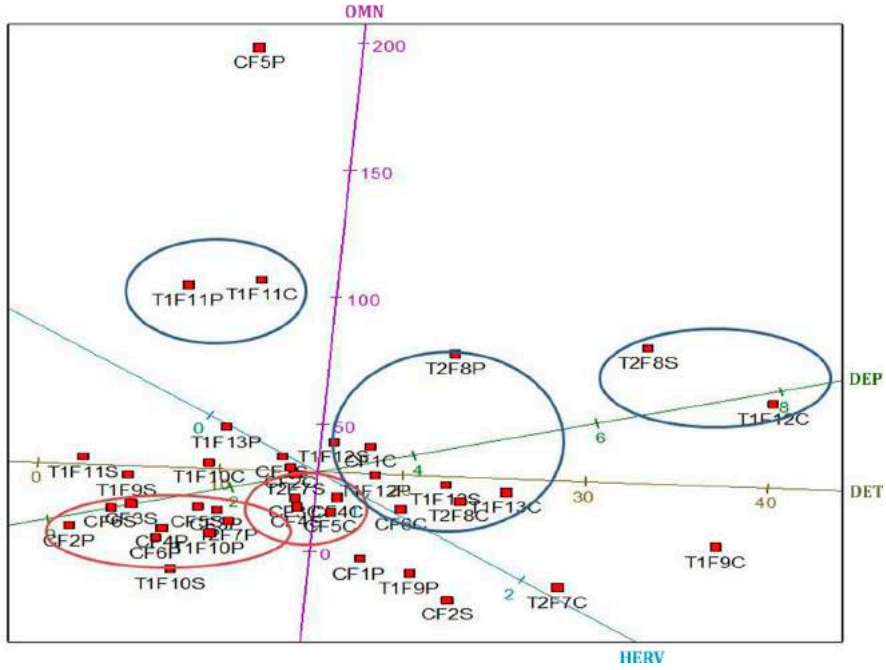
MANAGEMENT SYSTEMS	DETRITIVORES (#Ind)		
	MEDIA±STD	MAXIMUM	MINIMUM
Under coffee canopy			
CONVENTIONAL	19.0±15.2 a	39	1
TRANSITION I	24.0±13.8 a	38	9
TRANSITION II	29.0±2.1 a	30	27
Furrow			
CONVENTIONAL	6.0±2.6 b	9	2
TRANSITION I	10.0±9.3 b	26	3
TRANSITION II	30.0±9.0 a	36	23
Under banana canopy			
CONVENTIONAL	6.0±4.5 a	15	2
TRANSITION I	17.6±6.9 a	26	10
TRANSITION II	20.0±19.0 a	33	6
HERBIVORES (#Ind)			
Under coffee canopy			
CONVENTIONAL	1.0±0.8 a	2	0
TRANSITION I	1.0±1.8 a	4	0
TRANSITION II	2.0±2.8 a	4	0
Furrow			
CONVENTIONAL	1.0±1.6 a	4	0
TRANSITION I	1.0±0.8 a	2	0
TRANSITION II	1.0±0.7 a	2	0
Under banana canopy			
CONVENTIONAL	1.0±1.2 a	3	0
TRANSITION I	1.0±0.9 a	2	0
TRANSITION II	1.0±0.7 a	1	0
OMNIVORES (#Ind)			
Under coffee canopy			
CONVENTIONAL	19.0±17.5 a	47	1
TRANSITION I	47.0±40.4 a	109	2
TRANSITION II	12.0±9.9 a	19	5
Furrow			
CONVENTIONAL	12.0±8.5 b	26	0
TRANSITION I	30.0±21.3 b	59	0
TRANSITION II	53.0±38.1 a	80	26
Under banana canopy			
CONVENTIONAL	41.0±84.4 a	213	0
TRANSITION I	37.0±45.9 a	113	2
TRANSITION II	45.0±42.2 a	75	15
PREDATORS (#Ind)			
Under coffee canopy			
CONVENTIONAL	3.0±2.1 a	6	0
TRANSITION I	5.0±2.9 a	9	2
TRANSITION II	4.0±1.4 a	5	3
Furrow			
CONVENTIONAL	2.8±1.7 a	5	1
TRANSITION I	2.0±1.8 a	4	0
TRANSITION II	5.0±3.5 a	7	2
Under banana canopy			
CONVENTIONAL	2.0±1.5 a	3	0
TRANSITION I	2.0±1.2 a	4	1
TRANSITION II	3.0±1.4 a	4	2
COMPARISSON BETWEEN SAMPLING SITES			
	Coffee	**Furrow**	**Banana**
Wilks' Lambda	0.6203	0.0338	0.7419
Canonical	**No difference**	**Transition II =a conventional=b**	**No difference**

Equal letters indicate that there is no statistically significant difference ($p > 0.05$) and different letters indicate that there is statistically significant difference ($p < 0.05$).

Letras iguales indican que no hay diferencia estadística significativa ($p > 0,05$) y letras diferentes indican que hay diferencia estadística significativa ($p < 0,05$).

The Biplot analysis simultaneously depicted the components of plant arrangement, systems and the source of the systems (finches) in association with macrofauna groups (detritivores, omnivores, herbivores, predators) (figure 1). The results show a separatist tendency of the Transition I and II systems, with respect to the Conventional ones. As well as between the results of the furrow and the banana and coffee.

Once again, the trend that separates the composition of functional groups in Transition and Conventional systems highlights the effect of managing coffee agroecosystems, where in the first instance management is organic with agro-ecological principles that conserve the components of the agroecosystems and in the second case the systems use intensive management with inadequate practices that degrade soil properties and cause the loss of functional biodiversity (22, 23, 26).



Functional groups: DET - Detritivores, HER - Herbivores, OMN - Omnivores, DEP - Predators. The codes F1, F2, F3, F4, F5 and F6 are the Conventional system (C), F7, F8 are the Transition II system (T2) and finally F9, F10, F11, F12 and F13 are the Transition I system (T1).

Grupos funcionales: DET - Detritívoros, HER - Herbívoros, OMN - Omnívoros, DEP - Depredadores. Los indicativos F1, F2, F3, F4, F5 y F6 son los sistemas Convencionales (C), F7, F8 son los sistemas en Transición II (T2) y por último F9, F10, F11, F12 y F13 son los sistemas en Transición I (T1).

Figure 1. Biplot of functional groups of edaphic macrofauna by sampling sites and management typologies of coffee systems.

Figura 1. Biplot de los grupos funcionales de la macrofauna edáfica por sitios de muestreos y tipologías de manejo de sistemas cafeteros.

With regard to soil use, the Biplot analysis confirmed that there is a greater interaction of the soil communities in the coffee and banana systems, since these systems present crops of different sizes throughout the year, offering invertebrates plant cover, fresh food, low temperatures, shelter and favorable conditions for their development and reproduction, which is evidenced in the Transition I and II systems, while there is a positive effect on the growth and reproduction of the soil. These results have coincided with different scientific research (10, 24).

Conclusions

The functional composition of edaphic macrofauna under three components of the plant arrangement of coffee-growing systems was determined. In Conventional systems, the most abundant functional groups were predators, omnivores and herbivores; bioindicators of imbalance and disturbance of the soil, while in the Transition I and II systems the greatest abundance of detritivores was found; bioindicators of fertility and soil stability. The greater interrelationship of the functional groups of the edaphic macrofauna by management typologies was presented in the order Transition II>Transition I>Conventional, being the latter, the most disturbed systems and with low fertility, which makes them more prone to progressive degradation of soils, and by components of the plant arrangement in the order Banana>Coffee Crops>Furrows. From the agroecological approach, the functional groups of the edaphic macrofauna establish in the coffee systems interrelationships and key synergies for management above and within the soil, fundamental pillars in the restructuring and encouragement of processes between the components for agroecological conversion, towards sustainable and biodiverse systems. This research is a vital tool for agroecologists who wish to deepen in the efficiency of the biological processes that regulate the biodiversity and it is part of the agroecosystems complexity, under different management types in order to guarantee sustainability and resilience of coffee-growing systems or other agricultural sectors.

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La diversidad productiva y su influencia en los aportes orgánicos y la eficiencia energética, en sistemas extensivos del centro de Córdoba, Argentina

Productive diversity and its influence on the organic contributions and energy efficiency, in extensive systems of the Centre of Cordoba, Argentina

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Resumen

Los procesos de agriculturización-sojización y la retracción e intensificación ganadera en la provincia de Córdoba, redujeron la diversidad productiva de los sistemas agropecuarios, conduciendo a sistemas simples, frágiles, poco resilientes y muy subsidiados. La adopción del modelo de simplificación no es homogénea ya que algunos productores familiares introducen estrategias como una mayor diversidad de rubros, inclusión de la producción animal y manejos tecnológicos alternativos que afectan la circulación de materia orgánica y el flujo energético. Para analizar esta situación, se estudiaron 7 establecimientos de diferente diversidad productiva y trófica. Se registraron datos de uso del suelo y manejo tecnológico en un promedio de 3 campañas y se calcularon índices de diversidad espacial y temporal, aportes de materia orgánica y eficiencia energética. Los resultados indican que las diversificación productiva y la integración agrícola-ganadera pastoril tienen efectos favorables, especialmente en sistemas mixtos de cría, que optimizan la circulación de materia y flujos de energía. Los sistemas agrícolas presentan menor diversidad productiva, mayor eficiencia energética pero con un menor aporte de materia orgánica. En situaciones de intensificación ganadera se produce una disminución de la eficiencia energética y de los aportes orgánicos, afectando negativamente la sustentabilidad de los sistemas productivos.

Palabras clave
agrodiversidad • aportes orgánicos • eficiencia energética • intensificación ganadera

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Abstract

The processes of agriculturization-sojization and the retraction and intensification of livestock in the province of Córdoba, reduced the productive diversity of the farming systems, leading to simple, fragile, little resilient and highly subsidized systems. The adoption of the simplification model is not homogeneous and some family farmers introduce strategies such as a greater crops and pasture diversity, inclusion of animal production and alternative technological management that affect the circulation of organic matter and energy flow. To analyze this situation, 7 establishments of different productive and trophic diversity were studied. Land use and technological management data were recorded in an average of 3 campaigns and spatial and temporal diversity indexes, contributions of organic matter and energy efficiency were calculated. The results indicate that productive diversification and agricultural-pastoralist integration have favorable effects, especially in mixed breeding systems, that optimize the circulation of matter and energy flows. Agricultural systems have less productive diversity, greater energy efficiency but with a lower contribution of organic matter. In situations of livestock intensification there is a decrease in energy efficiency and organic contributions, negatively affecting the sustainability of production systems.

Keywords

agrodiversity • organic matter contributions • energy efficiency • livestock intensification

Introducción

Desde la década de los 90 se acentúa en Córdoba un fuerte proceso de agriculturización y sojización de la actividad agropecuaria, que incorpora a la actividad agrícola amplias superficies anteriormente ganaderas. La ganadería original se abandonó, relocalizó, o bien se concentró territorialmente adoptando una mayor intensificación (9, 18, 34). Estos procesos convergen en la disminución de la diversidad productiva de los sistemas, resintiendo algunos mecanismos homeostáticos emergentes de la diversidad biológica (36) como la regulación biótica, conservación de la fertilidad, minimización de pérdidas de agua y suelo y otros atributos sistémicos, dando lugar a sistemas simplificados, de mayor fragilidad frente a contingencias climáticas y biológicas y más exigentes en condiciones de fertilidad edáfica (25). Esto condujo a una incorporación creciente de insumos industriales y biotecnológicos, destinados a subsidiar a los procesos ecológicos mencionados aumentando los costos energéticos para mantener la funcionalidad y productividad de los sistemas (32). Esto significa un creciente costo energético de las intervenciones que no se compensa con el incremento de la productividad (3).

En la región central de Córdoba, los procesos de agriculturización, sojización y reconversión ganadera se asocian a los mismos procesos de degradación de suelo (erosión, agotamiento, deterioro de la estructura, contaminación y otros) señalados por Pengue (2009) para la región pampeana. Pero con el agravante de que la menor oferta de agua de la región

semiárida, reduce la productividad y los aportes de rastrojos, además de poseer condiciones meteorológicas más erosivas y los suelos de menor calidad y desarrollo. Si bien la sojización y la reducción e intensificación ganadera son tendencias generalizadas, es posible detectar, en algunos productores familiares, estrategias de "hibridación tecnológica" (27) ya que adoptan el modelo de simplificación e intensificación en forma parcial e introducen modificaciones de distinta índole (8). Estas estrategias se enfocan en objetivos más integrales y no solo en el logro de una mayor rentabilidad y están orientadas a una mayor diversificación productiva, integración agrícola-ganadera y manejos tecnológicos alternativos a la agricultura de altos insumos y monocultivo (24, 37). Los cambios introducidos afectan componentes estructurales del sistema y con ello, distintos procesos ligados a los ciclos de materiales, flujos de energía y regulaciones poblacionales, por lo tanto, modifican el metabolismo general del sistema y su capacidad productiva (17).

El ciclo de la materia orgánica está íntimamente ligado a la conservación y el mantenimiento de la productividad y la funcionalidad de los suelos. En los agroecosistemas simplificados el ciclo de la materia orgánica y de nutrientes involucra menos componentes bióticos, depósitos temporarios y circuitos de circulación (37) y considerables cantidades de elementos se pierden por exportación con la cosecha y como resultado de procesos de lixiviación y de erosión (20). Los sistemas con mayor complejidad, que incorporan el componente ganadero, tienen mayor diversidad productiva, mayores depósitos (vegetales, animales, reservas, suelo) y diferentes vías de circulación de materiales orgánicos e inorgánicos que reducen las pérdidas abióticas. Además, disminuyen la exportación de nutrientes, aseguran el aporte de materia orgánica desde distintos niveles tróficos, tienen mayor actividad biológica y mantienen altas tasas de reciclado de residuos vegetales y animales que favorece la fertilidad global (30).

El flujo de energía en un sistema productivo, medido a través de las entradas antrópicas y salidas productivas, permite cuantificar los subsidios de energía que se incorporan para alcanzar un determinado objetivo productivo. Los subsidios son intervenciones humanas dirigidas al medio físico y biológico para potenciar y direccionar la conversión de energía solar en productos. Los sistemas característicos del modelo dominante tienen un alto consumo energético por la incorporación de bienes, servicios e insumos externos, tanto en forma directa, por el uso de combustibles y derivados del petróleo, como en forma indirecta, por la energía implícita en la producción de agroquímicos, fertilizantes, maquinaria y semillas (15, 31). Esto significa un importante costo energético que afecta negativamente la eficiencia energética, expresada como la relación entre el producto obtenido y los subsidios entregados (salidas/entradas).

En este estudio se abordaron sistemas productivos familiares de la región central de Córdoba con diferentes estrategias de diversificación productiva -espacial y temporal- e integración agrícola-ganadera y se evaluó su efecto en la eficiencia energética y en el ciclado de la materia orgánica asociado a la conservación de la funcionalidad de los suelos.

Metodología

Los sistemas productivos seleccionados se localizan en la región central semiárida de Córdoba y representan diferentes grados de diversidad

productiva y complejidad trófica. Por su escala y características socioeconómicas corresponden a sistemas de agricultura familiar o según la tipología propuesta por Basco (1993), al tipo social agrario familiar capitalizado.

El estudio de estos sistemas se realizó aplicando la metodología de estudio de caso (39), y se incluyeron 3 sistemas agrícolas (SA1, SA2, SA3) y 4 sistemas mixtos: 2 con ganadería bovina de cría (SMC1, SMC2), 1 mixto de engorde (SME) y 1 mixto con tambo (SMT), de superficies entre 197 y 425 hectáreas. La información predial se recabó a través de entrevistas semiestructuradas y en profundidad (38), completando registros del uso de la tierra, del manejo tecnológico y de resultados productivos de cada rubro, considerando 2 a 3 campañas. Los croquis de los predios se dimensionaron con información satelital. Se efectuaron mediciones a campo con muestreos de biomasa productiva de las especies cultivadas y del aporte de biomasa de la vegetación espontánea y de restos orgánicos (kg de materia seca).

La diversidad productiva (diversidad vegetal de cultivos y pasturas presentes en el sistema) se calculó considerando la variedad y composición de rubros, la localización o distribución espacial de los mismos y las secuencias temporales (o uso de la tierra) en cada lote del sistema.

Para la primera de estas determinaciones, se utilizaron los índices clásicos de Riqueza, Equidad y Diversidad de Shannon (1, 20) calculados sobre la cantidad y/o proporción de los distintos cultivos y pasturas presentes en cada campaña. A la diversidad de Shannon se la denominó "estructural" ya que proporciona un valor numérico adimensional (de base logarítmica) que no tiene precisión espacial, ni temporal, excepto la de considerar las proporciones territoriales de cada componente. Para su cálculo, en una campaña (año agrícola) se tomó cada combinación de uso invernal y estival como una unidad específica anual. Así, por ejemplo, un lote con combinación de trigo-soja constituye una unidad diferente a un lote con combinación barbecho-soja Las pasturas perennes implantadas o naturales ocupan ambas estaciones. La proporción de superficies ocupadas por las diferentes unidades permiten calcular el índice.

Además, se calculó la diversidad espacial, definida por la localización o ubicación de las diferentes unidades en el espacio predial, determinando un "mosaico" de mayor o menor heterogeneidad. Se adaptó para ello, un índice propuesto por Boudry y Boudry-Burel (1982), donde cada unidad específica contribuye a la diversidad del mosaico según el número, tamaño, dispersión y perímetro de los lotes. En general, cuanto más fragmentado y distribuido esté ese mosaico, hay mayor diversidad espacial.

Un tercer aspecto de la diversidad productiva fue la diversidad temporal, que excede la campaña para aplicarse a todo el periodo analizado (2 a 3 campañas). Este índice considera las secuencias de uso (o historias de uso) en cada lote del territorio predial (31) y valora las secuencias con base en la presencia y alternancia de rubros vegetales a lo largo del tiempo. Emplea las mismas unidades de los índices anteriores, y la valoración se efectúa en función de la contribución a la diversidad biológica y al mejoramiento del medio biofísico. Los valores de cada lote se integran según su proporción territorial, para obtener el valor predial. Este índice valora la calidad de las rotaciones, ya que las repeticiones temporales (monocultivo) tienen un valor nulo comparado con las alternancias entre especies diferentes, particularmente si intervienen pasturas, por su mayor aporte a la diversidad y a las condiciones edáficas.

El ciclo de la materia de cada sistema productivo se analizó a partir de una modelización gráfica de su estructura trófica, identificando los distintos caminos o circuitos desde la producción de biomasa (componentes vegetales), su eventual almacenamiento (reservas forrajeras) y/o transformación (conversión) en la cadena trófica, hasta su salida como granos, carne o leche. Los valores anuales de componentes, depósitos, restos y salida de productos se cuantificaron en kilogramos de materia seca por hectárea y por año del sistema (kg ms/ha.año), exceptuando la biomasa animal y producción de carne que se expresó como kilogramos de peso vivo animal, por hectárea y año de sistema (kg PV/ha.año). En cuanto al circuito interno de los aportes de materia orgánica al suelo, se suman rastrojos, biomasa de malezas (controladas en barbechos), biomasa residual de pasturas, pérdidas vegetales durante el pastoreo, elaboración y consumo de reservas, además de los aportes por deyecciones animales (calculados según consumo y digestibilidad de cada alimento). Se calcularon estos aportes en función de proporciones, tasas y coeficientes obtenidos de diferentes fuentes bibliográficas (2, 4, 5, 10, 11, 12, 14, 16). La bibliografía sobre pérdidas de materia orgánica durante el pastoreo o elaboración de reservas, es bastante escasa y se debió recurrir a especialistas en la temática.

Según Morón (2001) la evaluación de aportes de materia orgánica es un indicador bastante integral por cuanto incide en la biodiversidad de los suelos y en los niveles de materia orgánica del recurso, regulando numerosas funciones edáficas. Este autor indica que la cantidad de aportes tiene, en sí mismo, mayor significación que la composición del material, frecuencia del aporte y sistema de manejo de suelo.

En el análisis energético se consideraron las entradas antrópicas de energía, el movimiento de la misma entre los distintos componentes estructurales del sistema y las salidas en forma de productos. Se cuantificaron las entradas de energía para cada rubro agrícola y cada actividad ganadera de los diferentes sistemas, de acuerdo con el método desarrollado por Pimentel *et al*. (1991), considerando el valor energético total de las distintas labores e insumos aplicados, utilizando los coeficientes energéticos correspondientes (13, 19, 29). La energía de salida se calculó empleando los equivalentes energéticos (Megajoules) de cada producto agropecuario destinado a la venta, por sistema y por año. A partir de estos resultados se calculó la eficiencia energética total del sistema productivo (energía producida/energía entregada). En relación con las entradas, se discriminó la energía involucrada en distintos procesos ecológicos, tales como la orientada a la regulación biótica (energía asociada al control de insectos, malezas y patógenos) y aquella dirigida a subsidiar la fertilidad del suelo (inoculación, fertilización, abonos). También la energía utilizada en labores (siembra, pulverizaciones, cosecha, elaboración de reservas y otros) y la ingresada en forma de semillas comerciales.

Para un análisis integral de las variables consideradas en los distintos tipos de sistemas productivos, se realizó un gráfico descriptivo multivariado (gráficos de ameba) donde se condensaron los valores de los distintos índices representativos de la diversidad, y del movimiento de la materia y energía. Esto se complementó con un análisis de componentes principales para analizar la interdependencia de variables medidas y determinar aquellas con mayor peso para explicar la variabilidad entre tipos de sistemas.

Resultados y discusión

Uso del suelo, productividad y aportes de materia orgánica

La principal estrategia determinante de la productividad vegetal de un sistema es el uso del suelo y en segundo lugar, la cadena trófica asociada a cada superficie, que define la conversión en producto animal. Los aportes al suelo (restos vegetales y deyecciones,) dependen de ambas estrategias. En la tabla 1 se detalla para cada sistema, el uso del suelo promedio de 2 a 4 campañas, considerando la superficie útil disponible y la proporción de superficies con finalidad agrícola (granos para venta). A su vez, los rubros vegetales se agruparon en pasturas y cultivos anuales. Estos últimos pueden tener finalidad agrícola, ganadera o ambas, mediante un uso directo (pastoreo natural o mecánico) o indirecto (reservas forrajera y granos para ración). Se presenta además la productividad anual discriminada en granos, carne y leche y el aporte de restos orgánicos, reduciendo la escala del sistema a 1 ha.

El 100% de superficie útil se distribuye entre pasturas y anuales estivales, ya que las invernales se asocian siempre a una estival determinando lotes con doble cultivo, y no hay descansos estivales. Las diferencias entre la superficie de estivales y superficies con doble cultivo (equivalente a anuales invernales) permiten calcular la superficie de anuales estivales con barbechos invernales.

En los sistemas mixtos el porcentaje de superficies con finalidad agrícola va disminuyendo y deja lugar a un gradiente creciente de ocupación de pasturas y anuales con finalidad ganadera, que se maximiza en el SME, que expresa la mayor productividad animal y menor producción de granos de todo el conjunto.

Tabla 1. Superficies (útil y agrícola); uso del suelo (pasturas y anuales estivales (E), e invernales (I)); la productividad (granos y leche en kg ms/ha.año; carne en kg peso vivo/ha.año) y aportes orgánicos (kg. ms/ha.año) se expresan reduciendo la escala del sistema a 1 ha.

Table 1. Surfaces (useful and agricultural); land use (pasture and annual summer (E), and winter (I)); productivity (grains and milk kg ms/ha.año; meat kg weight live/ha.año) and organic inputs (kg ms/ha.año) are expressed by reducing the scale of the system 1 ha.

Caso	sup. util	S. agríc.	Composición (% superficie)			Productividad anual/ha			Aportes
	ha	%	Pasturas	Anual E.	Anual I.	Granos	Carne	leche	kg ms/ha.año
SA 1	197	100	0	100	15	3091	0	0	**4522**
SA 2	425	100	0	100	15,5	6200	0	0	**9295**
SA 3	271	100	0	100	56	3390	0	0	**4608**
SMC 1	406	83	10,4	89,6	42	3982	12	0	**7512**
SMC 2	198	64	23,5	76,5	41	3715	35	0	**6879**
SMT	197	57	21,7	78,3	40	320	200	700	**3870**
SME	296	37	50	50	9	1657	748	0	**10100**

La productividad de granos sobre la base de la superficie agrícola del sistema (100% en SA y promedio de 73% en SMC) es menor en SA, lo que podría estar relacionado al efecto de mejores condiciones edáficas por los aportes de materia orgánica, menor incidencia de monocultivo y mejores rotaciones. El SMT tiene la menor productividad agrícola debido a que el principal cultivo destinado a grano para venta es soja, que produce $^{1}/_{3}$ del rendimiento de un maíz (figura 1, pág. 96).

Los aportes orgánicos de los SA tienen un promedio menor que los mixtos y las diferencias entre ellos obedecen a composición de especies cultivadas, rendimientos e índices de cosecha (relación entre biomasa de granos y biomasa total). En general, el cultivo de soja en el semiárido, produce menos de la mitad de rendimiento que el cultivo de maíz y tiene un coeficiente rastrojo/grano menor, lo que disminuye tanto la producción de granos como de rastrojos. Este efecto es más acentuado, cuanto mayor sea su proporción en el sistema.

Los aportes de materia orgánica se maximizan en el SME que tiene la mayor proporción de pasturas del conjunto (50% de la superficie), superando al resto de sistemas mixtos. Este caso valoriza el rol de las pasturas que aportan restos vegetales al ser pastoreada y suman también las deyecciones a campo durante el pastoreo y eventualmente, las pérdidas en la elaboración de reservas deshidratadas.

El SMT representa el menor valor de aportes al suelo de todos los casos analizados, incluso inferior a los agrícolas puros. Ello obedece a la conjunción de varios factores. Entre ellos: una baja proporción de pasturas que solo se usan para reservas deshidratadas, anuales invernales que se usan para corte y alimentación de animales confinados y una importante proporción de maíz que se destina a ensilado, que deja mínimos restos (equivalentes a menos de ¼ de lo que deja un maíz para grano). Esta intensificación ganadera que excluye el pastoreo biológico sustituyéndolo por pastoreo mecánico deja menores aportes y ninguna superficie recibe deyecciones, a lo que se suma la ausencia de reciclado de estiércoles.

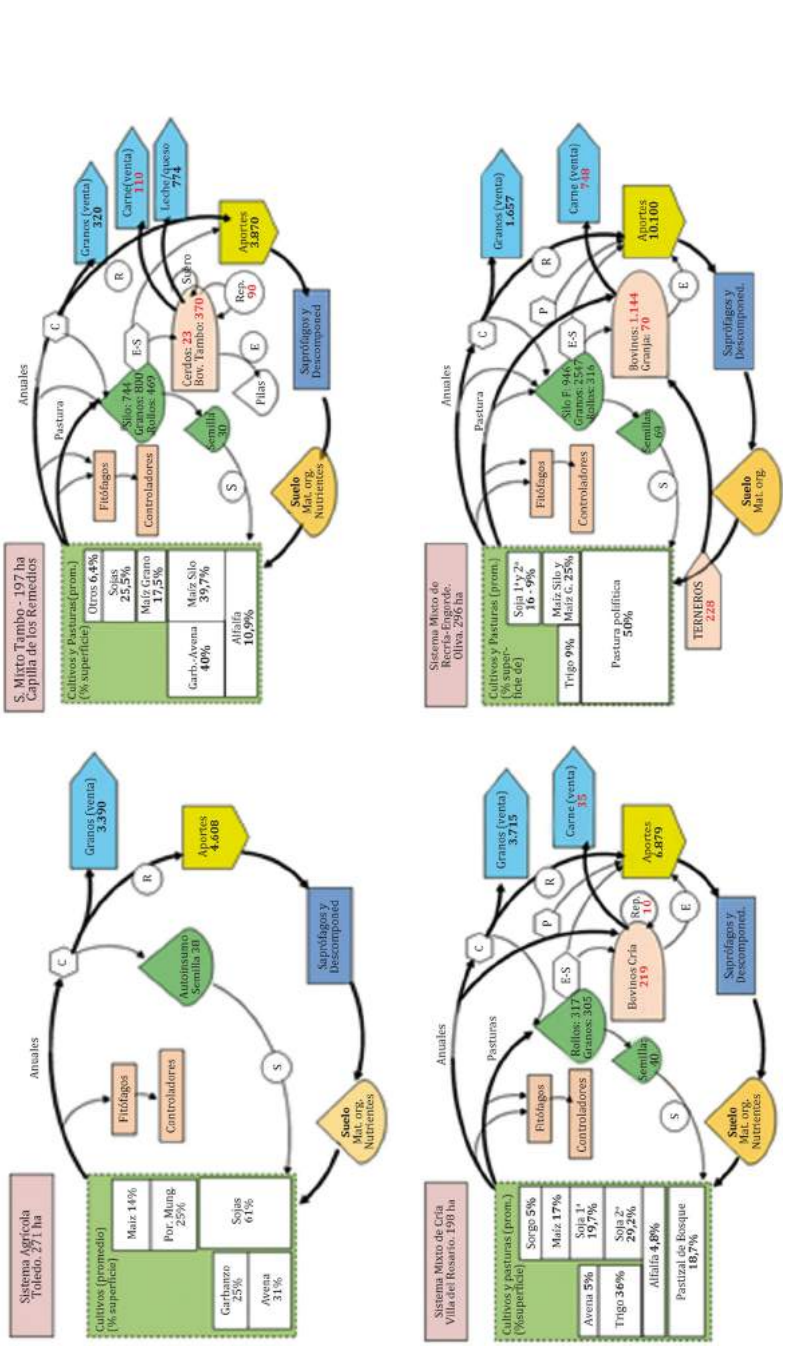
Diversidad productiva

En la tabla 2 (pág. 97), se observa que la riqueza de especies cultivadas crece con la presencia de ganadería, ya que intervienen pasturas y mayor variedad de cultivos anuales. El SMT tiene la mayor riqueza de todo el conjunto ya que incluye especies que no se presentan en otros sistemas o son muy poco frecuentes (papa, garbanzo, poroto Mung).

La dominancia de soja corresponde a la suma de unidades que la incluyen, tanto en la forma de barbecho-cultivo como en unidades de doble cultivo. Al considerarlas como unidades diferentes los valores de equitatividad en SA, no reflejan fielmente esta dominancia porque una proporción de soja se enmascara por el doble cultivo. El SMT tiene valores similares a los agrícolas, pero en este caso, hay una elevada dominancia de maíz (para silo y granos) que también se enmascara por el doble cultivo avena-maíz. En agrícolas, la dominancia es más evidente, porque hay un menor promedio de doble cultivo.

La sojización de los sistemas alcanza valores elevados. Considerando solo el periodo estival, en SA ocupa alrededor del 66% de las superficies, disminuye a un promedio de 55% en SMC y recién desciende a 25% en SME y SMT. La composición porcentual de cultivos puede verse en la figura 1 (pág. 96).

El Pastoreo (P) genera biomasa animal pero también aportes de materia orgánica en forma de restos vegetales, que caen al suelo durante el pastoreo y deyecciones (E).



Abreviaturas: S: semillas; C: cosecha; R: rastrojos; P: pastoreo; E estiércoles; E-S: elaboración y suministro de reservas; Rep: reposición de animales.
Abbreviations:. S: seeds; C: harvest; R: stubble; P: grazing; E: manure; E-S: Elaboration and provision of reserves; Rep: replacement of animals.

Figura 1. Estructura trófica de 4 casos: Arriba: Agrícola (SA3) y Mixto con tambo (SMT). Debajo: Mixto de cría (SMC2) y Mixto de engorde (SME). Valores biomasa vegetal en kg ms/ha.año y animal en kg Peso Vivo/ha.año.

Figure 1. Trophic structure of 4 cases: above: Agricola (SA3) and mixed with tambo (SMT). Below: Rearing (SMC2) and mixed for fattening (SME) mixed. Values biomass plant in kg ms/ha.año and animal in kg weight live/ha.año.

Tabla 2. Distintos Indicadores de la diversidad de los sistemas (valores adimensionales).
Table 2. Different Indicators of the diversity of the systems (dimensionless values).

Caso	Riqueza (n° especies)	Equitatividad (1-desvíos)	D. Estructural (proporciones)	D. Espacial (mosaico)	D. Temporal (secuencias)
SA 1	2,6	0,67	0,82	0,39	1,60
SA 2	3	0,45	0,99	0,29	1,55
SA 3	4	0,78	1,34	2,29	1,62
SMC 1	5,5	0,32	1,36	0,65	1,96
SMC 2	5,5	0,34	1,39	4,77	2,70
SME	4	0,48	1,20	1,92	2,72
SMT	6	0,61	1,60	0,39	2,07

Un cultivo anual (verdeo de invierno o verano) empleado para pastoreo puede aportar más restos que el rastrojo de un cultivo agrícola, especialmente si se usa en 2 o 3 pastoreos. Si un cultivo se emplea para cosecha de granos, el aporte es el de rastrojos, pero si el grano se usa luego para alimentación animal, se deben sumar a los aportes por las deyecciones derivadas de este consumo. El ensilado, es un caso especial que deja muy poco rastrojo en el lote y en su proceso de elaboración se producen pérdidas aeróbicas y anaeróbica, que en parte no vuelven al suelo (35). Las deyecciones originadas por su consumo no son altas por ser un alimento de alta digestibilidad.

Las reservas utilizadas para alimentación animal (silos, rollos y granos) reciben distintos flujos de biomasa vegetal (figura 1, pág. 96) y muestran importantes diferencias en composición y cantidad, maximizándose en los sistemas más intensificados (SME y SMT). La elaboración y suministro de reservas (E-S) generan diferentes aportes durante: la recolección de la biomasa; el procesamiento (emparvado, enrollado, ensilado) y el consumo por pérdidas eventuales. Luego del consumo, la fracción no asimilada retorna al suelo (o se acumula) como deyecciones sólidas y líquidas, mientras que lo asimilado se metaboliza en biomasa animal para reposición (Rep) y venta como en SMT y SMC, o solo venta como el SME.

En los gráficos de la figura 1 (pág. 96), se observa que el SME tiene 4 vías o circuitos que proporcionan aportes al suelo y tiene las mayores reservas alimenticias (provenientes, de 3 fuentes distintas). En los SMT y SMC, las pasturas perennes se usan solo para reservas deshidratadas, careciendo de los aportes que genera el pastoreo (P).

Los consumidores productivos son principalmente bovinos y representan valores bastante disímiles de biomasa animal: en el SME 1200 kg PV/ha.año, en el SMT un tercio y el SMC, un quinto, de esa cantidad.

Los aportes de residuos siguen un circuito interno por la cadena de detritívoros, generando condiciones edáficas que regulan la productividad vegetal del ciclo siguiente. Los SA tienen aportes menores que los mixtos de cría y engorde mientras que el SMT presenta valores mínimos debido a la falta de pastoreo y de reciclado de estiércoles. El reciclado solo se implementa en el SME. En los SMC las heces quedan en los lotes pastoreados, siendo la forma más eficiente de reciclado de materia.

La consideración de los aportes es un factor clave al analizar la sostenibilidad de los distintos sistemas, no solo por el rol biológico de los componentes en la regulación de la fertilidad, que según Gliessman (2002) debe maximizarse, sino porque estos aportes determinan los niveles de materia orgánica del suelo, regulando a través de esto, un amplio espectro de condiciones físicas, químicas, hídricas y biológicas (21) de alta incidencia en la conservación y productividad.

Energía

En la tabla 3 se presentan los valores energéticos de cada sistema, considerando las entradas (servicios, labores e insumos), salidas (productos) y la eficiencia energética (Salidas/Entradas). En el caso de las entradas (subsidios de energía) se discriminó la energía destinada en semillas (adquiridas), regulación biótica (controles de malezas, insectos o patógenos), fertilidad del suelo (fertilizantes, abonos, inoculantes) y en labores (siembra, pulverizaciones, cosecha, elaboración de reservas).

Las entradas de energía tienen menor variabilidad que las salidas en todos los sistemas en estudio a excepción del SMT por lo que se podría generalizar que el costo energético de operar un sistema mixto es similar a manejar un sistema agrícola, aunque tienen diferencias importantes en el destino de los subsidios. El costo energético en semillas disminuye en función de la presencia de especies que permiten su autoproducción, generalmente autógamas. En el caso de las labores, dado que todos los sistemas implementan siembra directa, los subsidios en siembra, pulverización y cosecha se asocian a la superficie de anuales. En el caso de los mixtos más intensificados (SME y SMT), se suman las labores destinadas a la elaboración de reservas y suministro de alimentos.

Los SA y SMC tienen un costo importante en regulación biótica (principalmente herbicidas) pero disminuyen en sistemas con mayor peso ganadero (SME y SMT), lo que podría estar asociado al efecto de las rotaciones agrícolas-ganaderas.

En cuanto a subsidios a la fertilidad del suelo, los SA tienen el mayor consumo de fertilizantes, que se asocia principalmente a las proporciones de maíz y se refleja luego en un mayor valor de salida energética por la mayor productividad.

Tabla 3. Valores energéticos de entradas y salidas, eficiencias y subsidios (Megajoules/ha.año).

Table 3. Energy values of inputs and outputs, energy efficiency and energy subsidies (Megajoules/ha.año).

Caso	Entr. Energ. MJ/ha.año	Sal. Energ. MJ/ha.año	Eficiencia Sal./Entr.	Subs. En. Semillas	Subs. En. Reg. Biot	Subs. En. Fertiliz	Subs. En. Labores
SA 1	6513,9	61588,4	**9,45**	1979,0	2461,9	532	1543,6
SA 2	10588,0	109678,2	**10,36**	2197,7	2978,3	3374,7	2058,8
SA 3	6901,6	63971,8	**9,27**	1502,6	2466,7	941,7	1984,2
SMC 1	7220,1	83696,7	**11,59**	2119,6	2518,1	726,8	1900,0
SMC 2	8649,3	82333,7	**9,52**	2572,6	2308,1	1456,5	2312,0
SME	10592,0	29672,1	**2,80**	700,4	1331,8	380,2	2622,8
SMT	67815,0	45468,7	**0,67**	2274,3	1984,2	765,9	2181,6

Se destaca el SME que no utiliza fertilizantes sintéticos y el mínimo valor registrado se debe exclusivamente, al reciclado de estiércoles (aprox. 1200 kg ms/ha.año).

Las salidas energéticas dependen del tipo de producto logrado, debido a que el contenido energético de los granos es superior al de carne o leche. En SA la productividad depende de los rendimientos unitarios y la superficie ocupada por cada especie.

Los sistemas con mayor proporción de soja producen menos energía que los que poseen mayores superficies destinadas al cultivo de maíz o sorgo. En la pirámide alimentaria que se establece en un sistema mixto, la productividad energética vegetal es mucho mayor que la productividad energética del estrato de herbívoros a raíz de la conversión alimentaria de la biomasa vegetal en producto animal (26). Por ello, en términos generales, hay una caída de la eficiencia energética en sistemas mixtos, proporcional a las superficies asignadas a la ganadería.

En el caso de los sistemas mixtos analizados, la eficiencia tiene una variabilidad importante. Los sistemas mixtos de cría con base pastoril no muestran diferencias con la eficiencia de un sistema agrícola ya que la incidencia de la actividad ganadera tanto en las entradas como salidas de energía es muy baja, mientras que el SMT es el menos eficiente debido a una alta intensificación ganadera, donde confluyen escasez de pasturas, ausencia del pastoreo directo, alto costo en elaboración de reservas e ingreso de insumos para alimentación animal.

El SME es semi intensivo, manteniendo una importante fase pastoril y producción propia de la mayoría de reservas y suplementos, lo que reduce el gasto energético comparado con SMT. Estos resultados son coincidentes con los hallados por Iermano (2015) en la zona centro este de la Provincia de Buenos Aires, donde los sistemas mixtos pastoriles tienen una eficiencia energética similar a los agrícolas y los sistemas ganaderos con mayor intensificación, muestran valores menores a uno.

Análisis integral

Se elaboró un diagrama radial para los sistemas analizados (figura 2, pág. 100) donde se representan los valores de diversidad, aportes de materia orgánica y la eficiencia energética. Los valores de cada variable se expresaron en porcentaje, según el mejor valor logrado en el conjunto de sistemas. Dado que las escalas representan las mejores condiciones en los porcentajes más altos, es posible lograr un orden de mérito sumando estos porcentajes, en cada uno de los casos. En la representación empleada, este orden es: SMC2>SMC1>SME>SA3>SA2>SMT>SA3.

Los sistemas agrícolas (SA) muestran alta eficiencia energética, por su exclusiva productividad de granos. Sus índices de diversidad tienen valores moderados a bajos, debido a la dominancia espacial y temporal del cultivo de soja, lo que influye también en bajos aportes de rastrojos y menor productividad unitaria de granos.

La calidad de rotaciones (diversidad temporal) es menor que en los mixtos.

En los sistemas mixtos de cría (SMC) se observa una alta eficiencia energética por la elevada producción de granos y bajo costo energético de su ganadería (auto-reposición de animales y bajo uso de reservas). Presentan valores altos de diversidad y aportes superiores a los agrícolas, por una composición vegetal más diversa y el pastoreo directo de anuales y pasturas. La diversidad temporal indica rotaciones de mayor calidad que los agrícolas.

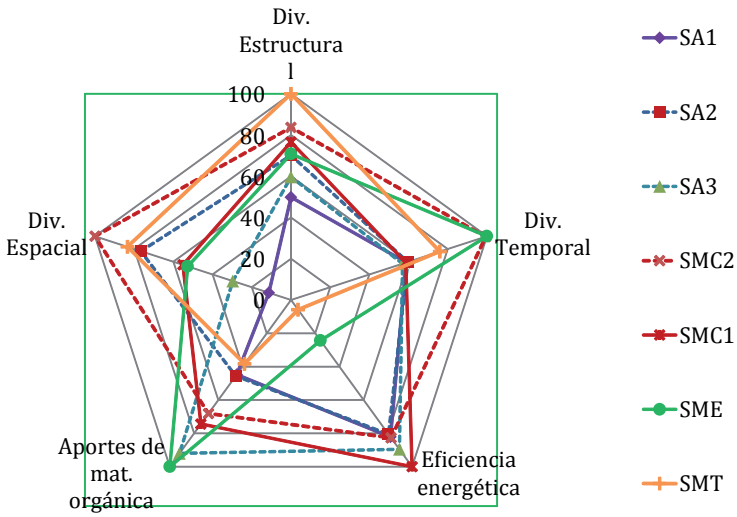


Figura 2. Diagrama radial de los 7 casos analizados donde se representan 3 dimensiones de la diversidad (Div.), eficiencia energética y aportes de materia (mat) orgánica al suelo.

Figure 2. Radial diagram 7 analyzed cases which represent 3 dimensions of diversity (div.), energy efficiency and contributions of material (mat) organic to the ground.

En el sistema mixto de engorde (SME), la diversidad estructural y espacial disminuyen debido al alto porcentaje de pastura, pero la diversidad temporal se optimiza por la rotación mixta (pastura-cultivos). Esto último permite lograr altos aportes de materia orgánica al suelo (restos vegetales y deyecciones originados por el pastoreo directo). Además de ello, se reciclan estiércoles a los lotes agrícolas lo que beneficia la conservación de suelos. La baja eficiencia energética deriva de una alta proporción de producción animal, alto costo energético en elaboración de reservas y compra de animales para engorde (terneros).

El sistema mixto de tambo (SMT) presenta alta diversidad estructural gracias a una mayor riqueza de especies y proporciones algo más equilibradas. Aún así, la dominancia de maíz influye negativamente en la diversidad espacial. El índice temporal presenta el menor valor de todos los casos, debido a la baja proporción de pasturas que imposibilita una rotación mixta.

El manejo tecnológico aplicado en la alimentación animal donde se sustituyen pasturas por cultivos anuales para producción de reservas, excluye el pastoreo de campo y no se efectúa el reciclaje de estiércoles, produce muy bajos aportes de materia orgánica al suelo. La intensificación, en este caso, disminuye la calidad de las rotaciones, determina bajo aportes y aumenta el costo energético para la producción de alimentos, determinando una menor eficiencia energética.

Análisis de componentes principales

En los resultados del análisis de componentes principales se reafirma la tendencia observada en el análisis integral. La CP1 que explica un 47,9% de variabilidad, diferencia los casos principalmente según los índices de diversidad, % superficie ganadera, entradas de energía auxiliar y eficiencia energética, ubicando a los SA en un cuadrante y a los SME y SMT en otro. Estos últimos presentan mayores valores en las 3 primeras variables y menor eficiencia energética. En el caso de la CP2 la variable más importante para discriminar los casos, es el aporte de materia orgánica, donde se destaca el SME, SMC2 y SA3, asociados a mayores valores.

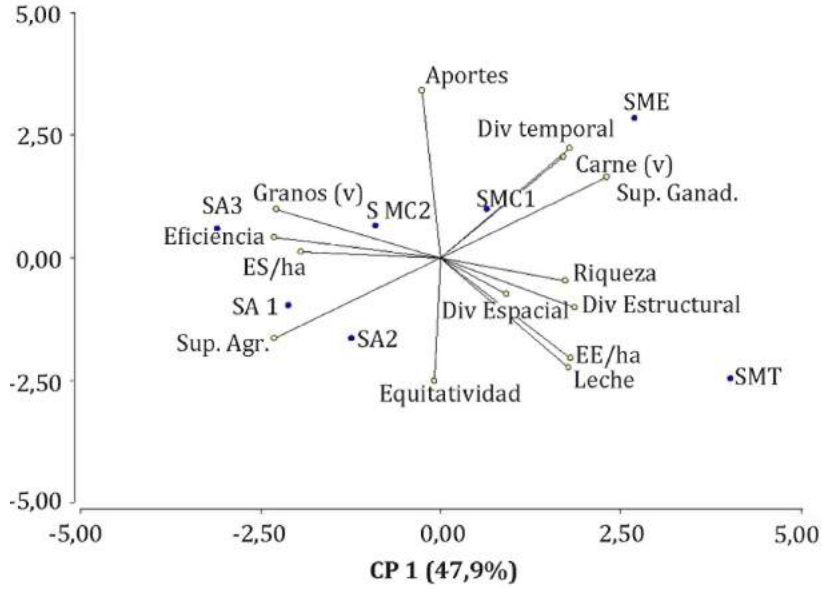


Figura 3. Análisis de componentes principales para los sistemas productivos estudiados.

Figure 3. Analysis of main components for the productive systems studied.

Conclusiones

Los resultados demuestran la importancia de implementar estrategias que promuevan la diversificación productiva ya que la composición, proporción, ubicación y secuenciación de cultivos permiten regular la productividad y los aportes de restos orgánicos.

Los SA si bien muestran una alta eficiencia energética, poseen dominancia de soja que influye en una menor diversidad espacial y temporal y se refleja en un menor aporte de materia orgánica al suelo.

La incorporación de la producción ganadera de base pastoril mejora los índices de diversidad espacial y especialmente el de diversidad temporal por la presencia de pasturas que permiten realizar la rotación agrícola-ganadera, aumentando los aportes de materia orgánica. Este tipo de sistemas logra mantener alta eficiencia energética cuando las superficies de pasturas son moderadas ya que no existe un costo energético alto en la producción animal. Cuando aumenta la superficie destinada a la producción ganadera con base pastoril (SME) disminuye la eficiencia energética pero se maximiza el aporte de restos, que reduce el uso de fertilizantes y permite una óptima conservación de suelos. La intensificación ganadera al reducir la superficie de pasturas por cultivos anuales para producción de reservas disminuye la calidad de las rotaciones y aumenta el costo energético para la producción y compra de alimentos para consumo animal, lo que disminuye considerablemente la eficiencia energética (SMT).

Los sistemas mixtos de cría logran una integración de la producción agrícola-ganadera favorable, alcanzando una alta eficiencia energética y buenos aportes de materia orgánica, manteniendo la base pastoril y la producción propia de terneros.

En este estudio, se ha verificado que tanto la diversificación productiva como la incorporación del componente ganadero, resultan estrategias alternativas al modelo de simplificación que benefician la sustentabilidad de los sistemas. Sin embargo, una fuerte intensificación ganadera que anula el pastoreo directo, resulta adversa a la sustentabilidad, tanto en la eficiencia energética como en la conservación del suelo.

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Estrategias de manejo para la transición hacia viñedos sostenibles en Mendoza

Management strategies for the transition to sustainable vineyards in Mendoza

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Resumen

En agro-ecosistemas de regadío frágiles como los de Mendoza, con suelos de baja fertilidad, escasas precipitaciones y elevada evapotranspiración estival, el cambio climático tendrá un impacto significativo. Las prácticas vitícolas convencionales mediante labranza y uso intensivo de agroquímicos, profundizan los desequilibrios ecológicos generando serios riesgos ambientales. La problemática requiere de un rediseño de estos sistemas y el replanteo de sus prácticas actuales. En la Estación Experimental Mendoza del INTA se trabajó en la transición hacia sistemas de manejo del viñedo con enfoque agroecológico. En una parcela demostrativa y experimental de vid, se establecieron corredores biológicos y diversos cultivos de cobertura, se estudiaron diferentes metodologías de compostaje y obtención de té de compost, bioles y su aplicación periódica al cultivo. Se ensayaron tecnologías alternativas para controlar malezas y programas preventivos de menor impacto para el control de plagas y enfermedades. Las prácticas propuestas permitieron aumentar la diversidad de especies en el viñedo, mejorar la fertilidad del suelo y alcanzar niveles productivos cercanos al manejo convencional. En el transcurso de nueve temporadas agrícolas, se logró mantener satisfactoriamente las condiciones fitosanitarias. Por último, los costos operativos se aproximaron a los de un manejo convencional.

Palabras clave
vid • agroecología • coberturas verdes • compost • bioles • fertilidad

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Abstract

In fragile irrigated agro-ecosystems like those in Mendoza, with low fertility soils, scarce rainfall and high summer evapotranspiration, climate change will have a significant impact. Agricultural practices with tillage and intensive use of agrochemicals generate serious ecological imbalances. This problem requires the redesign of these systems and a reconsideration of its practices. The Agricultural Experimental Station of INTA Mendoza worked on the transition to vineyard management systems with an agroecological approach. In a demonstrative and experimental vineyard plot, biological corridors and diverse cover crops were established, studying different methodologies of elaboration of compost, compost tea, bio-slurry and their periodic application to the crop. We evaluated alternative technologies for weed control and phytosanitary programs with lower environmental impact. The proposed practices allowed increasing biodiversity of species in the vineyard, improving soil fertility and achieving productive levels close to those of conventional management. In the course of nine agricultural seasons, the sanitary conditions of the vineyard were satisfactorily maintained. Finally, operating costs were close to those of conventional management.

Keywords
grapevine • agroecology • cover crops • compost • bioslurry • fertility

Introducción

Los valles cultivados de la provincia de Mendoza se encuentran insertos en ecosistemas áridos y semiáridos, con suelos de escasa fertilidad, bajas precipitaciones y elevados potenciales de evapotranspiración durante el verano. En estos agro-ecosistemas frágiles el cambio climático tendrá un impacto significativo (29).

Las prácticas vitícolas convencionales, mediante labranzas con arados de reja y vertedera, que en algunos casos son sustituidas por la aplicación de herbicidas, y el uso intensivo de otros agroquímicos, generan graves desequilibrios ecológicos: reducción de la infiltración de agua, aumento de la escorrentía, mayor riesgo de erosión y compactación del suelo, problemas de tracción en maquinaria, disminución de la fertilidad, pérdida de biodiversidad, perjuicios a insectos benéficos, contaminación del suelo, el agua y potenciales riesgos para la salud humana (8). Asimismo, la complejidad para controlar todos los factores que intervienen en los tratamientos fitosanitarios determinan una elevada ineficiencia en las aplicaciones de agroquímicos (13); en viñedos se verifica que la proporción del líquido pulverizado que queda en la vegetación objetivo es menor o igual al 55% del volumen total aplicado, cerca del 25% termina en el suelo y el 20% restante se pierde en el aire (1, 2, 16). Estas pérdidas por deriva además de reducir la eficacia del tratamiento y representar una significativa pérdida económica, se transforman en un serio peligro ambiental y de riesgo para la salud humana al contaminar suelo, agua y aire. Como ejemplo concreto se puede citar el aumento de metales pesados como el cobre y el cadmio, detectado en suelos agrícolas, mayormente vitícolas

de Mendoza, en comparación con suelos vírgenes no cultivados, lo cual se vincula a la aplicación de productos cúpricos para el control de hongos fitopatógenos (11).

Muestreos realizados por SENASA entre los años 2011 y 2016, tomando muestras en los principales mercados concentradores de frutas y hortalizas de Argentina, detectan presencia de pesticidas en cerca del 60% de las muestras analizadas, con alrededor del 10% superando el límite máximo de residuos. Además, se identifican varios principios activos para cada alimento (18).

Ante esta situación, se acrecienta la preocupación de los consumidores por los riesgos ambientales y la búsqueda de alimentos seguros y saludables (14). Los vitivinicultores preocupados por los diversos impactos de los agroquímicos y que buscan alternativas a las prácticas habituales, se encuentran con algunos problemas ante la transición hacia sistemas más sutentables: i) escasa diversidad parcelaria dentro de las fincas mendocinas; predomina el monocultivo de la vid, ii) baja fertilidad del suelo; escasos contenidos de materia orgánica, nutrientes y reducida actividad biológica, iii) limitadas alternativas de insumos de bajo impacto, que puedan sustituir a los agroquímicos tradicionales y de alto valor económico, iv) mayor necesidad de mano de obra para ciertas tareas, v) elevados costos operativos y vi) menores rendimientos.

La problemática de la viticultura regional requiere de un rediseño de los agroecosistemas vitícolas y un replanteo de sus prácticas agrícolas.

En la Estación Experimental Agropecuaria (EEA) Mendoza del INTA se trabajó en la evaluación de diferentes prácticas para la transición hacia sistemas sostenibles de manejo del cultivo.

Objetivo

Desarrollar tecnologías que faciliten la gestión de viñedos con un enfoque de manejo agroecológico, buscando aumentar la diversidad de especies, mejorar la materia orgánica del suelo, evitar el uso de agroquímicos, aumentando y sosteniendo la productividad en el tiempo.

Materiales y métodos

Parcela demostrativa de vid bajo manejo agroecológico

En un viñedo implantado en el año 1994 de 4,5 ha ubicado en Mayor Drummond, Luján de Cuyo, Mendoza (INTA EEA Mendoza), se inició en 2009 el proceso de transición hacia un sistema de cultivo con enfoque de manejo agroecológico (21).

Las plantas de vid fueron conducidas en un sistema de espaldero alto con poda corta (pitones de dos yemas dispuestos en cordones bilaterales) en hileras de 142 m de largo orientadas norte-sur (con 2.666 plantas ha^{-1}).

El riego fue superficial por melgas sin pendiente en el sentido de las hileras, con agua proveniente del río Mendoza (turno). En el transcurso de los años se redujo la dotación de agua para riego, lo cual se tradujo en una disminución de la frecuencia de riegos para determinados momentos de la temporada.

La parcela se aproximó a un rectángulo de 120 x 600 m e incluyó al viñedo y un sector inculto. En el límite oeste poseía un cerco de *Pyracantha coccinea* (crategо) que lo separó de una calle secundaria y una autopista, hacia el sur limitaba con el predio de una empresa, separada por una cortina forestal de álamos y olivos, mientras que el límite este poseía una fila de olivos que la separaban de una calle

y una zona residencial. El límite norte colindó con otros viñedos y al inicio de la experiencia no poseía cortina vegetal.

A partir de este viñedo de 15 años de antigüedad, manejado con prácticas habituales en la viticultura regional: uso de pesticidas para control de enfermedades, pulverización de herbicidas (mayormente glifosato) en la línea de plantas, aplicación eventual de fertilizantes de síntesis y labranzas periódicas; se propuso un cambio en la gestión abordando temas considerados esenciales para promover los principios agroecológicos: diversidad, fertilidad (en el suelo y el cultivo), actividad biológica (en el sistema) y protección integrada. Para ello, se decidió trabajar sobre diferentes estrategias que influyen sobre los temas definidos, estas son: establecimiento de corredores biológicos y cultivos de cobertura, utilización de enmiendas orgánicas, opciones para el control de malezas y búsqueda de pesticidas alternativos.

Se destinó un depósito de uso exclusivo de la unidad demostrativa para almacenar insumos y maquinarias (*e.g.* pulverizadora). En un sector inculto próximo al cultivo se estableció una playa para la elaboración de compost.

Inicialmente, año 2009; la parcela se registró en el proceso de certificación orgánica en el marco de la normativa Argentina (ley 25.127). En la temporada 2012/13, se alcanzó la certificación de uvas orgánicas correspondientes a 2,8 ha del cv. Cabernet sauvignon y 1,7 ha de cv. Sauvignon blanc, luego de tres temporadas de manejo de transición.

En 2017 se redujo el tamaño de la parcela a 2 ha del cv. C. sauvignon y se discontinuó la certificación formal, manteniendo las prácticas agroecológicas de manejo hasta 2018. En el periodo 2009-18 se registraron temperaturas mínimas medias anuales de 8,2°C y máximas medias de 22,5°C, con precipitaciones anuales promedio de 263 mm.

En el esquema de transición agroecológica definido por Tittonell (2014), se consideró que esta parcela de experimentación se ubicó dentro de la situación definida como la más crítica y vulnerable, caracterizada por la sustitución de insumos y en camino hacia el rediseño del sistema. En la unidad se experimentaron prácticas de manejo a escala de un productor vitícola medio a pequeño, y a su vez funcionó como viñedo demostrativo: fue visitado por productores, estudiantes y profesionales.

Estrategias de manejo del cultivo

Corredores biológicos

Se propuso el establecimiento de un corredor biológico en el límite norte de la parcela, compuesto por especies floríferas de bajo requerimiento hídrico, con mediano y bajo porte, para aumentar la biodiversidad y como barrera física (3 m de ancho y 310 m de largo) entre la parcela orgánica y el viñedo vecino bajo manejo convencional. En el año 2010, se trasplantaron más de 25 especies diferentes, nativas y exóticas (24).

Complementariamente, se incorporó una franja de cultivo con alfalfa, inicialmente de 10 m de ancho por 150 m de largo, y que se amplió en 2013 a 60 m de ancho, para separar la parcela de un sector inculto ubicado en el límite este.

Las especies del corredor biológico presentaron durante todo el año la floración de una proporción de las especies presentes (24). La flora proveyó de alimento y refugio a insectos benéficos con potencial para el control de plagas, favoreciendo su multiplicación y circulación. De la misma manera, la franja de alfalfa proporcionó condiciones óptimas a enemigos naturales, favoreció su establecimiento y reproducción.

Cultivo de cobertura

En el interfilar del viñedo orgánico se sembró, en septiembre de 2010, un cultivo de cobertura polifítico con especies herbáceas anuales: *Trifolium balansae, Tagetes sp., Calendula officinalis, Brassica alba, Lolium multiflorum, Bromus unioloides,* y perennes: *Festuca rubra, Lolium perenne, Lotus tenuis, Trifolium repens, T. fragiferum y T. pratense* (24).

Luego de tres temporadas de manejo con cobertura vegetal en todos los interfilares, en 2014 se decidió controlar el desarrollo de la cobertura hilera por medio: manejando entre hileras con especies perennes alternadas con interfilares con labranza periódica con rastra de cuatro cuerpos y doble efecto, buscando reducir la competencia entre la cobertura y la vid.

Los cultivos de cobertura se transformaron en soporte de diversos insectos benéficos, pero además contribuyeron a mejorar estructura y fertilidad del suelo, incrementando actividad biológica, favoreciendo biodiversidad, aumentando infiltración, reduciendo compactación, controlando erosión y permitiendo el ingreso anticipado de maquinaria al cultivo luego de una lluvia (21). Los cortes en franjas alternas permitieron mantener gran parte del ciclo a los cultivos en floración.

A partir de la temporada 2015/16 se trabajó con cultivos de cobertura anuales invernales interfilar por medio (*X Triticosecale, Hordeum vulgare* y *Vicia sativa*) y en la temporada siguiente se estableció un ensayo experimental para evaluar alternativas con diferente grado de competencia. En la experimentación se estudiaron los siguientes tratamientos: i-cobertura anual invernal: *Avena sativa* u *Hordeum vulgare, Secale cereale* y *Vicia sativa,* interfilar por medio, alternado con interfilar labrado periódicamente (TM); ii-cobertura permanente: *Lolium perenne* y *Trifolium repens,* en la mitad de los interfilares y labranza el resto (PM); iii-cobertura anual invernal todos los interfilares (TT) y iv-interfilares alternados con cobertura perenne y cobertura anual invernal (PT) (foto, pág. 110).

Los tratamientos fueron dispuestos en un diseño experimental de bloques completos al azar con cuatro repeticiones.

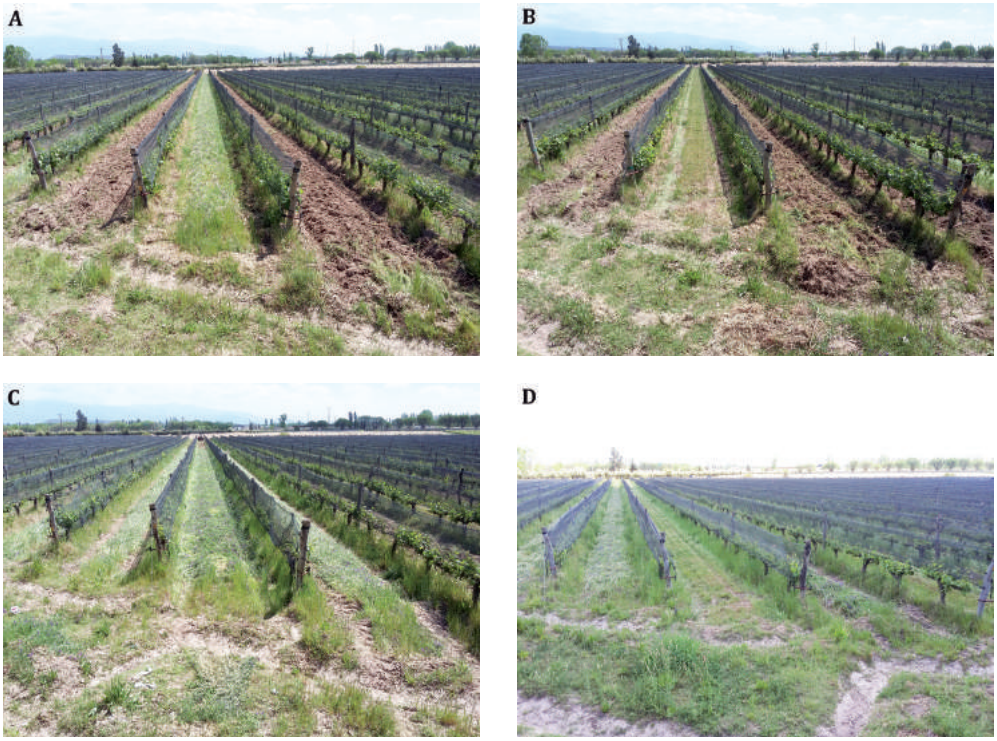
Los interfilares con cobertura anual invernal se manejaron mediante rolado primaveral a través de un rodillo de coberturas verdes, aplicado en la época de floración de las especies, y posteriormente mediante cortes periódicos con segadora, al igual que la cobertura permanente.

La superficie del interfilar efectivamente sembrada fue del 60% y en la línea de plantas se desmalezó mediante un prototipo de labranza intercepa. En todos los tratamientos se aplicó compost y se realizaron aplicaciones fitosanitarias de la misma manera que en el resto de la parcela demostrativa, tal como se detalla posteriormente. En el viñedo se evaluó producción, calidad de uva y peso de poda, mientras que en los cultivos cobertura se midió aporte de biomasa (datos no presentados).

Insectos benéficos y hormigas

Tanto en los corredores biológicos, la cobertura verde, como también en el viñedo, se observaron una gran diversidad de insectos. Sin embargo, no se efectuaron relevamientos sistemáticos, solo algunos muestreos puntuales (datos no presentados).

A partir del mes de noviembre, en algunos años con mayor intensidad que otros, se registró la presencia de coccinélidos de distintas especies, como *Hippodamia variegata, Chrysolina graminis, Adalia bipunctata, Eriopis connexa*, pasando el invierno bajo la corteza de las cepas y visitando los incipientes brotes.



(A) Cobertura temporaria la mitad de los interfilares: TM;
(B) cobertura permanente la mitad de los interfilares: PM; (C) cobertura temporaria todos los interfilares: TT; (D) cobertura temporaria y permanente alternada: PT.
La cobertura anual se observa recién rolada y la permanente segada.

(A) Temporary cover crop one interrow in half: TM;
(B) permanent cover crop in half of the interrows: PM; (C) temporary cover crop in each interrow: TT;
(D) alternating interrows with temporary and permanent cover crops: PT. Annual cover crops is observed recently rolled and permanent mowed.

Foto. Experimentación con cultivos cobertura de distinto grado de competencia con plantas de vid cv. Cabernet sauvignon, INTA EEA Mendoza.

Photo. Experimentation with cover crops of different levels of competition with grapevines cv. Cabernet sauvignon, INTA EEA Mendoza.

Asimismo, se encontraron numerosos pulgones parasitados con micro-himenópteros, principalmente en las especies leguminosas utilizadas como cobertura verde y en la alfalfa. También se observó actividad de diferentes especies de otros artrópodos como sírfidos, crisópidos y arácnidos.

La actividad de insectos benéficos es considerada una señal importante para el manejo integrado de plagas y enfermedades. Por ejemplo los coccinélidos, además de ser reconocidos predadores de áfidos, se ha comprobado que son importantes consumidores de hongos como el oidio (19).

En un estudio realizado por Chorbadjian y Kogan (2001) en viñedos de Chile, se evidenció que aquellos que poseían cobertura vegetal presentaron mayor número de artrópodos totales (fitófagos, benéficos y otros) que las viñas sin cultivo de cobertura, mientras que la proporción entre insectos benéficos/fitófagos fue muy superior en los viñedos con cobertura verde. Si en primera instancia se hiciera solo el relevamiento de algún insecto fitófago, se encontraría mayor presencia de la plaga en las parcelas que poseen cobertura verde, sin considerar que justamente allí es donde existe un mayor número relativo de insectos que pueden colaborar en el control natural de las plagas.

Durante 2014, se colectaron hormigas de forma manual y mediante trampas de caída, se clasificaron y se estudió el régimen alimenticio de las hormigas cortadoras, ya que representan un potencial riesgo de daños para los viñedos.

Compost

La aplicación de compost se implementó desde el inicio de la parcela orgánica demostrativa; ya que mejora la fertilidad física, química y biológica del suelo, incorpora materia orgánica, favorece la estructura del suelo, aumenta retención de agua, estimula actividad biológica y mejora la disponibilidad gradual de nutrientes (12). Anualmente se incorporaron cerca de 6 t de compost por hectárea, aplicado hilera por medio.

Los materiales iniciales utilizados fueron diversos y cambiaron según su disponibilidad: orujo fresco y agotado, hojas secas y segado de parques, estiércoles de vaca, caballo, cama pollo parrillero, guano gallina, cabra, cortes alfalfa, chala de ajo, aserrín, entre otros. Las técnicas también fueron modificándose a través de los años hasta ajustar la metodología de elaboración: desde pilas estáticas (2010), con ventilación pasiva (2011), volteos semi-mecanizados (2012/13), hasta volteos mecanizados (a partir de 2014).

El riego fue inicialmente por aspersión, aunque con baja eficiencia y escurrimiento superficial; y luego, a partir de 2014, se utilizaron cintas de goteo, logrando mayor eficiencia y homogeneidad en el riego.

En 2014 se comparó el trabajo realizado por una volteadora de compost del tipo comercial (desarrollada en INTA EEA Ascasubi) y un implemento tipo reja (desarrollada en INTA EEA Mendoza) y se evaluó la calidad final del compost obtenido.

Té de compost

El té de compost o extracto de compost (EC) es un preparado a base de compost fermentado en agua que posee nutrientes y microorganismos. Existen distintas metodologías de elaboración del té: con diferentes relaciones compost:agua, no aireados y aireados por diferentes lapsos.

En una primera experiencia local realizada en 2014, se caracterizaron los EC obtenidos con distinto grado de aireación y se evaluó su influencia sobre el crecimiento de plantas jóvenes de vid (22).

En la parcela demostrativa, el té de compost se elaboró a partir de 2015 en tambores de 220 litros con una relación en volumen compost:agua de 1:4, aireado mediante dos aireadores tipo pecera y difusores ubicados al fondo del recipiente, por un período de 24 horas. Anualmente, entre primavera y verano, se efectuaron entre tres y cuatro aplicaciones foliares sin dilución, con una dosis de 280 l ha^{-1} con plena vegetación.

En un ensayo comparativo a campo iniciado en el año 2016, se evaluaron los efectos de la incorporación de compost al suelo (CO) (dosis de 7 a 8 t ha^{-1}) y

la aplicación combinada de compost (al suelo con igual dosis) y extracto de compost (foliar, dosis de 280 l ha^{-1}) (CO+EC), contrastadas con un tratamiento testigo sin enmiendas (TE), en la producción y calidad de la uva. El compost tuvo una relación C/N de 17, 2,4 % de N, conductividad eléctrica (CE) de 2,2 dS m^{-1} y pH de 6,8, mientras que el té de compost presentó 2,9 mg l^{-1} de $N\text{-}NO_3$ y 4,0 mg l^{-1} de $N\text{-}NH_4$, 2,4 dS m^{-1} de CE y 7,9 de pH.

Biol

A partir de 2015 se complementó el plan de fertilización del viñedo a través del uso de biofertilizantes líquidos (bioles) incorporados al suelo. Con ello se buscó una aplicación localizada próxima a la zona de raíces y disponibilidad nutricional más rápida para las plantas. Además de su aporte nutricional, los bioles pueden contribuir con el aumento de la resistencia a enfermedades y mejorar las defensas ante un ataque de plagas, aumentar la actividad biológica del suelo y estimular la floración (10).

Los bioles pueden ser elaborados por el mismo productor de manera sencilla y se producen a base de una fermentación anaeróbica de residuos orgánicos, principalmente estiércoles y restos vegetales. En la parcela demostrativa se han utilizado para su elaboración los siguientes componentes: alfalfa fresca picada, estiércol de conejo fresco, bentonita, cáscara de huevo finamente molida, ceniza de madera y ceniza de hueso. Estos materiales se colocaron en un tanque plástico de 200 L con agua, tapado herméticamente y con una trampa de gases durante un período de 3 a 6 meses. Antes de su aplicación a campo, los bioles obtenidos fueron caracterizados presentando: 5,1 x 10^6 UFC ml^{-1} de microorganismos aerobios mesófilos (bacterias), sin presencia de hongos, levaduras, ni bacterias patógenas (*Salmonella sp.* y *Escherichia coli*).

En promedio registraron una concentración de N total de 11,3 g L^{-1}, 0,21 g L^{-1} de P y 3,59 g L^{-1} de K, la CE media fue de 15,1 dS m^{-1} y el pH 7,7. Finalmente, se realizó una aplicación primaveral mediante máquina con pastillas de alto volumen orientadas al suelo hacia ambos lados de la hilera de plantas, en dosis de 44 litros por hectárea diluido al 11%.

Evolución de la fertilidad del suelo

Se realizaron muestreos sistemáticos del suelo de la parcela orgánica en muestras compuestas antes del inicio de la transición en 2009, y en los años 2012, 2014 y 2018.

Se efectuaron análisis de fertilidad (N, P, K y MO) y salinidad (CE y pH). Complementariamente, se realizaron análisis microbiológicos. Se determinó abundancia de bacterias aerobias mesófilas, hongos totales y hongos formadores de micorrizas, microorganismos mineralizadores del nitrógeno, amonificadores, nitrificadores, fijadores de nitrógeno (15).

Gestión de malezas

Como se especificó previamente, en el sitio del interfilar de los viñedos bajo manejo agroecológico se proponen cultivos de cobertura y control de malezas en parte de la superficie o en una determinada época del año. Sin embargo, se presenta como desafío tener que reemplazar la aplicación de herbicidas en las hileras del cultivo, al pie de las cepas y en la base de los postes del sistema de conducción.

En la línea de plantación, los tres primeros años se utilizó un prototipo de flameado de gas licuado de petróleo (GLP) diseñado en INTA EEA Mendoza (21).

A partir de la temporada 2014/15, se efectuó desorillado tradicional y mediante un equipo intercepa automático disponible en el mercado. Paralelamente, se iniciaron trabajos para desarrollar un prototipo intercepa mecánico con el que se controlan las malezas en la línea de plantas de la parcela desde la temporada 2016/17. El equipo se encuentra en fase de desarrollo, pruebas y mejoras, posee dos discos cóncavos dentados dispuestos con cierta inclinación de ataque a la superficie del suelo, complementado en la parte trasera con una rueda sensora que produce el desplazamiento, de accionamiento mecánico, al interfilar de un disco plano horizontal.

Prevención de enfermedades y plagas

Al inicio de la transición de manejo se diseñó un programa de tratamientos preventivos de enfermedades fúngicas sobre la base de aplicaciones de azufre (espolvoreo y mojable), productos cúpricos (oxicloruro e hidróxido de cobre), extracto líquido de cítricos y control biológico de la polilla del racimo mediante *Bacillus thuringiensis*. Sin embargo, se sabe que el azufre puede afectar enemigos naturales, el cobre es un metal pesado y está restringido para cultivos orgánicos, mientras que en general los insumos alternativos ofrecidos para cultivos ecológicos son comparativamente más costosos. Por ello se buscaron opciones para reemplazarlos y alcanzar un programa fitosanitario más eficiente. Partiendo de la base que la idea en la agroecología es ir reduciendo el uso de insumos.

A partir de la temporada 2015/16 se efectuó un primer y único tratamiento primaveral con azufre y cobre, y posteriormente se realizaron aplicaciones periódicas de bicarbonato de sodio, bentonita y té de compost como preventivos fúngicos. Desde la temporada 2016/17 se utilizó la técnica de confusión sexual mediante la colocación de difusores de feromonas sintetizadas para control de la polilla del racimo. Este último tratamiento fue exigido por la autoridad fitosanitaria nacional.

Comparación de costos de manejo

Se realizó un análisis comparativo de costos operativos entre el cultivo de vid bajo manejo agroecológico (AE), ejecutado en la parcela demostrativa, y el manejo convencional (CO) realizado en el resto de las parcelas de INTA EEA Mendoza, para la temporada 2017/18.

Se tuvieron en cuenta gastos de combustible, mano de obra y otros insumos necesarios para las tareas anuales por unidad de superficie (hectárea).

Análisis estadísticos

Los datos obtenidos en las experimentaciones fueron sometidos a análisis de la varianza (ANAVA) y comparación de medias utilizando la prueba LSD de Fisher, mediante el software InfoStat/L (6).

Resultados y discusión

Hormigas

Los formícidos colectados, pertenecían a cuatro subfamilias, ocho géneros y 17 especies. Más del 90% de los formícidos colectados correspondieron a dos subfamilias: *Myrmicinae* y *Dolichoderinae*. Las especies más abundantes fueron *Dorymyrmex wolffhugeli* y *Pheidole bergi* representando entre las dos más del 50% del total.

Las *Dorymyrmex* son forrajeras generalistas mientras que el género *Pheidole* está integrado por individuos que para alimentarse atacan a otros insectos

o deambulan en busca de sustancias azucaradas (9), por lo que no representan un riesgo para el cultivo. Al evaluar comparativamente los índices de diversidad con los de una parcela bajo manejo convencional, la parcela con manejo agroecológico registró mayor abundancia de hormigas (tabla 1), aunque sin diferencias respecto de riqueza (5).

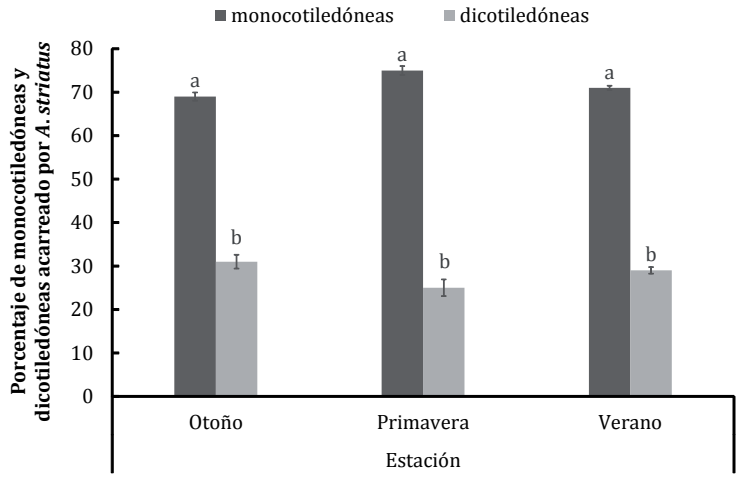
Debido a la abundancia de hormigas cortadoras de hojas (*Acromyrmex striatus*) en dicho viñedo se evaluó su actividad forrajera mediante el estudio de la composición de la dieta y sus preferencias de acuerdo con la oferta vegetal.

Se observó que *A. striatus* prefirió el forrajeo de material verde (recién cortado), en una proporción del 60 al 65% dependiendo de la estación del año, en comparación con el material seco y predominó la colecta de monocotiledóneas (figura, pág. 115). Por consiguiente, a pesar de representar un riesgo potencial para el cultivo de la vid, si este presenta oferta de cobertura verde en sus interfilares, el daño provocado por esta hormiga cortadora no sería tan significativo, así como tampoco los costos asociados al control de las mismas.

Tabla 1. Abundancia absoluta de formícidos en viñedos bajo diferentes prácticas de manejo.

Table 1. Absolute abundance of formicides in vineyards under different management practices.

		Parcela	
Familia	**Especie**	**Agroecológica**	**Convencional**
FORMICINAE	*Brachymyrmex* sp.	76	28
	Camponotus mus	207	33
	Camponotus punctulatus	212	145
MYRMICINAE	*Acromyrmex striatus*	91	10
	Acromyrmex lobicornis	0	0
	Pheidole bergi	1618	435
	Pheidole spininodis	1008	454
	Pheidole aberrans	34	8
	Pheidole triconstricta	4	0
	Solenopsis sp.*1*	651	136
	Solenopsis sp.*2*	133	32
	Solenopsis sp.*3*	217	68
	Solenopsis sp.*4*	106	13
	Strumigenys rogeri	0	0
DOLICHODERINAE	*Dorymyrmex wolffhugeli*	2200	747
	Dorymyrmex flevescens	15	0
	Dorymyrmex sp.*1*	1	0
	Forelius chalybaeus	920	25
PONERINAE	*Hypoponera opacior*	8	0
∑		7501	2134



Diferentes letras indican diferencias significativas (p≤0,05).
Different letters indicate significant differences (p≤0.05).

Figura. Porcentaje de monocotiledóneas y dicotiledóneas acarreado por *A. striatus* hacia los nidos, en diferentes estaciones del año dentro de un viñedo.

Figure. Percentage of monocotyledons and dicotyledons carried by *A. striatus* towards the nests, in different seasons of the year within a vineyard.

Por lo tanto, el uso de cultivos de cobertura podría ser un recurso para el manejo de *A. striatus* en el viñedo, siempre y cuando estas especies vegetales no compitan excesivamente con el cultivo.

Cultivos de cobertura y biofertilizantes

Cultivos cobertura

Investigaciones locales previas mostraron que el manejo con cultivos de cobertura perennes durante al menos cinco años logra mejorar la fertilidad del suelo (18).

No obstante, pueden competir con las plantas de vid por agua y nutrientes, en mayor o menor medida dependiendo de la especie (25, 26), reducir su expresión vegetativa y producción (20), modificar el microclima a nivel de racimos (22) y mejorar las características sensoriales de los vinos tintos (23).

La cobertura permanente establecida inicialmente en la parcela provocó excesiva competencia con el cultivo, lo cual se evidenció en una reducción del rendimiento inicial promedio desde las 8 a 9 t ha^{-1} hasta las 5 a 6 t ha^{-1} en el trascurso de dos a tres años.

Resultados preliminares para el ensayo de alternativas de manejo del suelo de baja competencia iniciado en 2016, mediante el uso de coberturas vegetales interfilar por medio, no manifestaron diferencias en producción y calidad de la uva luego del primer año de establecidos los tratamientos, mientras que a partir de la segunda temporada se detectaron los efectos del manejo del suelo: el tratamiento TM se diferenció significativamente de TT y PT (entre 5,5 y 5,8 t ha^{-1}), por una mayor producción de algo más de 8,0 t ha^{-1}, lo cual se estima próximo

al potencial productivo de la variedad para obtener vinos de cierta calidad para este terruño, presentando además un pH significativamente mayor a PM y PT, aunque los valores fueron muy próximos.

El tratamiento PM presentó una producción intermedia cercana a las 7,0 t ha^{-1}, sin diferenciarse estadísticamente del resto de los cultivos cobertura (tabla 2).

Compost

Cuando se compararon dos equipos para el volteo y aireación del compost (máquina volteadora y reja) se logró obtener un compost estable y maduro, similar composición final, en un lapso análogo de alrededor de cuatro meses. La máquina volteadora resultó ser más eficiente para compostajes de mediana a gran escala, mientras que la reja se adaptaría mejor para elaboraciones de menor escala (27).

Al comparar los sucesivos compostajes en el transcurso de las diferentes temporadas, de manera general, se puede observar que los volteos mecánicos y los riegos con cintas de goteo mejoraron la estabilidad final de los compost, obteniendo menores valores de relación C:N, menor salinidad, mayores contenidos de N, P, K y materia orgánica, en menor tiempo de compostaje (tabla 3, pág. 117).

Té de compost

El té de compost se utiliza como fertilizante líquido en aplicaciones foliares, porque aporta nutrientes solubles y como supresor de enfermedades de las plantas, ya que genera resistencia inducida, antibiosis y competencia, cuando se realizan aplicaciones preventivas (7).

En la experiencia realizada en 2014, se observó aumento de actividad de bacterias aerobias mesófilas y leve tendencia de incremento de hongos en los extractos más aireados.

Tabla 2. Efecto de cultivos de cobertura en la producción y calidad de la uva cv. Cabernet sauvignon, INTA EEA Mendoza. Resultados preliminares.

Table 2. Effect of cover crops in grape production and quality of cv. Cabernet Sauvignon, INTA EEA Mendoza. Preliminary results.

	Tratamiento	Rendimiento (t ha^{-1})	Azúcar (°Brix)	pH	Acidez (g l^{-1})	Fenoles totales (mg kg^{-1})	Antocianos (mg kg^{-1})
Cosecha 2017	TM	5,36 a	23,5 a	3,79 a	6,29 a	2446 a	1601 a
	PM	5,07 a	23,1 a	3,75 a	6,11 a	2368 a	1566 a
	TT	5,47 a	23,9 a	3,81 a	6,17 a	2408 a	1908 a
	PT	5,09 a	23,5 a	3,78 a	6,25 a	2723 a	1883 a
Cosecha 2018	TM	8,34 a	24,7 a	3,95 a	6,54 a	-	-
	PM	7,15 ab	24,8 a	3,84 b	6,73 a	-	-
	TT	5,53 b	24,7 a	3,90 ab	6,94 a	-	-
	PT	5,81 b	24,0 a	3,84 b	6,32 a	-	-

Referencias de tratamientos según foto (pág. 110).

References of treatments according to photo (page 110).

Letras distintas para cada temporada indican diferencias significativas entre las medias (p≤0,05; n=4).

Different letters for each season indicate significant differences between means (p≤0.05; n=4).

Tabla 3. Comparación de la composición final del compost, obtenido mediante diferentes metodologías de compostaje con diversos materiales iniciales, en el transcurso de las temporadas de experimentación.

Table 3. Comparison of the final compost composition, obtained through different composting methodologies with diverse initial materials, during the experimental seasons.

Año	Riego	Volteo	Material inicial
2010	aspersión	no	cama pollo, orujo, chala ajo
2011	aspersión	no, ventilación pasiva	guano gallina, orujo, chala ajo
2012	aspersión	sí, manual	cama pollo, orujo, chala ajo
2013	cinta goteo	sí, reja volteadora	guano caballo, cama pollo, orujo, hojas, chala ajo
2014	cinta goteo	sí, reja y máquina volteadora	guano de vaca, hojas, segado de alfalfa, orujo, aserrín
2015	cinta goteo	sí, reja volteadora	orujo fresco, orujo agotado, borras

Año	Tiempo (meses)	Relación C:N final	N (%)	P (%)	K (%)	CE (dS m^{-1})
2010	10	22	1,8	0,53	1,0	5,3
2011	10	23	0,6	0,01	1,3	6,1
2012	5	13	2,4	0,90	2,6	7,2
2013	5	17	2,0	0,45	1,0	3,5
2014	3,5	19	1,5	0,35	0,7	2,6
2015	4	17	2,4	0,29	0,8	2,2

La aplicación del té de compost en las plantas de vid indujo mayor crecimiento secundario (brotación de yemas laterales) y tendencia a menor longitud del brote principal (28).

En el ensayo comparativo iniciado en el año 2016, luego de dos temporadas, los resultados preliminares mostraron tendencias ligeramente significativas al aumento de la producción, del 14 al 16% y de la acidez de uva para los tratamientos CO y CO+EC, respecto del TE (tabla 4, pág. 118).

Los niveles productivos en este sector del viñedo son comparativamente más elevados aparentemente debido a una mayor eficiencia en los riegos, ya que son hileras más cortas que el resto de la parcela.

Tabla 4. Producción y calidad de uva para diferentes tratamientos de aplicación de compost (TE: testigo sin enmienda; CO: aplicación de compost al suelo; CO+EC: aplicación de compost al suelo y extracto de compost foliar). Resultados preliminares.

Table 4. Grape production and quality for different treatment of compost application (TE: control without amendment, CO: application of compost to the soil, CO + EC: application of compost to the soil and extract of foliar compost). Preliminary results.

	Tratamiento	Rendimiento ($t\ ha^{-1}$)	Azúcar (°Brix)	pH	Acidez ($g\ l^{-1}$)	Fenoles totales ($mg\ kg^{-1}$)	Antocianos ($mg\ kg^{-1}$)
Cosecha 2017	TE	7,47 a	23,7 a	3,79 a	6,47 a	1633 a	1559 a
	CO	8,23 a	23,6 a	3,83 a	6,21 a	1788 a	1843 a
	CO+EC	6,95 a	23,9 a	3,84 a	6,24 a	1689 a	1680 a
Cosecha 2018	TE	8,54 b	24,5 a	3,81 a	6,07 b	-	-
	CO	9,98 a	24,0 a	3,85 a	6,52 a	-	-
	CO+EC	9,74 a	23,7 a	3,83 a	6,49 a	-	-

Letras distintas para cada temporada indican diferencias significativas entre las medias ($p \leq 0{,}10$; n=4).
Different letters for each season, indicate significant differences between the means ($p \leq 0.10$; n=4).

Fertilidad del suelo

En la tabla 5 (pág. 119), se detalla la evolución de los parámetros de la fertilidad de suelo (de 0 a 30 cm) con los resultados de los análisis realizados al inicio (2009) y durante el manejo agroecológico (2012, 2014 y 2018). Allí se observa cómo la materia orgánica (MO) del suelo fue aumentando luego del cambio de manejo convencional a agroecológico. En el último año se apreció una disminución importante del porcentaje de MO, este hecho puede estar relacionado con los cambios en el manejo del suelo; pasando de tener cobertura permanente en todas las entrehileras a labranza interfilar por medio, en 2014 y cobertura temporaria invernal con rastreado hilera por medio, a partir de 2015.

Es importante destacar que las muestras de suelo fueron compuestas y se tomaron de los interfilares con y sin cobertura.

La concentración de fósforo (P) siguió una tendencia similar a la MO, alcanzando niveles muy altos en 2014 y pasando a tenores bajos en 2018, esta situación puede estar vinculada con los cambios de manejo expresados en los párrafos anteriores: la mayor cantidad de sistemas radicales en estado inicial de desarrollo determinados por las siembras de coberturas anuales, pueden establecer un mayor requerimiento de fósforo (17).

La concentración de nitrógeno (N total) fue oscilando entre valores altos y muy altos, en general con tendencia a incremento respecto del valor inicial, mientras que la concentración de potasio (K) fue aumentando hasta alcanzar niveles altos. La conductividad eléctrica se mantuvo dentro de valores que clasifican al suelo como no salino, aunque en el último año se registró un leve y despreciable aumento. Estos resultados confirman que los cambios en las prácticas de manejo modifican sensiblemente parámetros importantes que determinan la fertilidad de suelo.

Tabla 5. Resultado de análisis de suelo (muestras de 0 a 30 cm) inicial (año 2009) y durante el manejo agroecológico (años 2012, 2014 y 2018). La clasificación se indica entre paréntesis.

Table 5. Soil analysis results (sampling depth: 0-30 cm) initial (year 2009) and during agroecological management (years 2012, 2014, 2018). Classification is indicated in parentheses.

Parámetros / Año	2009	2012
MO (%)	1,28 (medianamente pobre)	1,39 (medianamente pobre)
N total (mg kg^{-1})	837 (alto)	1278 (muy alto)
P (mg kg^{-1})	2,11 (muy bajo)	5,32 (alto)
K (mg kg^{-1})	143 (pobre)	174 (bueno)
CE (dS m^{-1})	0,98 (no salino)	1,02 (no salino)

Parámetros / Año	2014	2018
MO (%)	1,69 (medianamente pobre)	0,79 (pobre)
N total (mg kg^{-1})	940 (alto)	1181 (muy alto)
P (mg kg^{-1})	7,29 (muy alto)	2,90 (bajo)
K (mg kg^{-1})	174 (bueno)	220 (alto)
CE (dS m^{-1})	1,01 (no salino)	1,27 (no salino)

Referencias: MO: materia orgánica; N total: nitrógeno; P: fósforo; K: potasio; CE: conductividad eléctrica del extracto de saturación.

References: MO: organic matter; N total: nitrogen; P: phosphorus; K: potassium; CE: electrical conductivity of saturation extract.

Una primera evaluación de los microorganismos presentes en el suelo y raíces de las plantas de vid reveló la presencia natural de hongos formadores de micorrizas en la parcela demostrativa con manejo agroecológico. En un relevamiento realizado en el año 2016 el porcentaje de micorrización para la parcela donde se aplicó compost al suelo fue del 92%, donde se combinó compost y pulverización de té de compost al follaje fue del 90% y en el testigo sin aplicación fue del 80%. Los tratamientos no mostraron diferencias significativas en la infección micorrícica de las plantas de vid. Las estructuras micorrícicas observadas fueron hifas principalmente y vesículas en menor cantidad, aunque no se encontraron arbúsculos.

Con respecto a otros microorganismos presentes en el suelo, tampoco se observó diferencias significativas entre tratamientos. La población de las bacterias aerobias mesófilas fue mayor que los hongos, con abundancias medias de $1{,}48 \times 10^7$ y $8{,}31 \times 10^4$ unidades formadoras de colonia (UFC) por gramo de suelo seco, respectivamente. Entre los microorganismos mineralizadores del nitrógeno orgánico del suelo, la abundancia de los amonificadores fue cercana a $2{,}14 \times 10^5$ número más probable (NMP) g^{-1} suelo, la de los nitrificadores $2{,}90 \times 10^3$ NMP g^{-1} suelo y finalmente, la de los microorganismos fijadores de nitrógeno $1{,}15 \times 10^7$ UFC g^{-1} suelo.

Control de malezas

El prototipo de flameado utilizado durante los primeros años requirió de personal especializado para su operación, siendo un equipo complejo, de elevado costo y cierto riesgo de operación. Realiza un efecto desecante foliar pero las malezas establecidas rebrotan al transcurrir un tiempo, siendo más efectivo para controlar dicotiledóneas. Con el prototipo de flameado fue necesario realizar tratamientos frecuentes y repasos manuales o mediante bordeadora, para lograr un control adecuado de las malezas, con alto consumo de GLP. Por todo ello, se decidió cambiar de estrategia hacia algún sistema de labranza de bajo costo.

El prototipo intercepa utilizado actualmente es relativamente efectivo para controlar las malezas en la línea de plantación, requiere escaso mantenimiento y es de bajo costo relativo. Eventualmente es necesario realizar un leve repaso manual del desmalezado.

Enfermedades y plagas

La incorporación de estrategias de gestión del viñedo para mejorar la biodiversidad mediante el establecimiento de corredores biológicos y cultivos de cobertura, como también la búsqueda de la mejora de la fertilidad integral del suelo y de las plantas de vid, mediante la incorporación de compost, té de compost y bioles, buscaron favorecer un agroecosistema más equilibrado, que albergue diversos artrópodos benéficos útiles para la predación y el parasitismo de plagas. Aunque hasta el momento no hay datos de relevamientos específicos, en la parcela demostrativa se observó una importante actividad de artrópodos valiosos para el control de plagas y enfermedades (coccinélidos, microhimenópteros, sirfidos, arácnidos, entre otros).

En experiencias en viñedos de la zona, se ha verificado una disminución de la incidencia de las principales enfermedades fúngicas cuando el viñedo posee cultivos de cobertura (datos no publicados), en concordancia con estudios realizados en otros viñedos del mundo (4). Este efecto se vincula con la mejora en el microclima en la zona de racimos, ya que se presenta vegetación menos densa, con mayor aireación e incidencia de radiación solar, lo cual determina condiciones desfavorables para el ataque de hongos.

Se buscó aumentar la actividad biológica, tanto en el suelo como en el follaje, para asistir en la supresión de enfermedades mediante resistencia inducida, antibiosis y competencia con patógenos.

La correcta nutrición de la vid ayudará además para que el cultivo pueda defenderse mejor ante la irrupción de enfermedades o plagas emergentes.

Resulta importante destacar que en el transcurso de las nueve temporadas de cultivo no se presentaron incidencias fitosanitarias de importancia, tanto plagas como enfermedades fueron controladas satisfactoriamente.

En los primeros años de transición se observaron algunos ataques puntuales de hormigas cortadoras, pero estos daños fueron disminuyendo con el transcurso del tiempo hasta mantenerse en baja incidencia, sin necesidad de efectuar controles específicos.

Una temporada en particular (año 2013/14) se presentó con mayor predisposición para el ataque de oidio, no obstante ello el viñedo agroecológico no fue mayormente afectado, a diferencia del resto de la viña con manejo convencional que presentó severa disminución de la producción, aparentemente por problemas de efectividad en las aplicaciones fitosanitarias.

Costos de manejo

El manejo de la parcela demostrativa agroecológica (AE) significó solo un 5,8% más costoso que el manejo convencional (CO) del viñedo vecino (tabla 6).

Tabla 6. Comparación de costos en dólares de insumos y mano de obra por hectárea y por año, para dos tipos de manejos del cultivo: agroecológico y convencional. Luján de Cuyo, Mendoza (18 de Junio 2018: $USD 1 = $ARS 28,5).

Table 6. Comparison of costs in dollars of inputs and workforce per hectare and per year, for two types of crop management: agroecological and conventional. Luján de Cuyo, Mendoza (June 18, 2018: $ USD 1 = $ ARS 28.5).

	Manejo agroecólogico			Manejo convencional		
Actividades [1]	**Insumos**	**Mano de obra**	**Total**	**Insumos**	**Mano de obra**	**Total**
Fertilización [2]	$ 352	$ 37	$ 390	$ 280	$ 8	$ 289
Tratamientos fitosanitarios [3]	$ 264	$ 49	$ 314	$ 399	$ 24	$ 423
Control de malezas [4]	$ 48	$ 86	$ 134	$ 110	$ 35	$145
Cobertura vegetal [5]	$ 102	$ 27	$ 128	-	-	-
Manejo del viñedo [6]	-	$ 1.153	$ 1.153	-	$ 1.146	$ 1.146
TOTAL	$ 767	$ 1.352	$ 2.119	$ 789	$ 1.214	$ 2.002

[1]Se considera que el cultivo agroecológico produce 7.000 kg ha^{-1} y el convencional 8.000 kg ha^{-1}.

[2]El manejo agroecológico incluye compra de compost y elaboración de biol. El manejo convencional incluye aplicación de urea y 18.46.00 en la misma proporción de nitrógeno que la aportada por el compost.

[3]Otros insumos (sin gasoil): difusores para confusión sexual de *Lobesia botrana*, azufre micronizado, oxicloruro de cobre, bentonita, bicarbonato y té de compost en el manejo agroecológico. Clorantraniliprole + abamectina, ametoctradin + dimetomorf, boscalid + pyraclostrobin, azufre micronizado y oxicloruro de cobre en el manejo convencional.

[4]El manejo agroecológico incluye cuatro pasadas con desorilladora mecánica (prototipo INTA EEA Mendoza) y un repaso manual con azadón por año. El manejo convencional incluye el uso de herbicida glifosato al 62,5% (2 l ha^{-1}) aplicado cuatro veces en la línea de plantas y tres rastreadas en todos los interfilares por ciclo de cultivo (que incluye la formación de surcos).

[5]En el cultivo agroecológico se incluye la siembra de la cobertura vegetal anual invernal en todas las hileras; rolado (hilera por medio) una vez al año; rastreado (hilera por medio), segado de la cobertura (hilera por medio) y surqueado (todas las hileras) dos veces al año. En el convencional no se considera el uso de cobertura vegetal, sino que se maneja con suelo descubierto.

[6]Incluye poda, desbrote, cruzado de brotes, despampanado, preparación manual del riego (limpieza de cupos), riego y cosecha para ambos tratamientos.

[1]It is considered that the agroecological crop produces 7,000 kg ha^{-1} and the conventional 8,000 kg ha^{-1}.

[2]Agroecological management includes purchase of compost and self-elaboration of bioslurry. Conventional management includes application of urea and 18.46.00 in the same proportion of nitrogen as that provided by compost.

[3]Other inputs (without gasoil): *Lobesia botrana* sexual confusion diffusers, micronized sulfur, copper oxychloride, bentonite, bicarbonate and compost tea in agroecological management. Chlorantraniliprole + abamectin, ametoctradin + dimetomorf, boscalid + pyraclostrobin, micronized sulfur and copper oxychloride in conventional management.

[4]Agroecological management includes four passes with a mechanical weeder (prototype INTA EEA Mendoza) and a manual hoeing per year. Conventional management includes the use of 62.5% glyphosate herbicide (2 l ha^{-1}) applied four times in the plants row and three disc harrowing in all the interrows per crop cycle (which includes furrow formation for irrigation).

[5]Agroecological cultivation includes seeding of winter annual cover crop in each interrow; rolled (a row of two) once a year; harrowing (a row of two), cover crop mowing (a row of two) and furrow formation (each row) twice a year. In the conventional crop, the use of vegetal cover is not considered, it would be handled with bare soil.

[6]Includes pruning, remove of shoots, shoot crossing, top cutting of shoots, manual preparation of irrigation (cleaning of channels), irrigation and harvesting for both treatments.

Los costos de mano de obra fueron 11% mayores en el manejo AE; este valor es relativamente bajo debido a que se considera la aplicación mecánica del compost y el control mecánico de malezas, tareas que si se hicieran de manera manual consumirían mayor cantidad de jornales.

El control de malezas fue 7% menor que el control químico con un herbicida sistémico en la línea de plantas sumado al rastreado de los interfilares utilizado en el cultivo CO. El costo de los insumos necesarios fue solo 3% superior en el manejo CO, lo que significa USD$ 22 más por hectárea que el cultivo AE.

Realizando las actividades mencionadas para cada tipo de manejo, y considerando un ingreso idéntico para ambas situaciones de USD$ 0,727 por kilo de uva, la ganancia bruta en el cultivo CO produciendo 8 t ha^{-1} sería de USD$ 5.816, mientras que para el sistema AE produciendo 7 t ha^{-1} sería de USD$ 5.089.

Teniendo en cuenta los costos mencionados, la ganancia neta sería de USD$ 3.814 y USD$ 2.970 por hectárea, respectivamente.

Sin embargo, con la producción agroecológica se espera obtener uvas que sean reconocidas con un precio diferencial de comercialización, ya sea por contar con la certificación orgánica (en el marco de la ley Argentina de producción orgánica) o bien por ser utilizadas para elaborar vinos de calidad e inocuidad con alto valor agregado (productos agroecológicos). Si esta diferenciación fuera del 17% equiparía la ganancia del sistema CO. Asimismo, como se evidenció en el ensayo de cultivos de cobertura (tabla 2, pág. 116), con un manejo del suelo de mínima competencia (coberturas anuales hilera por medio) a partir del segundo año se alcanzan rendimientos similares a los del manejo convencional.

Conclusiones

La parcela demostrativa permitió experimentar diversas alternativas de manejo bajo condiciones similares a las que se presentan en una propiedad vitícola de la escala más representativa de los viñedos del país (1 a 5 ha). Se probó maquinaria existente y nuevos equipos agrícolas, desarrollando y adaptando diferentes tecnologías para facilitar el manejo agroecológico a las condiciones de los viñedos de Mendoza. La parcela fue visitada periódicamente por productores, estudiantes universitarios, profesionales e investigadores interesados en la búsqueda de prácticas alternativas de manejo.

La transición de manejo convencional del viñedo hacia un sistema de gestión con enfoque agroecológico mediante el establecimiento de corredores biológicos, manejo de cultivos de cobertura y utilización de enmiendas biológicas permitió aumentar la biodiversidad y mejorar la fertilidad del suelo, logrando con el tiempo niveles productivos próximos a los del manejo habitual a través de un esquema de aplicación de agroquímicos, labranzas y fertilizaciones con productos de síntesis. Resulta imprescindible efectuar el seguimiento de la evolución de los parámetros de fertilidad edáfica, ya que su dinámica temporal es modificada por cambios en las prácticas de manejo.

Las prácticas propuestas confieren potencialmente diversos servicios ecosistémicos como el mantenimiento y mejora de la calidad del suelo y agua, reducción del riesgo de erosión hídrica y eólica, atenuación de la temperatura, secuestro de dióxido de carbono atmosférico y preservación de insectos benéficos para el manejo integrado de plagas y enfermedades. También influyen en el balance

hídrico de los viñedos, el micro y meso-clima, el rendimiento, la calidad de las cosechas y los vinos, entre otros factores.

Los tratamientos fitosanitarios con insumos alternativos de bajo impacto y el manejo cultural de las malezas permitieron mantener satisfactoriamente las condiciones sanitarias del cultivo luego de nueve temporadas agrícolas. Los costos operativos de manejo se aproximaron a los de un manejo convencional. Las estrategias de manejo planteadas pueden aumentar la resiliencia de los agroecosistemas vitícolas de zonas especialmente frágiles, promover la conservación de los recursos naturales, favorecer el equilibrio ecológico y la mitigación del cambio climático, reduciendo las necesidades de agroquímicos y con mayor armonía en zonas de transición urbano-rurales.

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Actual evapotranspiration and the pattern of soil water extraction of a soybean (*Glycine max*) crop

Evapotranspiración real y patrones de extracción de agua del suelo de un cultivo de soja (*Glycine max*)

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Abstract

Crop evapotranspiration knowledge during different phenological stages helps determine crop water requirements and water use efficiency. This study was intended to estimate evapotranspiration of soybean grown under field conditions using the water balance equation and to characterize root water extraction across different soil layers analyzing daily values of its availability. In order to estimate the crop daily water consumption, temporal and spatial variability (vertical) of soil water content up to a depth of 1.10 m was investigated. At the beginning of the experiment, measurements showed that the soybean crop extracted water from the upper levels, and as it continued to grow, water uptake at deeper levels increased. The highest water uptake occurred during reproductive growth stages, which matched the period of highest atmospheric demand. The crop showed a better response to atmospheric demand under water availability, whereas under stress conditions, both evapotranspiration and soil water content decreased.

Keywords
soil water balance • rainfed • Argentina • water stress • water uptake • soybean

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Resumen

Conocer la evapotranspiración de un cultivo durante sus distintos estadios fenológicos ayuda a determinar los requerimientos de agua del mismo y la eficiencia del uso de agua. Los objetivos de este trabajo fueron estimar la evapotranspiración de un cultivo de soja desarrollado bajo condiciones de campo, utilizando la ecuación de balance hídrico, y caracterizar la extracción de agua por parte de las raíces en las distintas capas del suelo, analizando los valores diarios de su disponibilidad. Para determinar los consumos diarios del cultivo se estudió la variabilidad temporal y vertical del contenido de agua en el suelo hasta 1.10 m de profundidad. Al comienzo de las mediciones, el cultivo de soja extrajo agua de los niveles superiores, y a medida que se desarrollaba, aumentó el consumo en niveles más profundos. El mayor consumo de las plantas se dio en los estadios reproductivos, coincidiendo con el período de mayor demanda atmosférica. El cultivo mostró una mejor respuesta a la demanda atmosférica bajo la disponibilidad de agua, mientras que, en condiciones de estrés, tanto la evapotranspiración como el contenido de agua en el suelo disminuyeron.

Palabras clave
balance de agua en el suelo • secano • Argentina • estrés hídrico • consumo de agua • soja

Introduction

The high growth rates of global population demand a substantial increase of food production. Soybean is one of the most important crops in terms of production, worldwide trade and harvested area (35). Argentine soybean harvested area has grown at an average rate of 7.3 x 10^5 ha/year from 1997 to 2016 which leads to 1.95 x 10^7 ha and 5.88 x 10^7 t in 2016 (21). The increased demand of agricultural food production requires a clever analysis of water needs over the crop cycle to ensure its maximum efficiency (36). Significant importance has been given to studies on evapotranspiration at different phenological stages, particularly in critical periods of yield determination (3, 15, 20, 46). These researches allow quantifying, under different management conditions, the amount of crop irrigation water needed to achieve optimal growth or the expected yield loss (10, 29).

Soil water balance is a method used for the estimation of actual evapotranspiration (ET_a) which considers the water balance within the soil depth explored by plant roots, and analyzes only the vertical components of water movements (26). Though this is not the method recommended by FAO (2006) for evapotranspiration estimation, it has virtually no restrictions for use. Besides, it facilitates decision-making on water management, since production in Argentina is mostly under rainfed agricultural systems (34). The use of this methodology requires rainfall and soil moisture measurements at appropriate scales in space and time. Soil moisture, the most difficult of both variables to measure, is critical for the evolution of meteorological variables, as its controls the water and energy exchanged by a surface with the atmosphere (23, 26).

The study on soil water extraction patterns (*WEP*) is used to obtain information about the spatial and temporal plant water consumption variability. Plant water uptake is affected by soil texture and vertical root distribution (5, 43). However, these authors (32) consider learning of *WEPs* is more useful than observing root density. Some researches focuses on the vertical distribution of water extraction in the soil profile (2, 5, 11), while others analyze temporal rate of water extraction (7, 14, 31, 38, 43).

Most water-extraction studies were performed in experimental plots or in the laboratory in controlled environments (39), which can hardly represent real field conditions. There is a gap between actual yields obtained by growers and the potential yield of the best-adapted crop varieties, under good management conditions and in absence of biotic and abiotic stresses (24). It is therefore important to record in productive plots the evolution of variables that are usually measured in controlled experiments, considering that their behavior should not change. Therefore, the objectives of this research are: (a) to learn of the daily ET_a in a field soybean plot, its variability compared to crop evapotranspiration under standard conditions, and its correlation to other meteorological variables, and (b) to study *WEPs* in order to investigate the relative contribution to ET_a of each soil layer.

Materials and methods

Sampling site and data collection

The study was carried out in the Unidad Integrada Balcarce [Unidad Integrada Balcarce] (UIB, Facultad de Ciencias Agrarias de la Universidad Nacional de Mar del Plata - Estación Experimental Agropecuaria Balcarce del Instituto Nacional de Tecnología Agropecuaria), located in the southeast of the Province of Buenos Aires. (37°45' S; 58°18' W), Argentina. The meteorological and edaphic variables need to estimate crop evapotranspiration were measured in a 19-ha soybean plot during the 2012-2013 summer season. The soil is classified as a typical Argiudoll, with a loamy clayey texture up to 0.30 m depth and between 0.80 to 1.10 m, and clayey between 0.30 and 0.80 m depth (41). The land has a slope of 1:50 oriented NE to SW. A caliche layer was found at 1.00 m to 1.20 m depth and the water table was considered to be beneath that level. The soybean variety used was DM 3810, Maturity Group III with indeterminate growth habit. The crop was sown on November 21, 2012, and emergence occurred on December 1. The crop was grown under rainfed conditions. Soy growing stages were identified on a weekly basis following phenological scale from these authors (22), the evolution of which is shown in table 1 (page 128).

The information used in this study included daily precipitation records and reference crop evapotranspiration (ET_0) estimated for the 2013 January-March period at the INTA-Balcarce Estación Experimental, located about 1 km away from the experimental site.

A database resulting from soil water content (*SWC*) readings measured with an array of Sentek EnviroSCAN capacitive sensors (Sentek Sensor Technologies, Stepney, Australia), and a Troxler neutron probe Model 4300 Depth Moisture Gauge (Troxler Electronics Laboratories Inc., Research Triangle Park, USA) was previously presented (12).

Table 1. Soybean Phenological Stages (21). Most significant events and dates are shown.

Tabla 1. Estadios fenológicos de soja (21). Se indican los eventos más significativos y las fechas de ocurrencia respectivas.

Phenological stage	Description	Date
	Sowing	November 21
VE	Emergence	December 1
V3	Third-node	January 7
R1 - V8	Beginning bloom	January 25
R3 - V12	Beginning pod	February 5
R5 - V15	Beginning seed	February 22
R7	Full maturity	April 6

The vertical spatial resolution of the database was a depth range of 0.01 m to 1.10 m and the temporal resolution was 15 minutes. The study period started on January 6 and ended on March 15, 2013 (69 days, phenological stages V3 to R5, table 1).

Methodology

Water balance within a soil thickness in a time interval Δt is expressed as Hiilel (1998):

$$SWC_t = SWC_{t-\Delta t} + PP_t - ET_t - Per_t - R_t \quad (1)$$

where:

SWC_t and $SWC_{t-\Delta t}$ = the soil water content measured at time points t and $t-\Delta t$, respectively

PP_t = precipitation

ET_t = evapotranspiration

Per_t = deep percolation

R_t = surface runoff, all of them for time t

Soil water content, precipitation, percolation and runoff measurements can be used to estimate soil water loss to the atmosphere by evapotranspiration, clearing this variable from Equation (1) and integrating at successive time intervals. Equation (1) could include other terms, which were not considered in this work such as irrigation, horizontal flow of water in soil and water movement from water table through capillary rise due to the fact that the crop grows under rainfed conditions, and the contributions of water horizontal fluxes and capillarity are neglegible.

For the estimation of *SWC,* high-resolution humidity profiles interpolated at 12:00 UTC (9:00 local time) were selected to coincide with the time at which precipitation was measured. This data was considered day-representative. A 24 h time interval (Δt) was used. Estimation of *SWC* for the soil profile ([0;1.1m]) was done by numerical integration of soil moisture profiles using the trapezoidal rule and expressing the result as water depth units (mm). Using the same methodology, the field capacity *(FC)* and permanent wilting point *(PWP)* values from this author (6) were integrated for the soil profile to estimate the available water *(AW)* (13).

In order to characterize soil water variability across different layers, four regular partitions were taken from the profile: 0-0.275, 0.275-0.55, 0.55-0.825 and 0.825-1.10 m. Partitions do not match with the soil stratigraphy (12). Soil water content (SWC_k) and soil water availability (AW_k) series for each layer

were obtained using the same method as for the entire profile with the information in table 2 (page 130). Decline in SWC_k was used to detect plant water uptake while increases were associated to water inflow. These values were integrated on a daily basis and for the V3-V8, R1-R2 and R3-R4 crop growth periods (table 1, page 128). Additionally, the percentage share contributed by each layer to the total water consumption, previously defined as *WEP*, was estimated. Also, average daily consumption was assessed for each individual level at the phenological periods mentioned before.

For the estimation of ET_a, Equation 1, (page 128), was solved for the total soil profile considering the *SWC* value integrated up to 1.10 m ($SWC_{0\text{-}1.10\ m}$). No estimations of surface runoff were done. Deep percolation and capillary rise from the water table were neglected due to their low incidence in the soil water balance. On days with precipitation, evapotranspiration was not estimated since temporal scales of processes represented by each term of Equation 1 (page 128), are different (37). The days in which an increase in $SWC_{0\text{-}1.10\ m}$ was found without previous rainfall record were also excluded.

A regression analysis between ET_a, crop evapotranspiration under standard conditions (ET_c) and $SWC_{0\text{-}1.10}$ was carried out in order to characterize the dominant drivers of actual evapotranspiration. Crop evapotranspiration under standard conditions was estimated as $ET_c = K_c\ ET_0$, where K_c is the crop coefficient (1). K_c values were obtained using the empirical equation developed by these authors (17) for soybean grown in Balcarce:

Water stress was studied for those cases where ET_a resulted lower than ET_c.

The Mann-Whitney U Test (9) was used to determine whether the crop was under water stress and to identify periods where plants suffered stress. *SWC* - ET_a/ET_c pairs were ordered by increasing magnitude of *SWC.* By using increasing *SWC* thresholds, the data set was divided into two groups that were sequentially tested to find the threshold value that best represented the difference between the two groups.

Results and Discussion

Soil water content

Measurements showed from the beginning a decreasing $SWC_{0\text{-}1.10\ m}$ (figure 1a, page 130), with occasional increases associated to precipitation events. At the early stages of senescence, this general decreasing trend reverted as a result of the gradual decrease of crop water uptake and the occurrence of long-duration precipitation events. The soil water content measured up to 1.10m depth was always higher than *PWP,* which was estimated at 206 mm.

At the beginning of the study period (January 6 through 25), $SWC_{0\text{-}1.10\ m}$ was above field capacity (369 mm; figure 1a, page 130). Its distribution in the profile showed that the largest soil water storage occurred at the 0.275 - 0.825 m depth range ($SWC_{0.275\text{-}0.55\ m}$ and $SWC_{0.55\text{-}0.825\ m}$, figure 2a, page 131 and table 2, page 130) reaching in some cases more than 100% AW_k for both levels (figure 2b, page 131) and for the entire profile ($AW_{0\text{-}1.10\ m}$; figure 1b, page 130).

$$Kc = 2.30 \times 10^{-8}(DAE)^4 - 7.29 \times 10^{-6}(DAE)^3 + 5.62 \times 10^{-4}(DAE)^2 + 4.93 \times 10^{-5}(DAE) + 0.32 \quad (2)$$

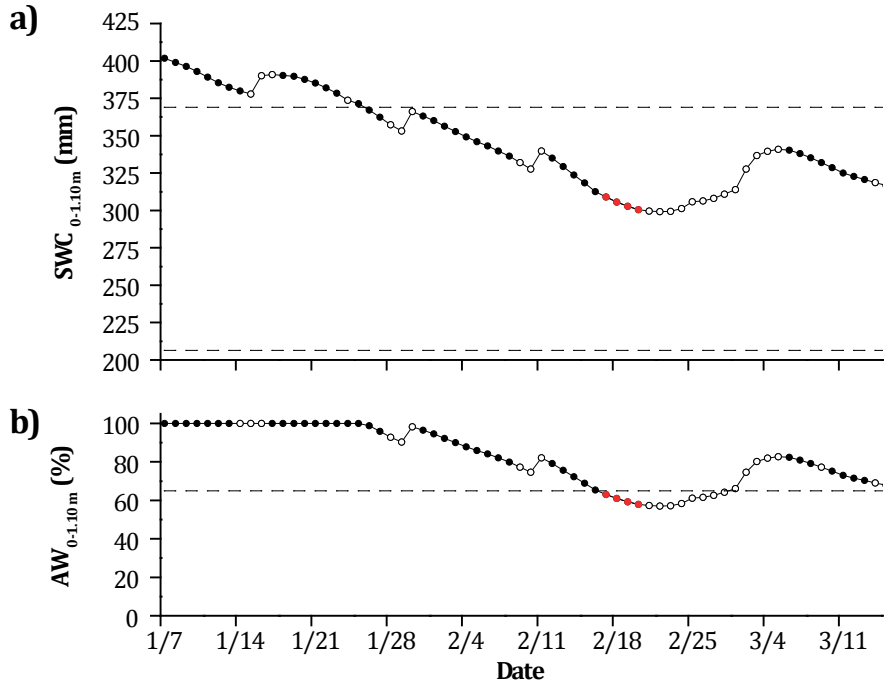
where:

DAE = days after emergence

Table 2. Field Capacity (FC_k), Permanent Wilting Point (PWP_k) and Saturation Point (SAT_k) expressed as depth of water (mm) for Balcarce at different soil depths (6, 39). Variables are expressed in average values for the defined layers.

Tabla 2. Capacidad de campo (CC_k), punto de marchitez permanente (PMP_k) y punto de saturación (SAT_k) expresados como lámina de agua (mm) para la localidad de Balcarce a distintas profundidades (6, 39). Los valores de las variables son promedios para las capas definidas.

Soil depth (m)	PWP_K	FC_k	SAT_k
0 - 0.275	41.3	94.9	145.6
0.275 - 0.55	56.0	102.0	162.3
0.55 - 0.825	57.6	95.6	169.0
0.825 - 1.10	52.3	77.0	143.0

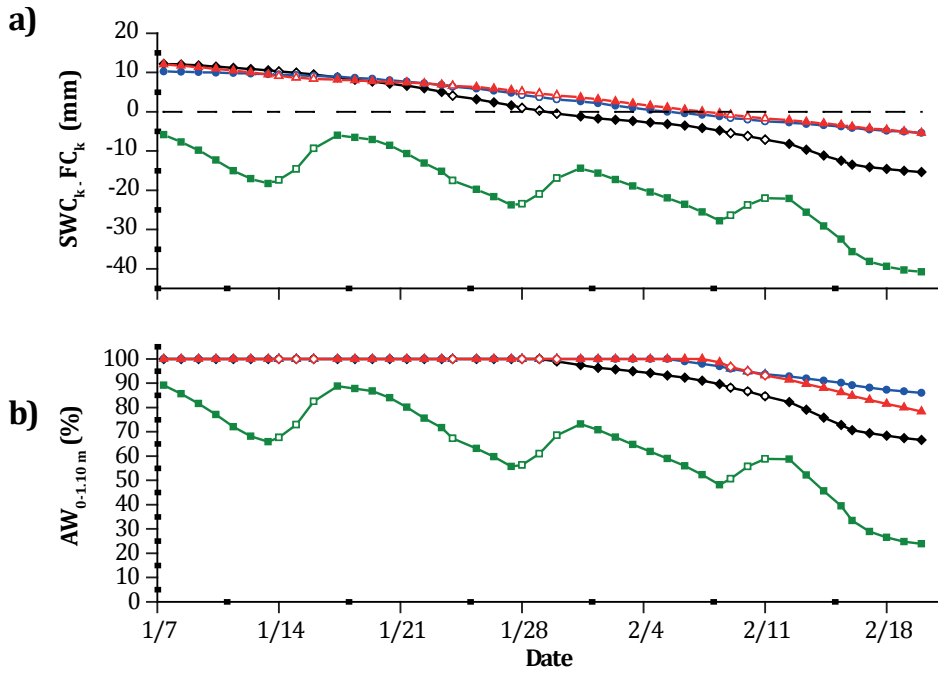


The dotted lines in (a) represent the field capacity (*FC*) and permanent wilting point (*PWP*) values. The dotted line in (b) indicates the $AW_{0\text{-}1.10m}$ threshold for plant stress (65% $AW_{0\text{-}1.10\,m}$); red icons show cases in that condition. The empty icons indicate the days not considered for the estimation of evapotranspiration (ET_a), where precipitations or an increase in the daily soil water storage occurred.

Las líneas punteadas en (a) representan los valores de capacidad de campo (*CC*) y punto de marchitez permanente (*PMP*). Los íconos rojos y la línea punteada en (b) indican el valor a partir del cual la humedad del suelo se encuentra en condiciones de estrés (65% $AU_{0\text{-}1,10\,m}$). Los íconos vacíos indican los días que no se consideraron para las estimaciones de evapotranspiración (ET_r), por ser días con precipitación o con aumento del almacenaje diario de agua en el suelo.

Figure 1. Water Content Evolution ($SWC_{0\text{-}1.10\,m}$; a) and soil water availability ($AW_{0\text{-}1.10\,m}$; b), integrated for each date in the entire soil profile.

Figura 1. Evolución del contenido de agua ($CAS_{0\text{-}1,10\,m}$; a) y porcentaje de agua útil ($AU_{0\text{-}1,10\,m}$; b), integrado para cada fecha en el total del perfil del suelo.



The empty icons indicate the days not considered for the estimation of evapotranspiration (ET_a), where precipitations or an increase in the daily soil water storage occurred.

Los íconos vacíos indican los días que no se consideraron para las estimaciones de evapotranspiración (ET_r), por ser días con precipitación o con aumento del almacenaje diario de agua en el suelo.

Figure 2. Difference between the soil water content and the field capacity value (SWC_k- FC_k; a) and soil water availability (AW_k; b) for the 0-0.275 (green squares), 0.275-0.55 (black diamonds), 0.55-0.825 (blue circles) and 0.825-1.10 m (red triangles) layers.

Figura 2. Diferencia entre el contenido de agua en el suelo y el valor de capacidad de campo (CAS_k- CC_k; a) y del porcentaje de agua útil (AU_k; b) para los estratos de 0-0,275 (cuadrados verdes), 0,275-0,55 (rombos negros), 0,55-0,825 (círculos azules) y 0,825-1,10 m (triángulos rojos).

A number of edaphic, biological and meteorological factors can explain this behavior. In the first place, precipitation in December was 239.9 mm, which included heavy rainfall events on December 19 and 24 (77 mm and 88 mm, respectively).

In addition, precipitation recorded on January 1 and 5 was 26.5 mm and 20 mm, respectively. Secondly, there is an assumption of shallow rooting during the heavy precipitation period (31, 43) because crop emergence occurred in the early days of December.

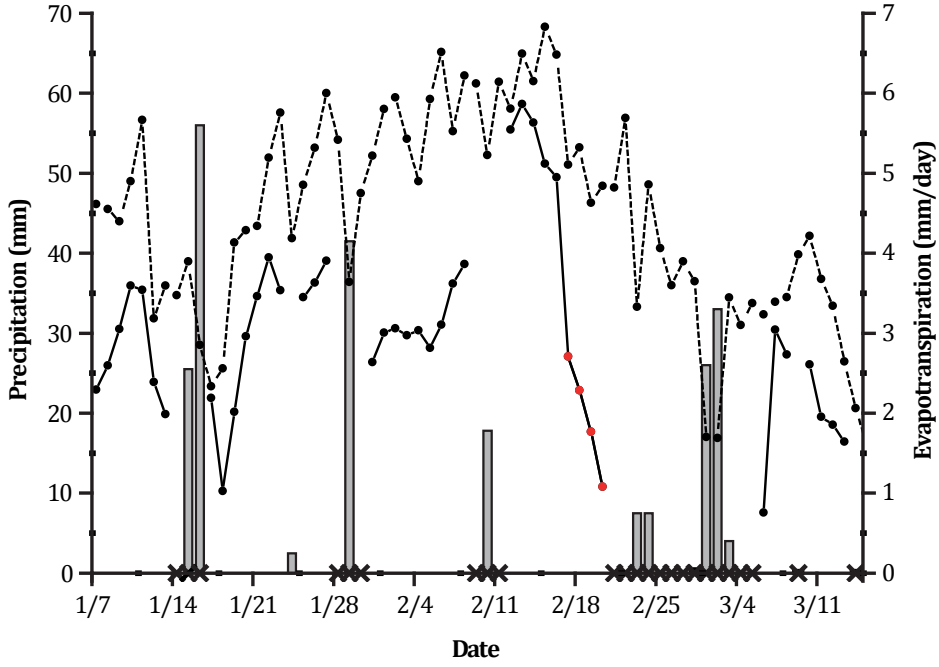
Lastly, the presence of clay in the intermediate soil layers (41) and caliche in the lower levels may have slowed down water movement and reduced percolation to deeper levels. These factors could prevent precipitation from rapidly flowing out of the system by percolation or evapotranspiration.

Actual Evapotranspiration

ET_a increased gradually to reach a maximum value between February 12 and 15, and then decreased (figure 3, page 133). The highest estimated value was 5.9 mm/day on February 13. The highest ET_c value recorded for soybean was 6.8 mm/day on February 15 (77 DAE). This result is similar to that obtained by these authors: Della Maggiora *et al.* (2000), for a soybean crop in Balcarce of approximately 7 mm/day 70 DAE. The mean and median ET_a values for the complete period were both at 3.0 mm/day, with a standard deviation of 1.2 mm/day and an interquartile range of 1.3 mm/day.

ET_a was always lower than *ETc*, even during the periods when $AW_{0\text{-}1.10m}$ was 100%, which suggests that there has been another factor limiting evapotranspiration other than water availability. The dry biomass value for the V3/V4 stage was 308 kg/ha, with a row spacing of 0.38 m and a plant density of 383000 plants/ha. For a soybean crop, at the same development stage and in the same region, under controlled water conditions and without limitations, Della Maggiora *et al.* (2006), obtained 324 kg/ha with a row spacing of 0.70 m and plant densities between 240000 and 309000 plants/ha. But Andriani *et al.* (1991) found values above 500 kg/ha in rainfed conditions with a row spacing of 0.70 m and plant densities between 270000 y 330000 plants/ha. This information confirmed that the crop growth rate declined during the growing season. It failed to achieve complete coverage thus limiting the evapotranspiration.

The results for the different phenological stages show that the $SWC_{0\text{-}1.10m}$ value decreased to a rate of about 5.5 mm/day until February 21 (figure 1a, page 130), whereas ET_a and ET_c increased until February 13 and 15, respectively. Until then, the crop developed from the V3 stage up to pod formation at R3, passing through the flowering stage (table 1, page 128). As already mentioned, the high water requirements of plants at these stages plus the increased atmospheric demand (table 3, page 134) contributed to a higher evapotranspiration causing therefore a reduction in soil water content. Grain filling at R5 started on February 22. At this stage, plants start to translocate nutrients to the pods in formation (40) and senescence starts. As of this moment, ET_a and ET_c started to decrease (figure 3, page 133), and as a result of the 63 mm rainfall between February 25 and March 7, $SWC_{0\text{-}1.10m}$ increased (figure 1, page 130). ET_a values obtained from Equation 1 (page 128), showed a better correlation with ET_c (r^2=0.62, p<0.01; figure 4a, page 135) than with ET_0 (r^2=0.43, p<0.01).



Crosses indicate the days not considered for the estimation of evapotranspiration (ETa), where precipitations or an increase in the daily soil water storage occurred. Red dots indicate the cases where soil water availability in the 0 - 1.10 m ($AW_{0\text{-}1.10\,m}$) profile was below 65%.

Las cruces indican los días que no se consideraron para las estimaciones de ET_r por ser días con precipitación o con aumento del almacenaje diario de agua en el suelo. Los puntos rojos indican los casos en los que el agua útil en el perfil 0 - 1,10 m ($AU_{0\text{-}1,10\,m}$) fue inferior a 65%.

Figure 3. Soybean crop evapotranspiration under standard conditions (ET_c, dotted line), actual evapotranspiration (ET_a, solid line) and precipitation (grey bars).

Figura 3. Evapotranspiración de un cultivo de soja bajo condiciones estándar (*ETM*, línea punteada), evapotranspiración real (ET_r, línea llena) y precipitación (barras grises).

Table 3. Actual Crop Evapotranspiration (ET_a), Reference Crop Evapotranspiration (ET_0) and soybean crop evapotranspiration under standard conditions (ET_c), average (mm/day) and accumulated (mm) for Balcarce, for each phenological period.

Tabla 3. Evapotranspiración real del cultivo (ET_r), evapotranspiración de cultivo de referencia (ET_0) y evapotranspiración para un cultivo de soja bajo condiciones estándar (ETM) promedio (mm/día) y acumulada (mm) para la localidad de Balcarce, para cada período fenológico.

	Phenological stage		
	V3-V8	**R1-R2**	**R3-R4**
ET_a (mm)	59.6	28.5	60.6
ET_0 (mm)	82.0	53.2	86.7
ET_c (mm)	68.9	54.6	96.9
ET_a (mm/day)	3.3	2.6	3.8
ET_0 (mm/day)	4.6	4.8	5.1
ET_C (mm/day)	3.8	5.0	5.7

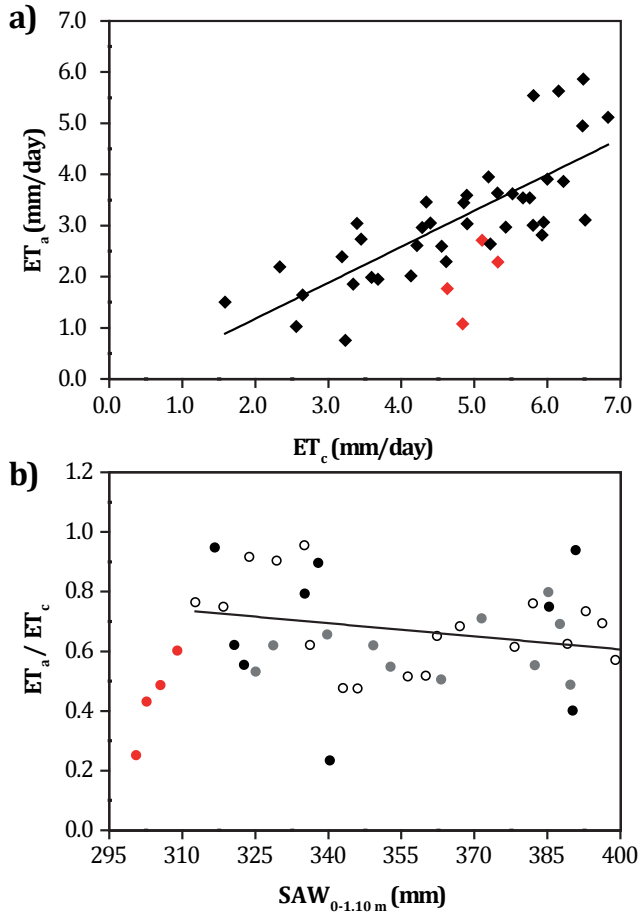
This is an indication that the K_c coefficient, which summarizes the evolution of crop characteristics, had a significant impact on the results.

Mechanisms controlling the relationship between evapotranspiration and water availability in the soybean crop can be diverse. The most important one is associated with the stomatal regulation, controlled by hormone signals coming from the roots and by leaf turgor loss (11, 30, 45).

Some authors suggest an effect resulting from the reduction of soil hydraulic conductivity associated to drying, particularly in clayey soils with high impedance to water flow (19, 38, 39). An additional difficulty for water extraction in this type of soils is associated to root clumping (14). It has also been suggested that there is another effect associated to root contraction caused by drying, which increases the resistance to water flow in the soil-root interface (44).

In association to these mechanisms, the behavior usually found in some evapotranspiration and soil moisture variables is a process regulated by a limiting factor (8, 27, 33, 42). However, in other studies (7, 14, 19) there is no clear evidence of this kind of response.

According to the proposed method, the threshold value established for water stress of $SWC_{0\text{-}1.10\ m}$ = 312 mm was used to identify groups with $p < 0.01$. This threshold value correlates to an approximate $AW_{0\text{-}1.10\ m}$ value of 65%, and a mean matric potential for the soil moisture profile of -1.4 MPa, as per retention curves from these authors (18). The data set for water stress included findings of four consecutive days (February 17-20, around R4 stage), during which $SWC_{0\text{-}1.10m}$ and ET_a/ET_c decreased simultaneously (red dots in figure 1 (page 130), figure 3 (page 133) and figure 4b (page 135).



Red icons indicate the cases where soil water availability in the 0 - 1.10 m (AU0-1.10 m) profile was below 65%. These were not included in the estimation of linear regressions shown. Black, grey and empty dots show first, second and third tercile for the reference crop evapotranspiration (ET_0).

Los íconos rojos indican los casos en los que el agua útil en el perfil 0-1,10 m fue inferior a 65% ($AU_{0\text{-}1,10\ m}$). Esos casos no fueron incluidos en las estimaciones de las regresiones lineales mostradas. Los puntos negros, grises y vacíos indican pertenencia al primer, segundo y tercer tercil de la evapotranspiración de cultivo de referencia (ET_0).

Figure 4. Actual Evapotranspiration (ET_a) as a function of soybean crop evapotranspiration under standard conditions (ET_c) (mm/day; a) with its linear least squares adjustment (ET_a = 0.70 ET_c - *0.23 mm,* r^2 = 0.62, p < 0.01). ET_a /ET_c, ratio as a function of soil water content for the 0-1.10 m soil thickness ($SWC_{0\text{-}1.10\ m}$, mm; b) with its linear regression (ET_a/ET_c = *-0.001 mm^{-1}* $SWC_{0\text{-}1.10\ m}$ + *1.19,* r^2 = 0.07, p > 0.16).

Figura 4. Evapotranspiración real (ET_r) en función de la evapotranspiración de un cultivo de soja bajo condiciones estándar (*ETM*) (mm/día; a) con su respectivo ajuste lineal por cuadrados mínimos (ET_r=0,70 *ETM - 0,23 mm,* r^2 = 0,62, p < 0,01). Cociente entre ET_r y *ETM,* en función del contenido de agua en el suelo para el espesor de suelo 0-1,10 m ($CAS_{0\text{-}1,10\ m}$, mm; b) con su respectiva regresión lineal (ET_r/ETM = *-0,001 mm^{-1}* $CAS_{0\text{-}1,10\ m}$ + *1,19,* r^2 = 0,07, p > 0,16).

Functional dependence was not studied in this case because there were not enough available data. Other authors found similar behaviors. These authors (8) detected reduced evapotranspiration in soybean with *AW* values below 48% to 36%, while these others (33) found similar results but only with AW values below 25%. These authors (27) found a decrease in evapotranspiration for pre-dawn plant water potential values of -0.33 MPa. A study aimed at modelling water deficit in Balcarce (15) used *AW* thresholds of 60% for the three decades (10-day periods) of flowering, and 40% for the rest of the cycle. These authors (14) established an *AW* threshold of 62%, and a pre-dawn water potential of -1.4 MPa for the same location, with results similar to those obtained in this study.

However, the different methods used to define *FC*, *PWP* (42), the differences found between different types of soils and evapotranspiration references used in the different studies make comparison of results difficult.

Under no-stress conditions (black, grey and empty dots in figure 4b (page 135), E_a/ET_c showed no significant dependence on $SWC_{0\text{-}1.10\ m}$, and presented high variability (a median of 0.62, interquartile range of 0.22). These authors (7, 45) found a relative reduction in ET_a under potential and high demand conditions, while these others (19) did not find a similar response. Results seem to indicate that the atmospheric demand had no impact on the E_a/ET_c ratio, as represented by the distribution of black, grey and empty dots (terciles of ET_0) in figure 4b, page 135.

Other possible sources of evapotranspiration variability that have not been analyzed can be water balance components not considered in this study, such as surface or subsurface runoff (associated to land slope), deep percolation and ponding. In this regard, an increased $SWC_{0\text{-}1.10\ m}$ was found in days without precipitation (a total of 12 days between January 7 and March 15, with a median increase value of 1.4 mm and interquartile range of 1.4 mm), which could be associated to ponding or horizontal water movement phenomena. These were not taken into account for ET_a estimations.

Water Extraction Patterns (WEP)

Considering the net consumption per level over the different phenological stages, the upper layer (0-0.275 m) is where the crop met most of its water needs (figure 5a, page 137).

The third and fourth layer contributed alternately with smaller amounts of water at different crop phenological stages: 0.55-0.825 m at V3-V8 and R3-R4, and 0.825-1.10 m at R1-R2. It was also found that the vertical profile of water consumption (%) for the vegetative phases was less homogeneous (highest consumption in the first stage), while water uptake was more equally distributed among layers during R1-R2. This might be an indication that, as there is less root development at the beginning of stage V, the crop restricted water extraction to upper levels.

Then, with an increased root development at R1-R2, and with less water content in the surface layer, the relative contribution of deeper layers increased.

At R3-R4, water consumption by the crop increased as a result of a higher atmospheric demand (table 3, page 134) and because the crop was passing through phenological stages in which there is a high demand of water (16). Water use values for this stage were the highest of the three stages under study (figure 5b, page 137).

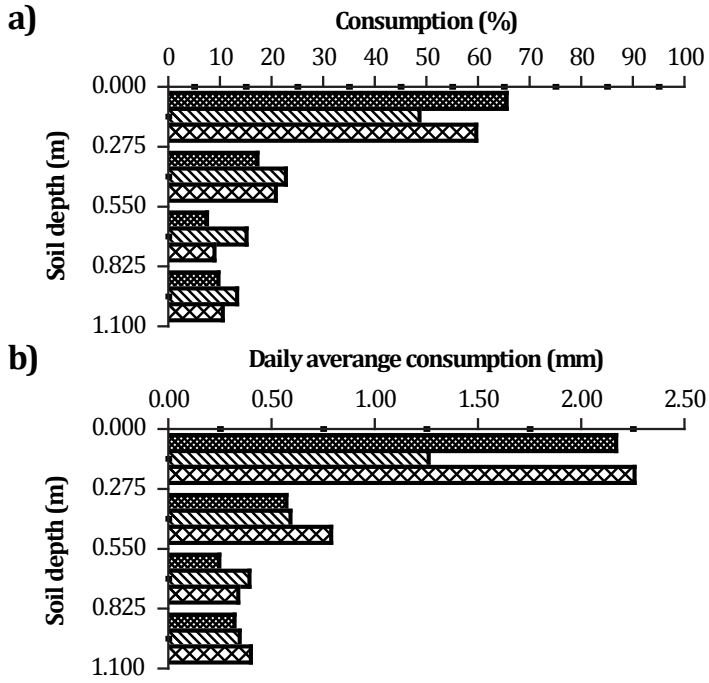


Figure 5. (a) Individual layer share of total water consumption (%) and (b) daily average consumption (mm) in each layer, for V3-V8 (dense grid pattern), R1-R2 (striped pattern) and R3-R4 (spaced grid pattern) phenological periods.

Figura 5. (a) Contribución de cada capa al consumo total de agua (%) y (b) consumo medio diario (mm) de cada capa, para los períodos fenológicos V3-V8 (patrón cuadriculado denso), R1-R2 (patrón a rayas) y R3-R4 (patrón cuadriculado espaciado).

However, consumption percentage increases again in the upper soil level (figure 5a, page 137). As the top soil layer is recharged (figure 4, page 135; 10/2), roots will take up water again from that layer regardless of the moisture in deeper levels (28). Vertical distribution of *WEP* values were in general similar to those found by these authors (25) for the two-year trial on soybean under irrigation in the same location, where most of the water supply came from the upper soil layer.

The water stress event occurred at the end of R3-R4. It consisted in a reduction of water storage, which was reflected in a lower AW_k value even in the two deepest levels (figure 2, page 131), which can be associated to a dominant effect of water extraction by plants (43).

From February 17 to 20, the crop gradually extracted a higher volume of water from deeper soil layers (figure 6), as moisture decreased in the surface layer.

Other authors (2, 4, 11) also reported that higher water extraction records moved to deeper soil levels, as moisture decreased in upper layers.

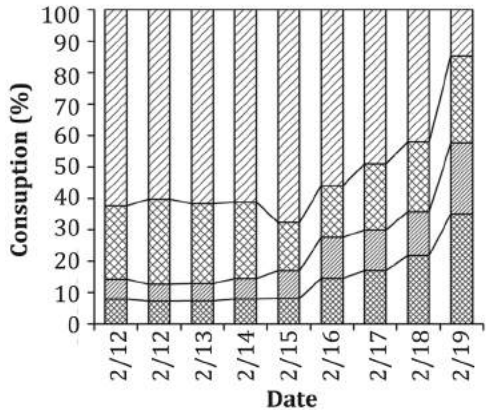


Figure 6. Individual layer share of total water consumption (%) 0-0.275 (spaced striped pattern), 0.275-0.55 (spaced grid pattern), 0.55-0.825 (dense striped pattern) and 0.825-1.10 m (dense grid pattern) strata, for February 12 to 20, which includes the water stress period.

Figura 6. Contribución de cada capa al consumo total de agua (%) para los estratos de 0-0,275 (patrón rayado espaciado), 0,275-0,55 (patrón cuadriculado espaciado), 0,55-0,825 (patrón rayado denso) y 0,825-1,10 m (patrón cuadriculado denso), para los días 12 al 20/2, que incluyen el período en que el cultivo de soja sufrió estrés hídrico.

Conclusions

This research work studied the ET_a and *WEP* in a soybean crop grown in the productive area of Balcarce, Buenos Aires Province, Argentina, using the soil water balance equation. *SWC* decreased over crop growth stages until the start of grain filling (V3-R5), showing a clear correlation between the root *WEP* and the crop phenological stages. After a stress event in R4, *SWC* increased gradually again until the end of cycle of the crop, as a result of soil water recharge through precipitations and a reduced plant water uptake.

Furthermore, ET_a values under no-water stress conditions were in average as much as 40% lower than the estimated value under standard conditions (ET_c). These results indicate that crop growth and development conditions in a productive scenario can show evapotranspiration values different from those found in an experimental context, even if the crop has no water constraints. This could explain why in some cases crop yields in productive conditions are lower than in controlled conditions, shown in this study by a reduced production of above-ground biomass.

WEPs were similar to those found in field experiments under controlled conditions. The crop modifies the water extraction pattern based on the water availability in the different strata, with a preference for upper rather than deeper layers. When water availability in upper levels declines, plants increase extraction from deeper levels to supply their water needs.

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Finite (Hausdorff) dimension of plants and roots as indicator of ontogeny

Dimensión finita (de Hausdorff) de plantas y raíces como indicador de ontogenia

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Abstract

The architecture of plants responds to endogenous processes and to the influence of environmental factors. The allometric study of architecture has been a challenge for biology. We define a new *finite (Hausdorff) dimension* of plants, that considers both the aerial part and the roots, and compute examples. This new finite dimension was introduced recently and, in contrast to the classical Hausdorff dimension, is not zero on finite sets. We propose the finite dimension, as a function of time, as a "signature" of the plant or root. Our first results suggest that the signature is specific to each plant species and its growth period, and constitutes an objective metric that allows to study its ontogenesis in detail.

Keywords
fractal dimension • finite (Hausdorff) dimension • plant architecture • plant development

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Resumen

La arquitectura de las plantas responde a procesos endógenos y a la influencia de factores ambientales. El estudio alométrico de la arquitectura ha sido un desafío para los biólogos. En este trabajo definimos una nueva *dimensión finita (de Hausdorff)* de plantas, considerando su parte aérea y de raíces y calculamos algunos ejemplos. Esta nueva dimensión finita fue introducida recientemente y, a diferencia de la dimensión clásica de Hausdorff, no es cero en conjuntos finitos. Proponemos que la dimensión finita, como función del tiempo, es una "firma" de la planta o raíz. Nuestros primeros resultados sugieren que la firma es específica para cada especie de planta y su período de crecimiento, y constituye una métrica objetiva que permite estudiar detalladamente la ontogénesis.

Palabras clave
dimensión fractal • dimensión finita (de Hausdorff) • arquitectura de plantas • desarrollo de plantas

Introduction

The morphological structures of plants can be thought of as the translation of a program of endogenous development that influences the relative disposition of the aerial and subterraneous axes that express the plants' architecture (12). This is the expression of endogenous processes and of the plants' answer to the external factors that influence them along their lives (26). Plant architecture is defined as the three-dimensional organisation of the plant's body. Plant architecture determines their ability to compete for resources (22). For the parts of the plant that are above ground, this includes the branching pattern, as well as the size, shape and position of leaves and flower organs. For example, among other factors, the aerial architecture determines the ability of plants to use light, and the root system architecture, their ability to explore the soil (12). In this way, modelling and analysing the allometries of plants allows us to interpret their adaptive strategies and their ability to respond to environmental factors, and even to devise productive managerial strategies (16). Different approximations have been used in the quantitative study of architecture. However, the identification of allometric variables that synthesise the architecture of plants and can be followed during ontogenesis and used to make comparisons between species or between environments, is still under discussion.

There is a long tradition in biology, going back at least to the 1890's, of computing allometries (9). A prominent example is Kleiber's law (15, 16):

$$R = aM^{3/4} \quad (1)$$

where:
R = the metabolic rate
a = a constant
M = the body mass, the so-called "quarter-power scaling" of metabolic rate to body mass.

In the late 1990's West *et al.* (1997, 1999a, 1999b), proposed a model to explain the exponent ¾ in Eq. (1), by assuming a fractal-like design of resource distribution networks and exchange surfaces. The exponent can

be interpreted as a "fractal" dimension. The model of West *et al.* (1997, 1999a, 1999b) stimulated further investigation and a renewed interest in computing the "fractal" dimension of plants, including a debate about the quality and analysis of the supporting empiric evidence. In any case, it is clear that this model has had a profound influence and dominated the subject in the last two decades. Also, a large body of work has been produced aimed at clarifying the situation (10, 11, 18, 19, 20, 21, 24, 25).

Even in the case of roots there is a large literature on "fractal" dimension, starting in the late 1980's with the pioneering papers of Tatsumi *et al.* (1989) and Fitter and Stickeland (1992). The shared point of view is that root systems are "fractals" -meaning self-similar objects- and merit, therefore, the study of their "fractal" geometry and dimension. By "fractal" dimension they mean the *box-counting* dimension of Falconer (2003), which they approximate and compute in various ways. In fact, a troublesome issue found in the literature, common to the "fractal" dimension of plants and roots, is the difficulty in comparing results obtained with different methods and choices (5). This issue is even worse for roots, due to the difficulty in accessing root systems. Another issue is the very tentative interpretations of the values obtained (28). It is worthy of note that Tatsumi *et al.* (1989) and Fitter and Stickeland (1992) consider the ”fractal” dimension not only as a single number, but as a function of time.

We refer the reader to the extensive bibliography on allometries in biology, and on the model of West *et al.* (1997, 1999a, 1999b), and content ourselves with the brief discussion and short literature list we have presented. This is motivated by the fact that our work, while sharing the interest in dimension with these works, is quite different. Indeed, in contrast to the literature we have mentioned, we make *no assumptions* on the plants; in particular, no self-similarity assumptions. Our aim is simply to compute the finite dimension of plants and roots (as in Eq.(2)) and try to understand what it says about them.

In this paper we define a new dimension for plants and roots, called *finite dimension* and denoted $\dim_f$, and compute a few examples. The finite dimension is a real number which is computed every time the plant or root is measured; thus, we consider $\dim_f$ as a function of time rather than as a single number. Our approach is new in two ways: (a) we use a new definition of "fractal" dimension, called *finite Hausdorff dimension*, denoted $\dim_{fH}$, and (b) we model plants and roots by means of (mathematical) trees. Schematically, every time the plant or root is measured, we have: (2)

$$\Pi(t)\rightarrow T_{\Pi}(t)\rightarrow \dim_f(\Pi)(t):=\dim_{fH}(V(T_{\Pi})(t))$$

where:

$\Pi(t)$ = denotes the plant or root Π measured at time t

$T_{\Pi}(t)$ = mathematical tree associated to $\Pi(t)$

$\dim_f(\Pi)(t)$ = *finite dimension of* Π at time t = defined to be the finite Hausdorff dimension of $V(T_{\Pi})(t)$, the set of nodes of $T_{\Pi}(t)$

This process will be explained in more detail in Section "The model". Finite Hausdorff dimension was introduced in Alonso (2015), was further studied in Alonso (2016), and was used in Alonso (2018) to study glycans and their structure.

There are several advantages with this new dimension in relation to the "fractal" dimension reported so far in the literature. One is that the process of measuring, while laborious, is rather error-free.

This makes results easy to compare and reproduce. Another is that, while its meaning is not completely transparent, we have a good understanding of what the finite dimension measures, and what its *variation in time* means (cf. Section "What $\dim_f(\Pi)$ measures"). This turns $\dim_f$ into an objective and reasonably well understood measure from which we can *deduce* information about ontogenesis. In fact, we regard finite dimension not as a number but as a sort of *signature* of each species, intimately related to its ontogenesis.

Metodology

Finite Hausdorff dimension

We know from elementary geometry, that any finite set of points has dimension 0, a segment or a line has dimension 1, a square or an open subset of the plane has dimension 2, and "space" has dimension 3. In the 1880's finite dimensional vector spaces were defined in full generality, extending dimension to any nonnegative integer: 0,1,2,...,*n*,.... But associating a dimension to more complicated sets (like the Cantor set, for instance) had to wait for Felix Hausdorff's definition (1919) of what we now call Hausdorff dimension, denoted $\dim_H$. Hausdorff's definition is a far-reaching generalisation that associates a dimension to a vast class of subsets of Euclidean space (7). But there is a price to be paid: $\dim_H$ has often irrational values, not integers. This is at the origin of the word "fractal". However, when it comes to classical geometric objects like the ones mentioned at the beginning of this section, $\dim_H$ gives the well-known values. In particular, the Hausdorff dimension of finite sets is zero.

The importance of finite sets has increased in time, among other things thanks to computers, which can only handle finite sets. *Finite Hausdorff dimension*, $\dim_{fH}$, is a variation of Hausdorff dimension introduced recently (1). It is defined *only* on finite sets, but its values can be any real non-negative number, or infinity. The point of this new dimension is that it is non-trivial on finite sets and, hence, can discriminate among them, just as the classical dimension discriminates among continuous sets. Rather than giving a rigorous account of the definition and properties of finite dimension, we content ourselves with a quick overview in section "The finite Hausdorff dimension of trees and roots" below and for more detail, refer the reader to Alonso (2015, 2016) which treat, respectively, the general case and that of graphs, which is the case we need for plants and roots.

The model

Natural trees and mathematical trees

Graphs consist of vertices (or nodes) and edges (that join certain pairs of vertices). A mathematical tree, is a *simple, connected, non-directed* graph without *circuits*. *Simple* means that between any two nodes there is *at most* one edge connecting them; *connected* refers to the fact that any two nodes v,w can be joined by a *path*, *i.e.* there is a sequence of edges $e_1 ... e_n$, where e_i joins nodes v_{i-1}, v_i, and $v = v_0$, $v_n = w$. A *circuit* is a path with distinct edges, whose initial and last nodes coincide, *i.e.* $v_0 = v_n$.

We model natural trees and plants Π by means of mathematical trees T_Π, as follows. Nodes correspond to ramification points, endpoints (of leaves or branches or twigs, as the case might be), and "the point" where Π goes into earth. We abuse language and do not distinguish between "nodes" (*i.e.* vertices of T_Π) and "points" (*i.e.* the corresponding points of Π: ramification, end or contact with earth). Edges

represent the space between consecutive nodes, and are assigned a length equal to the actual length, measured on the plant Π, between these consecutive nodes. In our model, the thickest trunk and the thinnest twig are equally modelled by a segment that joins the corresponding nodes. In other words, we model the length but not the thickness of internodal segments. This explains the step $\Pi \to T_{\Pi}$ of Eq. (2) (page 144).

The intrinsic distance

Next, we define a distance on $V(T_{\Pi})$, the set of nodes of T_{Π} (2). The length of a path in T_{Π} is define to be the sum of the lengths of each of its edges. The distance $d(v,w)$ between nodes v,w is the minimum length of all paths joining v and w (we might add that, since T_{Π} is a tree, there is exactly one *shortest* path joining v,w). This definition of $d(v,w)$ gives the *intrinsic* distance; informally, this is the distance "inside" the plant, *i.e.* the distance travelled by an ant on the plant, not the *extrinsic* distance, *i.e.* the bird's distance obtained by connecting v and w by a straight segment lying in the 3-dimensional space that surrounds the plant. Using the intrinsic distance is an important modeling decision; clearly this distance lies closer to the plants "own" distance (say, when transporting nutrients) than the ambient distance which is, perhaps, more "natural" for us humans. A practical implication of this decision is that the spatial structure of a plant or root system is completely ignored by the finite dimension.

Researchers using the box-counting dimension must deal with a difficult problem: transforming the spatial structure of plants and roots to a planar structure from which to approximate de box-counting dimension, and hope the final result is somewhat independent of the method used. Using finite dimension, we avoid completely this difficulty -which is even worse for root systems.

The end-result is that we replace a plant Π by a mathematical tree T_{Π}, and endow the nodes $V(T_{\Pi})$ with the intrinsic distance obtained by measuring the internodal distance in the plant itself. Thus $V(T_{\Pi})$ is now a finite metric space whose finite Hausdorff dimension can be calculated. We define:

$$\dim_f(\Pi) := \dim_{fH}(V(T_{\Pi}))$$

As explained earlier, we repeat this procedure at each designated time in order to make the finite dimension a function of time. In practical terms, we have to define the position of nodes in the plant itself, and measure the internodal distances. This may be laborious but it is straightforward and relatively error-free. In any case, it is more exact than existing methods of computing various "fractal" dimensions of plants.

The finite Hausdorff dimension of trees and roots

We explain the definition through an example and refer the reader to Alonso (2015, 2016) for details. Consider for example the tree T_{Π} of figure 1 (page 147). The figure suggests that the longest distance one can travel inside T_{Π} is $d(E,G) = d+b+e+g$ (we assume this is the case). This distance is called the *diameter* of the mathematical tree, denoted $\Delta(T_{\Pi})$; in this situation, we say that the diameter is *attained* at E,G. As usual, we abuse notation and call $\Delta(T_{\Pi})$ the diameter of the plant itself. Note that "diameter" for us means the longest possible distance between nodes of a mathematical tree, not to be confused with the diameter of trunks, branches or twigs, which for us are zero, as explained in Section "The model" The finite dimension is computed by solving for s the equation: (3)

$$a^s + c^s + d^s + f^s + g^s + h^s = \Delta(T_{\Pi})^s = (d + b + e + g)^s$$

The numbers *a,...,h* are measured internodal distances and are known. The left-hand side of Eq. (3) (page 146), is computed as follows: first we find a *2-cover* of the vertices of T_{Π}, *i.e.* the smallest number of adjacent nodes that cover $V(T_{\Pi})$. These are represented by six red ellipses in figure 1. Then we raise the length of the edge enclosed by each ellipse to the power s (s is the only unknown in Eq. (3)), and add over all elements of the 2-cover. The right-hand side is the diameter raised to the power s. It can be shown (1) that, in the cases of our interest here, there will be exactly one value s_0 that solves Eq. (3); this value is defined to be the finite dimension, $\dim_f(\Pi) := s_0$.

What $\dim_f(\Pi)$ measures

The left-hand side of Eq. (3) represents the s-dimensional volume (s-volume, for short) of Π. Recall that the area (*i.e.* 2-volume) of a disc of radius r is $(\pi/4)\Delta^2$, and the volume (*i.e.* 3-volume) of a ball of radius r is $(\pi/6)\Delta^3$, where $\Delta = 2r$ is the diameter of the disc or ball. Thus, we think of an expression like a^s as an "s-volume" because this term, up to multiplication by a universal constant, is actually an s-volume in Euclidean geometry.

The right-hand side of Eq. (3) represents the s-dimensional volume of the ball of radius half the *diameter* of Π. It is the s-volume the plant would have if it were a solid ball of the given diameter.

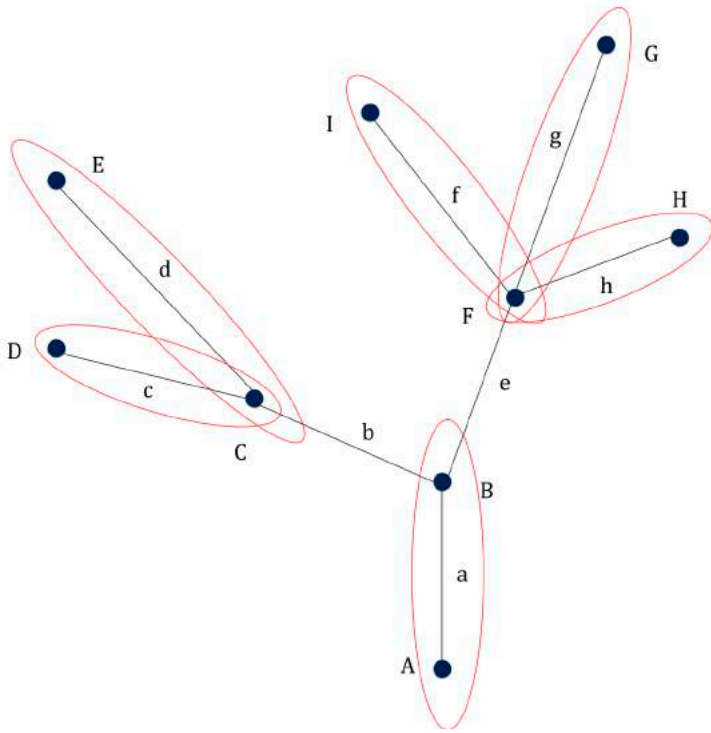

Figure 1. A metric mathematical tree T_{Π} with 9 vertices, and a 2-cover.
Figura 1. Árbol matemático métrico T_{Π} de 9 vértices, con un 2-cubrimiento.

We interpret the sum of the lengths of the elements of the 2-cover, $a + \cdots + h$, as the *actual mass* of the plant (even when it is not equal to the sum of all the edges), and the diameter, $\Delta(T_\Pi) = d + \cdots + g$, as the *reference mass*.

Thus, the finite dimension is 1 when the actual mass equals the reference mass. Mathematically, Eq. (3) (page 146), is such that s_0, *i.e.* the finite dimension, is < 1 when the actual mass is *less* than the reference mass, *i.e.* when the tree is "sparse". And it is > 1 when the actual mass is *larger* than the reference mass, *i.e.* when the tree is "dense".

Rather than the exact number $\dim_f(\Pi)$, we focus attention on $\dim_f(\Pi)(t)$, the finite dimension as a function of time. Indeed, it is the variation in time of $\dim_f(\Pi)(t)$ that is of interest for us. Let $t_1 < t_2$ be two time points. Our measurements will generally increase from t_1 to t_2, so that $\Delta(t_1) < \Delta(t_2)$ (*i.e.* the reference mass will grow), and also the actual mass will grow, as the plant continues to grow and ramify. This does not necessarily mean, however, that $\dim_f(\Pi)(t_1) < \dim_f(\Pi)(t_2)$; in fact, all three possibilities can occur. When $\dim_f$ decreases [increases, or stays the same, respectively], the reference mass grows more than [less than, or at the same rate as, respectively] the actual mass. If we think of the ratio of reference to actual mass as a sort of "density" of the plant, we can interpret these three situations by saying that the "density" decreases, increases or stays the same. Note that claims like "the reference mass grows more than the actual mass" can equally well be read as "the actual mass grows less than the reference mass", etc.

Experiments

The finite dimension of the aerial architecture of Lecanophora heterophylla (Cav.) Krapov. and *Larrea cuneifolia* Cav.

We measured simultaneously 9 plants of *Lecanophora heterophylla* and of *Larrea cuneifolia*. Measurements were taken approximately once a week, from about a week after germination. Plants were kept at field capacity, the substrate was sandy loam. Maximum and minimum mean daily temperatures during the experiment ranged between 17.6° and 7.8°C, relative humidity was 59.7%, and there was no precipitation during the experiment. The seeds were harvested in Mendoza's piedmont. Experiments were conducted at the experimental field of IADIZA (Insituto Argentino de Investigaciones de Zonas Áridas) (32°53' S; 68°57' W), an institute of Centro Científico Tecnológico (CCT), Mendoza-CONICET (Argentina). To measure internodal distances we used a caliber (Mitutoyo, Model N° CD-6"PSX, with resolution: 0.01mm), and a powerful magnifying glass.

Both species belong to the native flora of the dry regions of Argentina's West, in the biogeographical Monte province. They were chosen to simultaneously show different forms of growth, by comparing two different forms of life: herbs and bushes. The way each species develops determines the velocities at which those parts of the plant that we measure (bifurcations, nodes, internodes, leaves) appear. Moreover, different parts of plants grow at a different pace, usually in different times of the year (16). All this information on the plant, the time and rate of appearence of the different parts, their number and growth rate, is combined by $\dim_f(\Pi)$ to produce, in the end, a single number (each time one measures): the finite dimension.

Lecanophora heterophylla belongs to the family *Malvaceae* and is endemic to the dry regions of Argentina; it has great ornamental potential, and is found between 0 and 2,500 meters above sea level. It is distributed through dry and half-dry habitats from Río Negro to Tucumán. These plants have erect and cylindrical stems, and deep roots.

Larrea cuneifolia (*jarilla* in Spanish) is a bushy species of the family *Zygophyllaceae*, endemic of South American deserts, with a wide distribution from Central-Western Argentina to the Center of Patagonia, between 0 and 3,000 meters over sea level. It is a bush up to 2 m high, with branches oriented pointing North and South, in such a way that the leaves are exposed to the morning sun, on the one side, and to the afternoon sun on the other.

The finite dimension of roots of Leptochloa crinita (Lag.) P.M. Peterson & N.W. Snow

To understand the development of roots we used as model *Leptochloa crinita*. We computed the finite dimension of the roots of two samples, of one root each, with different water regimes: one was permanently kept at field capacity, and the other was subjected to periods of stress, receiving a unique initial watering, where plants were watered at 50% of field capacity. The experiment took 92 days from sowing time. Measurements started a week after germination, and roots were recorded every 2-3 days, through an acrylic wall (prismatic plant pots with a transparent side).

Leptochloa crinita is of the family *Poaceae*, very common in South America. It is one of the most important grasses of the Monte biogeographic province. Grasslands of *Leptochloa crinita* extend from sub-humid to desertic areas in Argentina, Paraguay and Uruguay (23). This grass, which is well adapted to natural conditions of habitat stress, provides pastures of good quality and is often used to regenerate pastures. Its fodder production varies substantially according to the environment it lives in (6) and efforts are being made to select varieties with improved features for fodder production (17).

Results

Lecanophora heterophylla

The (average) finite dimension of *Lecanophora heterophylla* and of *Larrea cuneifolia*, together with the corresponding standard error bars, is summarised in figure 2 (page 150), from day 1 (first measurement) to day 49. We see that $\dim_f$ grows steadily up to day 30, then falls drastically on day 35 and thereafter remains between 1.50 and 1.65. The *diameter* of each plant is always attained at endpoints of leaves, not at endpoint of leave to soil. We could say that, in this case, the diameter measures the size of the "foliage" or "crown". All through the measurement period the diameter grew essentially at constant pace.

The pronounced dip between days 30 and 35 means (cf. "What $\dim_f(\Pi)$ measures") that the reference mass has grown more than the actual mass, *i.e.* the plant has become "sparser". In actual fact, all plants in the sample lost their cotyledon leaves in this period, thus producing an important *decrease* of the plant's actual mass (while the diameter (*i.e.* the reference mass) continued to grow). As explained in Section "What $\dim_f(\Pi)$ measures", the increasing values of $\dim_f$ in 1 - 30 mean that the actual mass increases faster than the reference mass, *i.e.* the number and sizes of leaves (the actual mass) increases faster than the size (the diameter, or reference mass) of the plant. Summarising, *Lecanophora heterophylla* starts as a "foliage sparse" plant with "wide" crown and small "size" (initially it consists of two cotyledon leaves), and gradually becomes denser: initially the crown grows faster than the "size", but later crown and "size" grow roughly at the same pace.

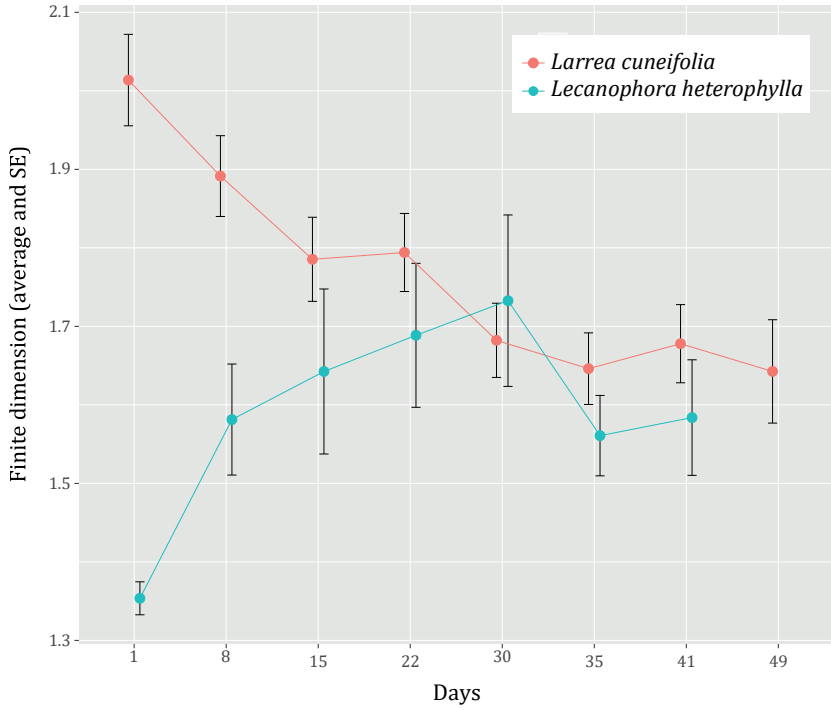

Figure 2. Average finite dimension of *L. cuneifolia* and *L. heterophylla*, with standard error bars.

Figura 2. Dimensión finita promedio de *L. cuneifolia* y *L. heterophylla*, con error estándar.

Larrea cuneifolia

We see from figure 2 that *Larrea cuneifolia* does the opposite: its finite dimension decreases almost linearly in 1-30 and then remains in the range 1.6-1.7. In contrast to the previous case, the diameter of the plants is always attained at the endpoint of some leave and the soil point. Thus, for *Larrea cuneifolia* the reference mass is essentially the hight of the plant. That $\dim_f(\Pi)$ falls in 1 - 30 says that the reference mass grows faster than the actual mass, *i.e.* the height of the plants, grows faster than the number and sizes of branches and leaves. In the period 30-49, reference and actual mass (*i.e.* hight and "size") grow at essentially the same pace. To summarise, *Larrea cuneifolia* starts as a "foliage dense" plant and gradually becomes sparser: initially the hight grows faster than the "size", but then hight and "size" grow roughly at the same pace.

Roots of *Leptochloa crinita*

The two roots were treated differently: the red one in figure 3 (page 151) was subjected to water stress, while the blue one received water at field capacity. The finite dimension of both roots decreases in 8 - 24. The first five values of the red line are at the top of the diagram which means, for the software used (the statistical software R), that the value is infinite.

This says that the root is not ramified in this period. When it does ramify the finite dimension becomes finite and goes down. Not surprisingly, since both are of the same species, the blue one also decreases in this period and, indeed, reaches almost the same minimal value (around .84). We should also remark that these roots are far longer than wider, so that their diameters are achieved at soil-point to deepest endpoint.

Most interesting is the different behaviour of the roots in the period 24 - 78. While the red one (with little water) grows steadily, at almost constant pace, the blue one (with lots of water) goes up and down, almost periodically. The interpretation, using "What $\dim_f(\Pi)$ measures", is that the plant that received less water in this period (red line) prioritises lateral growth over growth in depth. The plant that received water at field capacity (blue line), on the other hand, keeps changing priorities: growth in depth over lateral growth, and then the opposite, and so on.

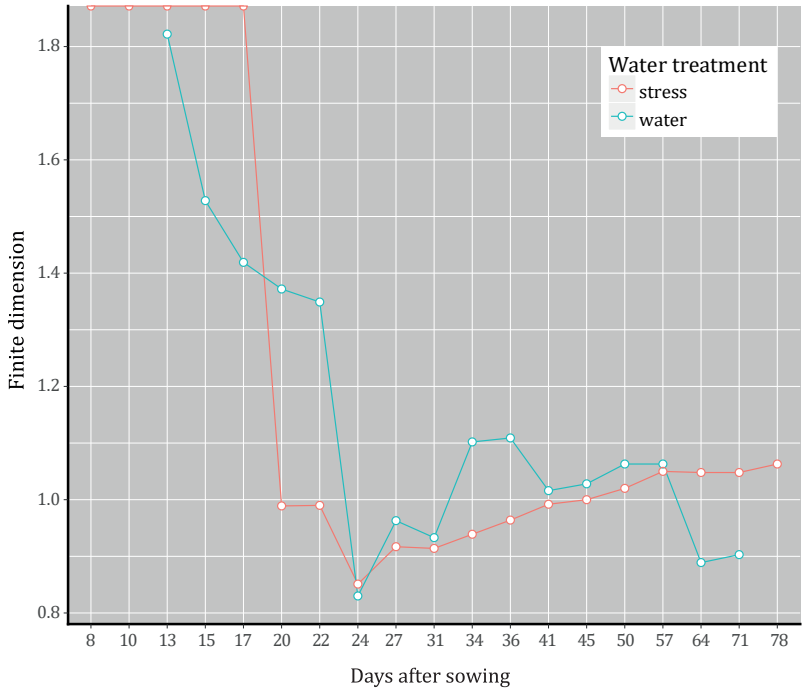

Figure 3. Finite dimension of roots (*Leptochloa crinita*), with different water treatments.

Figura 3. Dimensión finita de raíces (*Leptochloa crinita*), con distintas condiciones de riego.

Conclusions and future work

The results reported here show that $\dim_f$ closely follows the ontogenesis of the aerial part and roots of the plants studied. Moreover, they clearly show the different growth strategies followed by plants. This is why we believe that $\dim_f(\Pi)$ is a good, objective measure of ontogenesis that, moreover, is reproducible because it is calculated with the same methodology for every type of foliage of plant or root. Our results suggest that each species has its own distinctive "signature".

This is a first, pioneering work in applying finite Hausdorff dimension to the study of plants. Our results are tentative because we have little empiric evidence. We have to study longer series of measurements, other species, other roots. This way we could prove or disprove our claims above, as well as elucidate, for instance, how $\dim_f(\Pi)$ reacts to different management practices, *e.g.* pruning. Silvicultural treatments applied to woody plants in arid zones still have a high degree of uncertainty (4, 16). We hypothesise that, after each practice, a plant tries to regain its "normal" finite dimension, as expressed in its finite dimensional "signature". These questions will surely lead us to a deeper understanding of how plants grow, and result in better management practices. In addition, if we can associate different functional traits with the finite dimension, it could become a tool to improve species in search of specific traits.

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Optimal design of drip irrigation submains: presure-compensating emitters

Diseño óptimo de sectores de riego por goteo: emisores autocompensados

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Abstract

For a drip irrigation system to be successful, it must be well designed, properly installed, managed and maintained. In plots with steep slopes and irregular topography that have little land leveling capacity and/or that require very efficient agricultural machinery, drip irrigation designs generally use pressure-compensating emitters. This work develops a methodology and implements it in a computer tool that makes it possible to optimally determine in drip-irrigated plots with pressure-compensating emitters: a) telescopic sizing of the submain manifold pipe, b) supply valve pressure and c) subunit's intake valve location, when considering all hydraulic-economic aspects in the design phase. Techniques of optimal sizing of pipe networks and simulation of hydraulic networks under pressure are linked to economic analyzes of total annualized costs. Finally, the practical usefulness of the proposed methodology is shown with three examples of complex real cases where pipe design costs are reduced by 16-34% and energy costs by 37-51%.

Keywords
drip irrigation • design • submain • pressure-compensating emitters

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Resumen

Para que un sistema de riego por goteo tenga éxito, debe estar bien diseñado, adecuadamente instalado, apropiadamente manejado y mantenido. En parcelas con grandes pendientes, topografía irregular, con poca capacidad de nivelación y/o en situaciones donde se pretende tener una alta eficiencia de la maquinaria agrícola, los diseños de riego por goteo generalmente recurren a la utilización de emisores autocompensantes. El presente trabajo desarrolla una metodología y realiza su implementación en una herramienta informática que permite en sectores de riego por goteo con emisores autocompensantes determinar de manera óptima: a) el dimensionado telescópico de la tubería secundaria (porta-laterales), b) la presión en válvula de alimentación del sector y c) el punto de alimentación del sector (ubicación de dicha válvula); considerando todos los aspectos hidráulicos-económicos involucrados en la fase de diseño. Para ello, se vinculan técnicas de dimensionado óptimo de conducciones y simulación hidráulica de redes a presión con análisis económicos de costos totales anualizados. Finalmente, se demuestra la utilidad práctica de la metodología desarrollada mediante ejemplos reales de aplicación, donde los costos de diseño se reducen entre 16-34% y los costos de energía entre 37-51%.

Palabras clave
riego por goteo • diseño • subunidades • emisores autocompensantes

Introduction

The success of a drip irrigation system depends essentially on proper design, selection and installation (25) and correct management and maintenance (12). In order to ensure its financial sustainability, the benefit of the irrigated crop must cover the high capital costs which this method entails (17). Optimal design of drip irrigation is important to increase the investments in and benefits derived from irrigation (16). Such design should aim at minimizing total annualized piping and energy costs (32, 33) and ensuring water distribution uniformity (4, 6, 17, 19)

In steep slope plots (20) with irregular topography and little land leveling capacity and/or that require highly efficient agricultural machinery, drip irrigation systems generally use pressure-compensating emitters. They deliver a practically constant discharge over a wide range of pressures (20, 21, 35), called effective pressure compensation range, between 5 and 35 m, and can lower the limits by ± 2 or raise them by ± 5 m (3), depending on each manufacturer. Pressure regulation is achieved by means of an elastic membrane that covers the flow path (36). A pressure-compensating emitter can be described by the following pressure-discharge function (26):

$$q = \begin{cases} kh^x, 0 \langle h \leq h_0 \\ q_0, h_0 \langle h \langle h_{max} \end{cases} \quad (1)$$

where:

q = flow of the emitter $[L]^3 [T]^{-1}$

K = characteristic discharge coefficient of the emitter $[L]^{3-x} [T]^{-1}$

h = emitter pressure [L]

x = emitter discharge exponent

q_0 = constant discharge for the compensation range $[L]^3 [T]^{-1}$

h_0 = minimum pressure of the compensation range [L]; this is defined as:

$$h_0 = \left(\frac{q_0}{K} \right)^{1/x} \quad (2)$$

For a good pressure compensating emitter, most designers will try to keep the pressures throughout the field in a range between 7-24.5 m (5). Montalvo (2005) recommends for safety reasons a minimum pressure value for the emitter that is at least 2 m higher than the lower limit of the compensation range and a maximum pressure of 25 m. It is necessary to keep pressures within that range to avoid: a) disconnection of the drip laterals of the manifold, b) breakage of the drip irrigation lateral or exhaustion of their service life, and c) high energy consumption.

Several authors address the hydraulic-economic optimization to design irrigation subunits. Saad and Mariño (2002) developed a linear optimization model for rectangular subunits, with telescopic pipes placed in the direction of the slope gradient which minimizes the annualized equivalent irrigation and pumping costs and maximizes distribution uniformity. Valiantzas (2003) and Valiantzas *et al.* (2007) derived a simple equation to calculate the length and available diameters of pipes within a subunit that minimizes total annualized pipe and energy costs. Dercas and Valiantzas (2012) presented two simple analytical methods to calculate the adequate diameters of the main pipes based on hydraulic-economic analyzes.

In Irricad software (2013), the pipe sizing is carried out using a Linear Programming optimization (LP) in conjunction with hydraulic grade lines analysis method. Pipe sizes are optimized based on the annualized cost of pipes and energy (18). Intake valve position and pressure are defined by the user without optimization criteria.

Carrión *et al.* (2013) and Carrión *et al.* (2014) devised a methodology and computer tool (PRESUD-Presurized Submain Design) applied to turbulent drippers for the optimal design of submains. The criterion used to optimize the design was to reduce the total annualized costs of water per irrigated unit area (C_T). They adopted a double iterative process for the design of the submain manifold pipe and the intake valve pressure similar to the ones introduced in the present article. Their approach is valid only for rectangular plots, does not incorporate the telescopic design of submain manifold pipes and does not identify the optimum intake valve location.

Moreno *et al.* (2016) expands the PRESUD tool as PRESUD-IR to optimize the design of triangular or trapezoidal plots. It incorporates an extension of the algorithm above mentioned by Carrión *et al.* (2013 and 2014); at a third iteration intake valve location is determined by considering the location of each emitter as a possible feeding point for the submain. The optimum intake valve location is the one that maximizes distribution uniformity and minimizes the C_T. This update does not incorporate the telescopic design of submain manifold pipe and is not suitable for irregularly shaped subunits.

For determining the optimum intake valve location in the submain, previous works (20) stated that the location must be aligned to the slope and pressure loss in manifold pipes of a single diameter so as to balance the minimum pressures on both sides of the valve. For telescopic manifold pipes, this depends on the selected diameters and lengths. Finally, in order to optimize the feeding point of an irrigation submain it is necessary to improve irrigation uniformity and considerably reduce the cost of pipes.

Rodrigo López *et al.* (1992) said that when the average slope of the land in the direction of manifold pipes is less than 3%, it is usually more economical to feed the subunit through an intermediate point so as to ensure that pressure variation is almost the same in the manifold pipe of the upstream and downstream feeding point.

Although there are some general recommendations and criteria for the optimal design of irrigation submains with pressure-compensating emitters, there is no methodology to define, both in topographies and/or arbitrary geometries, the pressure and the valve's intake point and the telescopic sizing of the submain manifold pipe that will lead to the best possible hydraulic-economic design.

Objective

To develop a methodology for subunits with pressure-compensating emitters to optimize: a) telescopic sizing of the submain manifold pipe; b) the intake valve's input pressure to the subunit; c) the intake valve location, by considering all hydraulic-economic aspects involved in the design phase so as to minimize total annualized costs per irrigated unit area. The resulting methodology is applied within the design module in drip irrigation plots in the GESTAR computer package (2), thus providing a new advanced functionality.

Materials and Methods

For irrigation submains with pressure-compensating emitters, the methodology uses as initial design condition an admissible range of design pressures based on the criteria proposed by Burt and Styles (2007) and Montalvo (2005). However, the user will be able to modify the minimum and maximum valve's pressure for the design (admissible range of design pressures) according to the characteristics and knowledge of the submain.

The Darcy-Weisbach formula is used to calculate pressure losses in pipes and laterals where: 1) the friction factor (f) is determined by approximation to the Basius equation (in the case of laterals) and to the Colebrook's equation for manifold pipes; and 2) the Christiansen reduction coefficient is used to calculate pressure losses in pipes or lateral according to the number of outlets as pressure-compensating drippers conform to this model.

For optimal sizing of telescopic submain manifold pipe, the method developed by González and Aliod (2003), and González (2006) is applied. It is an optimization algorithm (LMM/KPH -LM) that uses an improved Lagrange Multipliers Method (LMM) (27) in conditions of Know Pressure Head (KPH), in combination of a Labye-type Method (LM) (22) for standardization of the continous diameters obtained in LMM. At each connection point of every lateral to the submain manifold pipe, the optimization algorithm must supply a minimum required pressure so that the most unfavorable pressure-compensating dripper of the respective lateral reaches its minimum operating pressure.

The Nodal Analysis method (1, 9, 10, 11) is used for hydraulic simulation of the irrigation submain, once it is already designed, which includes a set of matrix analysis techniques, extended to consider the specifities of pressure irrigation systems, that incorporates the integral-differential hydraulic modeling for drip laterals where the emitters discharge flow can depend on the local pressure (10, 15, 34). It makes it possible to perform a detailed quasi-stationary hydraulic-energy simulation either for turbulent or self compensating drippers.

The baseline data to be selected includes: a) for the hydraulic calculation of the subunit, the inner diameter (DI; mm), length (L_l; m), slope (S_o; m/m), separation (distance between rows; S_l; m) and laterals per crop row (N°lat/row); flow (q_o; L h^{-1}) and emitter spacing (S_e; m), maximum (PVmax; m) and minimum design pressure (PVmin; m) of the subunit, as well as the design pressure step range (PV Step; m); b) for the economic calculation of the subunit, gross crop water requirements (Nb; m^3 ha^{-1} $year^{-1}$) per year, pumping equipment efficiency (Ep; %/100), transmission ratio (Tr, this represents the additional amount of water that must be applied during the highest demand period taking into account the inevitable deep percolation, with values ranging between 1.0 and 1.1 (20)), cost of energy (C_e; € kwh^{-1}); water price (C_w; € m^{-3}); interest rate (i; %/100), service life (N); maintenance cost as a percentage of the irrigation system purchase cost (C_m; %/100). Figure 1 (page 159), shows the flow diagram of the design optimization process.

The proposed algorithm first determines if the lateral should be fed from one end or through an intermediate point by checking if Pmin = minimum design pressure of the submain is higher than Pminobj = minimum target design pressure. It also evaluates the intermediate feeding point of the lateral using the methodology defined by Keller and Bliesner (1990) according to the procedures mentioned in Schilardi *et al.* (2017).

Figure 1 (page 159), shows a nested iteration process to optimally determine the valve pressure (Pvop) and optimal intake valve location (Nop). The calculation sequence begins at the first possible intake point (N1) with an outer iterative process (position iteration), where optimal telescopic sizing of the submain manifold pipe is carried out at different intakes pressures which are stablished in a second inner iterative process (pressure iteration). Thus, for each possible intake position and for each possible valve pressure head, from the minimum (Pvmin) to the maximum design pressure (Pvmax) in an incremental range defined by the user (PV Step - Ex: 1 m), the optimal sizing of the submain manifold pipe is carried out with the above mentioned process (LMM-LM/KPH).

A variant of the LMM method for unknown pressure head (LMM /UPH) was not used to find in a single sequence pipe sizing and optimal valve pressure, avoiding the pressure iteration cycle, since it depends on the *a priori* identification of the most unfavorable point of the network, which is subject to uncertainty.

As additional conditions for the design of the irrigation submain, the speed in manifold pipes is restricted to a range of maximum and minimum admissible values, which are determined by the user, usually between 2.5 and 0.5 m s^{-1}.

For each pressure and position iteration (figure 1, page 159) an optimal design of the submain manifold pipe is obtained and its annualized total costs per unit of irrigated area (C_T; € ha^{-1} $year^{-1}$), life-cycle, total investment cost (C_a, € ha^{-1} $year^{-1}$), maintenance costs (C_m - 5% of Ca; € ha^{-1} $year^{-1}$), energy costs; if pumping is required (C_e; € ha^{-1} $year^{-1}$) and water cost (C_w; € ha^{-1} $year^{-1}$) associated to the subunit are calculated as shown in the following equation (Carrión *et al.* 2013) which states:

$$CT = 1.05C_a + C_e + C_w \quad (3)$$

$$CT = \frac{\frac{i(1+i)^N}{i(1+i)^N - 1}}{S} + 1.05\frac{\frac{i(1+i)^N}{i(1+i)^N - 1}}{S} + \frac{\frac{9.81Q_{OS}H_OR_n}{Ep3600Q_{OS}eA}En_c}{S} + \frac{R_nTr}{EU}P_w \quad (4)$$

where:
C_i = total investment cost (€)
S = irrigated area (ha)
i = interest rate
N = service life (years)
Q_{os} = design flow ($m^3\ s^{-1}$)
Ho = subunit intake pressure (m)
Ep = pumping efficiency (0.65 on average)
Rn = annual net crop water requirement ($m^3\ ha^{-1}\ year^{-1}$)
Enc = average cost of energy consumed, (€ kwh^{-1})
Ea = general application efficiency for the irrigation system (%/100)
Tr = transmission ratio for maximum demand period (20)
Pw = price of water, excluding energy costs for pressure supply (€ m^{-3}).

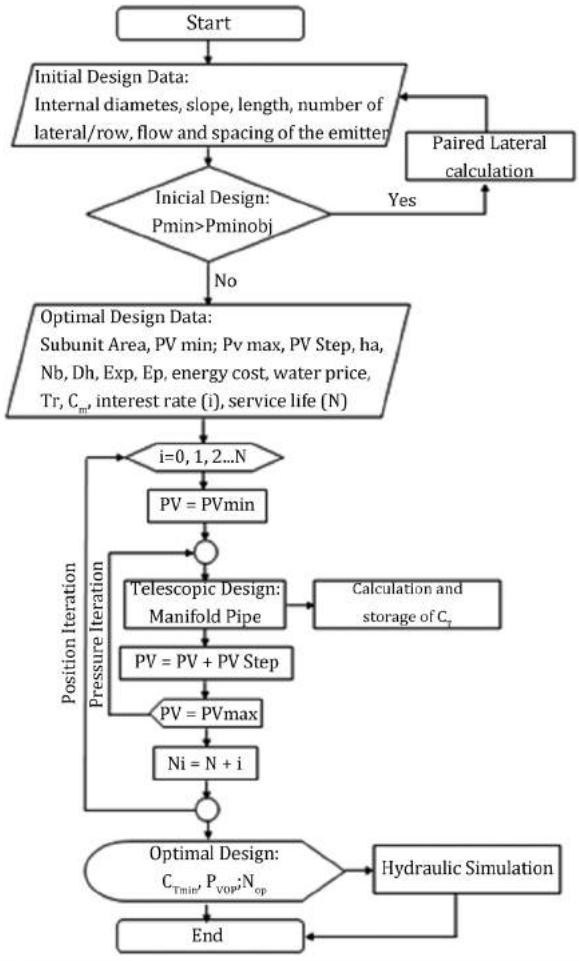

Figure 1. Subunit optimization process with pressure-compensating emitters (GESTAR).

Figura 1. Diagrama de flujo del proceso de optimización de sectores con emisores autocompensantes (GESTAR).

Though in the case of pressure-compensating emitters the values of C_w and C_m are constant, they are incorporated into the calculation process to be compared with designs made with other software and/or with designs that include turbulent emitters. The aforementioned cost approach does not consider explicitelly the extended length of the main pipe necessary to conect the optimum intake point to plot network. Nevertheless, this cost and all storage and infrastructure inevestments required to make the subunit operational can be included in Cw and, if it is appropriate, also the energy costs asociated to head losses in the plot network and collective network that drive water to the subunit.

Once the double nested iterative process is completed, the optimal joint design (telescopic sizing of the submain manifold pipe, pressure and valve intake location) is determined as the alternative that minimizes the total annualized cost per unit of irrigated area. A soon as the optimal design has been completed, its hydraulic simulation can be performed to predict the detailed pressure distribution and verify the proper hydraulic operation of the submain. The described methodology was implemented within the drip irrigation design module, in the GESTAR software package, using the Visual Basic 6.0 programming language, providing a new advanced functionality.

Results

The methodology is applied to three examples of irrigation submains with pressure-compensating emitters for their optimal design, where the geometrical and topographic configuration is heterogeneous. The results obtained are compared with previous designs developed with another commercial computer tool (Irricad Pro) as a feasibility test.

The submains irrigate vineyards that share the following characteristics: a) distance between rows: 2 m, b) distance between plants: 1 m, c) emitter flow: 1.60 L h^{-1}, d) emitter spacing: 0.60 m, e) emitter range of compensation: 4-40 m, f) external diameters of the lateral: 16 mm, g) internal diameter of the lateral: 15.5 mm, h) number of laterals per row: 1 lateral, i) emitter manufacturing variation coefficient: 4%. For each example, the layout of the main pipe runs parallel to every manifold pipe; this gives the possibility of connecting the valve at any point along the submain manifold pipes.

Based on the external diameters (mm), table 1 shows the costs per linear meter of the possible pipes that are used in the LMM/KPH-LM algorithm for the submain manifold pipe optimization.

For the hydraulic-economic optimization of the submains, the following variables have been considered: a) energy price: 0.06 € kwh^{-1}, b) water price: 0.10 € m^{-3}, c) cost of the lateral: 0.33 € m^{-1}, d) interest rate: 7%, e) service life: 25 years, f) pump efficiency: 65%, g) annual net irrigation requirements: 6,000 m^3 ha^{-1}, h) transmission ratio: 1.05, and i) maintenance cost: 5% of annualized material acquisition cost (C_a).

Table 1. External diameters of pipes and their corresponding cost per linear meter.
Tabla 1. Diámetros externos de tuberías y su correspondiente costo por metro lineal.

DE (mm)	50	63	75	90	110	125	140	160	200
€/m	1.33	1.39	2.62	2.71	3.12	3.89	4.97	3.36	10.4

Figure 2 and table 2 show the geometrical and topographic characteristics and the results of the previous hydraulic design of the three submains, where the valve's pressure (Pv), submain flow, length, diameter and total cost of secondary pipes are shown.

Figure 3 (page 162), shows the new form created with the GESTAR tool to introduce the necessary data for the optimal hydraulic-economic design of the submains. Table 3 (page 162), summarizes the results of the hydraulic design optimization of the submains and shows: the number of hydraulic designs (Designs No.), the optimized design valve pressure (Pv); the length, diameter and total cost of the manifolds optimized pipes.

While the cost of the lateral for each design alternative (initial and optimized) is the same, significant cost savings are achieved by placing the valve at an intermediate point of the submain manifold pipe where the flows are divided and design is optimized using the proposed algortihm LMM/KPH-LM (13). Thus, in Example 1 a 22% manifold pipe cost reduction is achieved, while in Example 2 cost reduction is in the order of 34% (because the pipe throughout its entire length is on an ascending slope) and in Example 3 it is 16%.

Table 4 (page 162), summarizes the main results of the hydraulic-economic optimization of the irrigation submains and shows: the intake point of the subunit (X/Lp), where X is the distance downstream of the submain manifold pipe with respect to the connection of the valve and Lp is the total length of the submain manifold pipe (20).

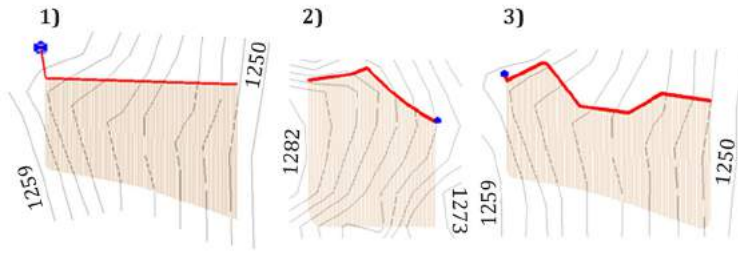

Figure 2. Geometric and topographic form of submains (contour lines every 1 m).
Figura 2. Forma geométrica y topográfica de sectores de riego (curvas de nivel cada 1 m).

Table 2. Hydraulic design characteristics of the irrigation sector according to Irricad Pro.
Tabla 2. Características hidráulicas de diseño de los sectores de riego ejemplo según Irricad Pro.

Ej.	S	Flow	Pv	Diameter (mm) and length (m) of pipes				Total length	Total Cost
	ha	m^3h^{-1}	m	90	75	63	50	m	€
1	2.30	30.67	28		66	56	78	200	354.5
2	1.94	25.87	24	49	70	38		157	369.8
3	2.09	27.87	24		50	65	118	232	376.6

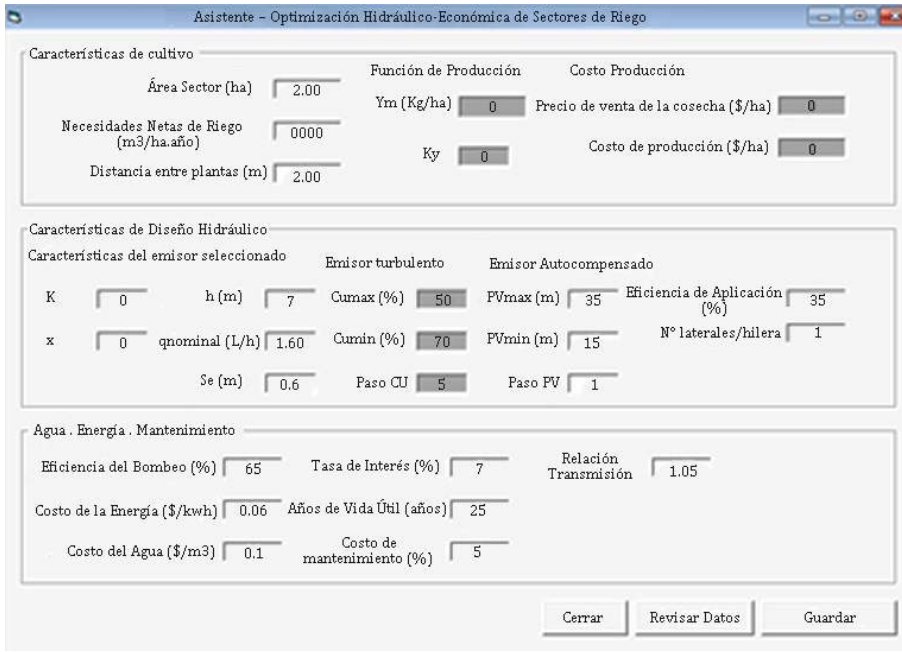

Figure 3. GESTAR form for data entry for economic hydraulic optimization of submains with pressure-compensating emitters.

Figura 3. Formulario GESTAR de ingreso de datos para la optimización hidráulica y económica de sectores con emisores autocompensados.

Table 3. Optimized hydraulic design features of the irrigation submains (Gestar).

Tabla 3. Características de diseño hidráulicas optimizadas de los sectores de riego (Gestar).

Ej.	Area	Designs	P_{Vop}	Diameter (mm) and Length (m) of pipes					Total	Total Cost
	ha	N°	m	110	90	75	63	50	m	€
1	2.30	1648	15			2	68	122	202	276.2
2	1.94	924	15	2		22.25	43.13	87.51	157	240.9
3	2.09	1648	15			2	72.31	157.59	232	315.4

Table 4. Main hydraulic operation characteristics of the optimized Gestar design for the irrigation submains (Gestar).

Tabla 4. Principales características de funcionamiento hidráulico del diseño optimizado de los sectores de riego (Gestar).

Ej.	X/Lp	Pv (m)	Pmax (m)	Pmin (m)	Vmax (m/s)	Vmin (m/s)	C_e	CeOpt.
1	77	15	14.46	7.48	2.47	0.041	49.30	23.77
2	72	15	14.90	6.62	2.50	0.102	42.26	26.41
3	92	15	15.24	7.49	2.50	0.041	49.30	26.41

The optimized valve pressure is also presented: P_{Vop} (m), the maximum and minimum dripper simulated pressures: Pmax, Pmin; the maximum and minimum secondary pipe simulated speeds: Vmax, Vmin (m/s); the annualized energy cost per unit of irrigated area (C_e; € kwh^{-1}) of the initial design; and the annualized energy cost of the optimized design.

Figure 4 graphically details the results of the hydraulic simulation of the optimal design for each irrigation submain. It shows the real hydraulic operation of the submain and the optimal design pressure in all the emitters (7-24 m). The high distribution uniformity is only dependent on the manufacturing variation coefficient of the emitter because it is a pressure-compensating emitter.

In terms of energy optimization of optimized designs, a 51% saving is achieved in Example 1 with respect to the annualized energy cost per unit area; a 37% saving is achieved in Example 2 and a 46% saving in Example 3 by reducing the valve pressure value to almost half of the initial Irricad design. Tarjuelo *et al.* (2010) stated that energy costs per unit area can reach approximately 40-50% of the total annualized cost when considering all the submains of a drip irrigation system. However, this depends on the characteristics of each irrigation design.

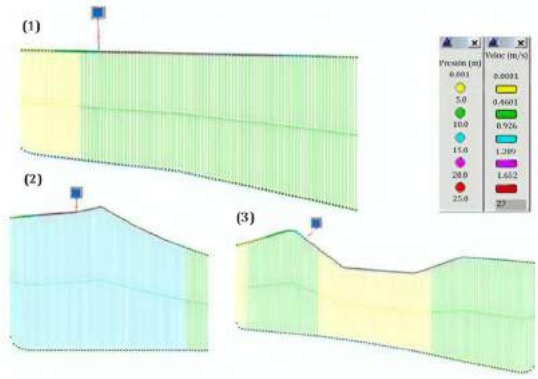

Figure 4. Hydraulic optimal design simulation in irrigation subunits (the color ramp is related to the speed of the water at the inlet of the lateral - Gestar).

Figura 4. Simulación hidráulica del diseño óptimo en sectores de riego (la escala de colores está relacionada con la presión y velocidad del agua al ingreso del lateral - Gestar).

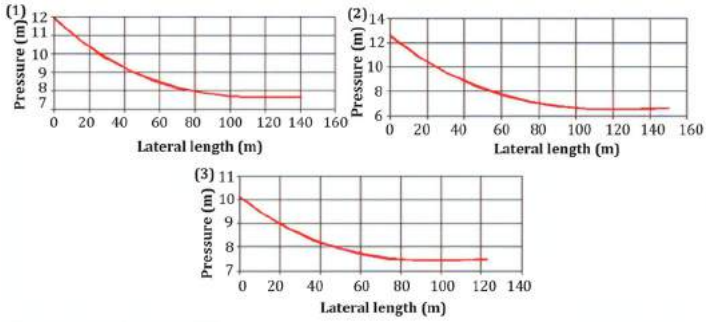

Figure 5. Pressure distribution for the critical lateral of each example (Gestar).

Figure 5. Distribución de presiones para el lateral crítico en cada ejemplo (Gestar).

Conclusions

A computational methodology was developed for the optimal hydraulic-economic design of irrigation submains using pressure-compensating emitters which makes it possible to optimize in each specific topographic and geometric sector: a) the telescopic sizing of the manifold pipe; b) the intake valve pressure and c) the submain's intake valve location, by minimizing the total annualized cost per unit of irrigated area. To this end, the optimal sizing algorithms of the pipes (13, 14) were used and generalized in a double nested iterative process.

The methodology was implemented in the design module of a drip irrigation plot, in the GESTAR computer package, using the Visual Basic 6.0 programming language which provided a new advanced functionality.

The hydraulic simulation techniques of pressure networks in the software (1, 2, 9, 10, 11, 15, 34) make it possible. If deemed appropriate, is posible to perform a final interactive verification and modification of the resulting design.

The practical usefulness of the methodology is shown with three examples of complex real cases where pipe design costs are reduced by 16-34% and energy costs by 37-51%. Results show that it is feasible to optimize the feeding point for any design condition and that the methodology is a useful decision-making tool for the design and management of drip irrigation systems with pressure-compensating emitters. Optimal design solution calls for deep knowledge of the design area as well as of agronomic management of the irrigated crop.

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Vineyard zoning of *cv* Bonarda argentine (*Vitis vinífera* L.), from Sentinel satellite images and three vegetation indexes

Zonificación de un viñedo de *cv* Bonarda argentina (*Vitis vinífera* L.) a partir de imágenes del satélite Sentinel y tres índices de vegetación

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Abstract

This study describes the results and conclusions obtained from vineyard zoning in Mendoza by using three vegetation indexes (NDVI, EVI and SAVI). Such indexes were calculated from spectral signals received by the Sentinel-2A satellite in the days close to the 2017 harvest. With this information, maps from the plot of land with the zoning given by each index were obtained. Based on the NDVI zoning, a stratified sampling was carried out. On each stratum, 14 plants were marked and the production variables total weight of grape per plant, number of grape bunchs per plant and the weight of 50 berries were measured. The results showed that NDVI and SAVI led to similar classifications in terms of vineyard zones (strata), while EVI captures high vigor levels with less sensitivity. There was a correspondence between the production variables and the strata of high, medium and low vigor. The three indexes clearly showed two different vineyard areas in terms of production. Consequently, these indexes may contribute to rationalize viticulture practices, adjusting the intensity of such practices to the characteristics and needs of each of these vineyard areas.

Keywords
precision agriculture • Sentinel 2 • zoning • vegetation indexes • grapevine • Bonarda argentina

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Resumen

El presente trabajo describe los resultados y conclusiones obtenidos en la zonificación de un viñedo de Mendoza, empleando tres índices de vegetación (NDVI, EVI y SAVI) calculados a partir de las señales espectrales captadas por Sentinel 2A, en los días próximos a la cosecha de 2017. A partir de esta información, se obtuvieron los mapas de la parcela con la zonificación dada por cada índice. En base a la zonificación dada por NDVI, se realizó un muestreo estratificado y en cada estrato se marcaron 14 plantas sobre las que se midieron las variables productivas peso total de uva, número de racimos por planta y peso de 50 bayas. Los resultados muestran que NDVI y SAVI conducen a clasificaciones similares en cuanto a zonas (estratos), mientras que sugieren que EVI capta con menos sensibilidad niveles de alto vigor. Hubo correspondencia entre los valores de las variables productivas con los estratos de alto, medio y bajo vigor. Los tres índices diferenciaron claramente dos zonas del viñedo en lo relativo a su producción, en consecuencia, los mismos podrían contribuir a racionalizar las prácticas de cultivo, adecuando su intensidad a las características y necesidades de cada una de estas zonas del viñedo.

Palabras clave

agricultura de precisión • Sentinel 2 • zonificación • índices de vegetación • vid • Bonarda argentina

Introduction

The study of the spatial variation in the vine, which has been associated to the vigor variation, tends to focus on foliar area measurement, production variables (berry weight, yield, among others) and also on variables related to the quality (polyphenol concentration and anthocyanin content of grapes, among others) (2, 7, 11).

For premium winemaking, it is essential to identify stratum of homogenous variability in a vineyard in order to contribute to wine quality and/or quantity improvement according to the needs of the winery (6).

Currently, remote sensing systems, geographic information systems and GPS receivers, allow the identification, measurement and mapping of the special variability of the grapevine. Such technologies are globally known as precision agriculture or site specific crop management (2, 14, 15) and they allow to carry out sampling plans conducted by areas or strata, to estimate the vintage precision and to perform a selective grape harvest (19).

In this context, free-to-use images given by the Sentinel-2A are currently available. Such satellite belongs to a constellation of 2 identical satellites that were launched by the European Space Agency (ESA) and they have been working since August 2015 (2A) and October 2017 (2B) in order to carry out different missions. The main goal of these satellites is to provide earth, ocean and atmosphere data. One of these missions works specifically to monitor the environment, the vegetation status and, mainly the agricultural and forestry practices, among other goals. In this way, the capacity of SPOT and Landsat missions were continued and broaden.

The optical images from the Sentinel-2 satellite may revolutionize the research on the Earth's surface since it has never been possible before to get images of such a good spatial, temporal and spectral resolution for free (13).

Different vegetation indexes could be calculated from the spectral answer of a surface received by the satellite remote sensing systems (IV). Such indexes are the product of simple relations between the reflectance in different bands of the electromagnetic spectrum, seeking to enhance the contribution of the vegetation and to mitigate the contribution of other elements such as the soil, the lighting or the atmosphere.

From 1974, a great number of vegetation indexes has been developed, among which the NDVI (Normalized Difference Vegetation Index) has been the most used one because of its easy way of calculation and interpretation (3). This index allows to calculate the plant vigor on a particular phenological stage. The vigor indicates the condition of the crop. In the particular case of vineyards, a strong temporal relation between this index and the leaf area index was found (10), while the relation with productivity and whit enological quality parameters (researched in a great number of studies) has been less clear (11, 12, 21).

The main problem of the NDVI index is the influence of the ground reflectance when the vegetation coverage is very poor (wide planting standards or early farming stages,) hindering the interpretation of the obtained values. Consequently, other indexes have been proposed, such as Soil Adjusted Vegetation Index (SAVI), which includes a soil-adjusted constant (L) that reduces the resulting reflectance and is able to compensate the "soil effect" in these cases (8).

Another improved vegetation index is the EVI (Enhanced Vegetation Index), which corrects distortions in the reflected light caused by clouds and aerosols that can block the satellite's view. The EVI data does not become saturated as easily as the NDVI when viewing rainforests and other areas of the Earth with large amounts of chlorophyll and it allows a better differentiation of the structural variations of the canopy (3, 9).

The purpose of the present study was to analyze and compare the zoning given by each of the three vegetation indexes abovementioned from the spectral signal of a vineyard. Such spectral signal was obtained by the remote sensing of a new satellite, better in spatial and temporal resolution in relation to other satellites that provide free-to-use images (Lansad 8).

Likewise, an attempt was made to establish whether zoning obtained agreed to the spatial variability of the production variables.

Materials and Methods

Study area

This study was carried out during the 2016-2017 production cycle in a pergola trellis system of Bonarda argentine grape cultivar, located at Chapanay division, Department of San Martín, in the East region of Mendoza province, Argentina. This area has a warm temperate climate with cold nights. During the harvest season evaluated, it was recorded an average temperature of 38.4°C maximum, 21.96°C medium and 6.18°C minimum with an average precipitation of 86.26 mm (5).

Image acquisition

Satellite images from the Sentinel-2A that were taken on the 26th of January of the year 2017 were used. Such images were taken during the veraison growing period (4). It was worked with satellite images provided by the Geological Service of the United States, whose link is: https://earthexplorer.usgs.gov/.

The bands used were the blue, red and near-infrared ones respectively with a spatial resolution of 10 m.

The obtained image of the plot of land for this study had a total of 661 pixels, each of which represents an area 10 m x 10 m.

Zoning

From the obtained image, the software QGIS2.16 (16) was used in order to calculate the vegetation indexes NDVI, EVI and SAVI.

The formulas used to calculate the NDVI, SAVI and EVI indexes are the following:

$$NDVI = \frac{NIR - R}{NIR + R} \quad (1)$$

where:
NVDI = Normalized Difference Vegetation Index
NIR = reflectance in the near-infrared
R = reflectance in the red.

$$SAVI = \frac{NIR - R}{NIR + R + L}(1 + L) \quad (2)$$

where:
SAVI = Soil Adjusted Vegetation Index
NIR = reflectance in the near-infrared band
R = Reflectance in the red band
L = constant to adjust the vegetation-soil line to the origin, 0.5

$$EVI - 2.5\text{x}\frac{(NIR - R)}{(NIR + C1 \times R - C2 \times B + L)} \quad (3)$$

where:
EVI: Enhanced Vegetation Index
NRI = reflectance in the near-infrared band
R = reflectance in the red band
B = reflectance in the blue band; the used coefficients in the algorithm are L = 1, C1 = 6, C2 = 7.5.

Lately, an unsupervised classification was carried out. Such classification was obtained from the emerging data of the calculated pixels, based on the division of the range of values at equal intervals. From this classification, three areas (strata) associated to different levels of vigor (high, medium and low) for each calculated index arise (figure 1, page 171).

Sampling design

Taking the NDVI as a reference classification given its wide diffusion in precision viticulture (1), and taking into account that the strata produced by the preceding classification are approximately the same size, a stratified sampling was carried out, with n = 7 pixels/stratum.

The same technique was applied for the strata corresponding to the EVI and SAVI classifications.

The corresponding area of each pixel was located through a satellite navigation system (GPS +GLONASS) and, for each of them, two plants were chosen and identified.

Of a Samsung J7 phone (2016) - GPS + GLONASS + BEIDOU-, with the Mobile topographer application, which allows autonomous positioning with an error radius of 1 to 3 m, contrasted with official GPS points of the National Geographic Institute of Argentina.

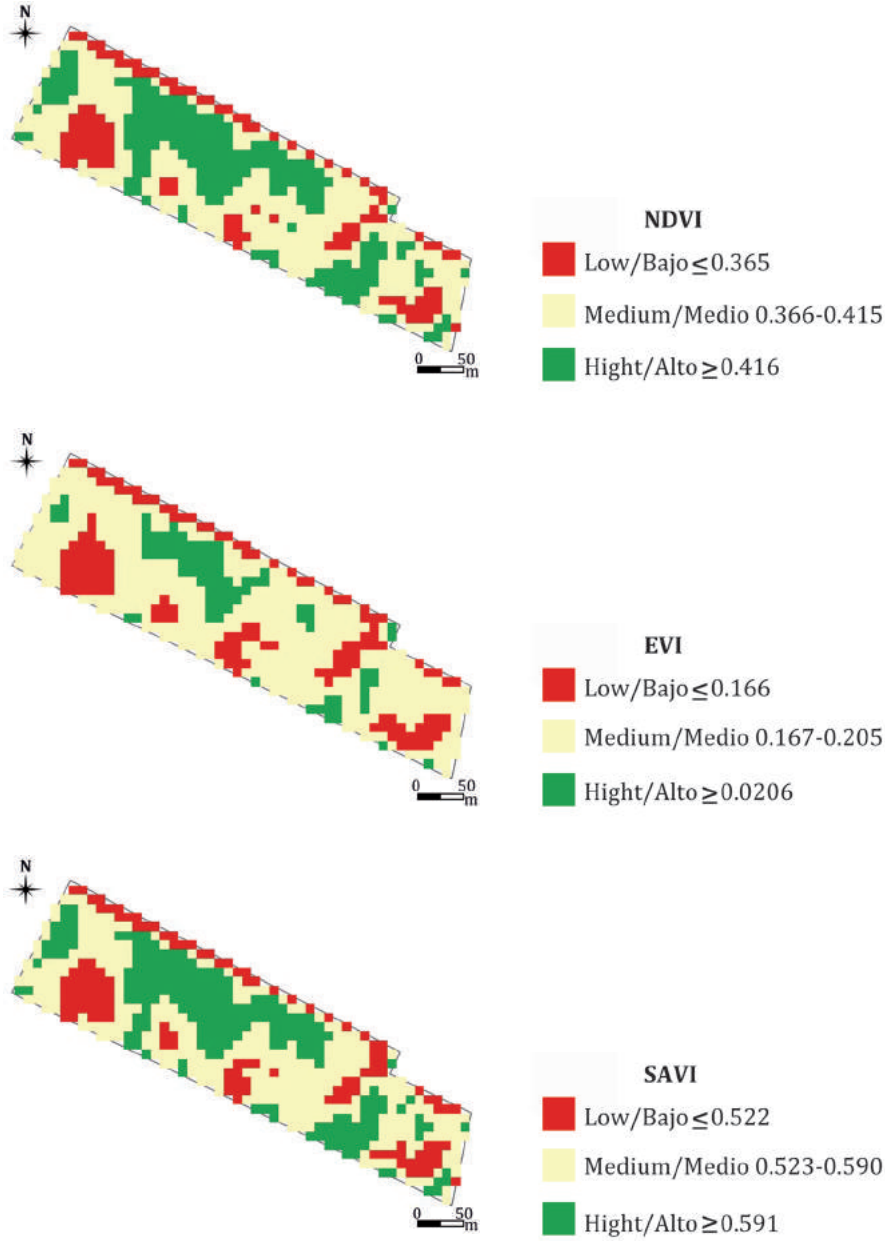

Figure 1. Maps of the studied plot with the zoning given by each vegetation index with the values of the same for each degree of vigor (stratum).

Figura 1. Mapas de la parcela estudiada con la zonificación dada por cada índice de vegetación con los valores de los mismos para cada grado de vigor (estrato).

All clusters of the plants selected as samples were harvested at the moment harvest opportunity, all the bunches of the plants sampled were collected.

The production variables evaluated were: total number of clusters, total weight of the grape and the weight of 50 berries, codified in this study as NRP, PTU and P50b respectively.

Then, a statistical description of the data sampling for each index (arranged in table form) was carried out. After that and during the inference stage, the statistical software R (17) was used to carry out a Multivariate Analysis of Variance (MANOVA) for each obtained index. In this study, the production variables obtained as response variables and, the stratum as explicative variable were included.

Two multiple comparison tests were carried out in order to determine the interaction between the strata corresponding to each index: Tukey's and Bonferroni's HSD (Honestly Significant Difference) tests with a significance level of 0.05.

Results

In relation to the zoning given by each one of the vegetation indexes, it may be noticed on figure 1 (page 171) that the maps obtained were similar among them.

The same situation was evident when analyzing the number of classified pixels in each stratum any for three or two of them (figures 2, 3 and 4, page 173).

Regarding the information shown in the Venn diagram, it is observed that for the "high" stratum (figure 2, page 173) of the indexes, NDVI-SAVI agreed a 98% on the pixel classification, while NDVI-EVI just on the 44.5% of the cases. However, this does not modify the average calculated per stratum of the measured variables PTU and P50b as it may be observed on table 1 (page 174).

From the same diagrams, it is observed that regarding "medium" and "low" strata (figure 3 and figure 4, page 173), the percentages of coincidence between the three vegetation indexes are superior to the 85% and 98% respectively.

One of the statistical assumptions for the MANOVA was omitted from the NRP variable due to multivariate normality problems.

Both Tukey's and Bonferroni's DHS tests gave identical results in the determination of interactions between the strata corresponding to NDVI, EVI and SAVI indexes.

A summary of such tests with the average parameters and the standard deviation for each variable and each stratum is shown on table 1 (page174).

From the analysis of table 1 (page 174), it may be seen that the three vegetation indexes showed significant differences for the NRP and P50b production variables between the high and low vigor areas.

Likewise, it can be seen in the same table that NDVI and SAVI capture the same information for the measured production variables and in all the strata. Such indexes establish meaningful differences between the high and low vigor strata in the two studied variables.

However, when considering the medium vigor area in relation to the high or low vigor, the interpretation is less clear since the response differs for different variables.

In the case of the total production per plant, NDVI and SAVI detect meaningful differences between the strata of high and medium vigor and between high and low vigor. EVI just detects differences between the strata of high and low vigor.

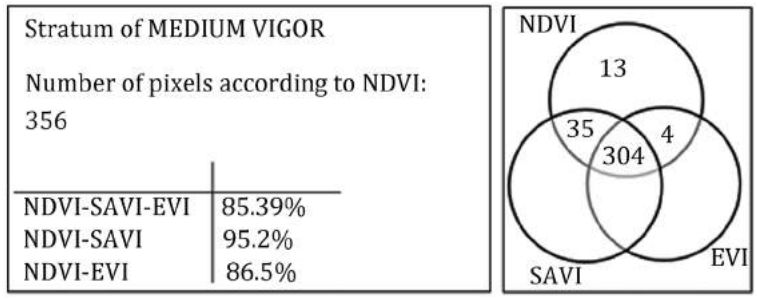

Figure 2. Venn diagrams corresponding to the distribution of pixels in the "high" stratum, according to the NDVI, EVI and SAVI indexes.

Figura 2. Diagramas de Venn correspondientes a la distribución de píxeles en el estrato "alto", según los índices NDVI, EVI y SAVI.

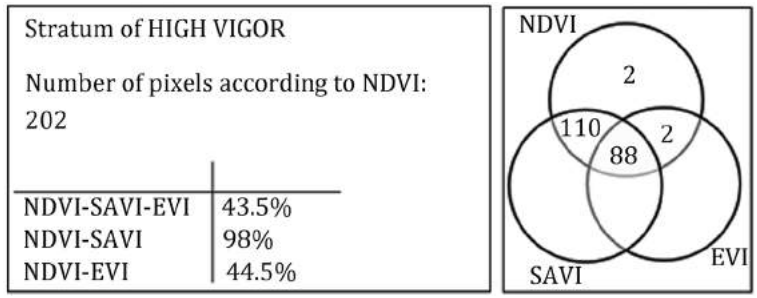

Figure 3. Venn diagrams corresponding to the distribution of pixels in the "medium" stratum, according to the NDVI, EVI and SAVI indexes.

Figura 3. Diagramas de Venn correspondientes a la distribución de píxeles en el estrato "medio", según los índices NDVI, EVI y SAVI.

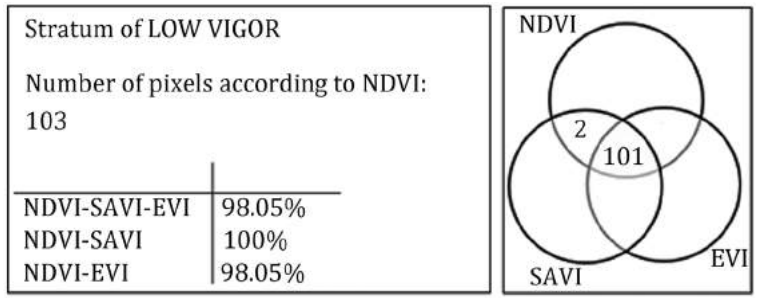

Figure 4. Venn diagrams corresponding to the distribution of pixels in the "low" stratum, according to the NDVI, EVI and SAVI indexes.

Figura 4. Diagramas de Venn correspondientes a la distribución de píxeles en el estrato "bajo", según los índices NDVI, EVI y SAVI.

Table 1. Average values of productivity in each block and multiple comparison by means of Tukey's HSD test.

Tabla 1. Valores medios de productividad en cada bloque y comparación múltiple de medias por test de Tukey HSD.

		Índices de Vegetación					
		NDVI		EVI		SAVI	
Variable	Stratum	n	Average ± DS	n	Average ± DS	n	Average ± DS
PTU (kg)	High	14	12.23 ± 4.5 a	8	12.69 ± 5.3 a	14	12.16 ± 4.5 a
	Medium	14	8.20 ± 2.1 b	18	9.28 ± 3.1 ab	14	8.27 ± 2.1 b
	Low	14	6.16 ± 3.2 b	16	6.48 ± 3.1 b	14	6.16 ± 3.2 b
P50b (g)	High	14	103.2 ± 12.5 a	8	104.5 ± 12.8 a	14	101.5 ± 11.3 a
	Medium	14	92.99 ± 14.5 ab	18	96.66 ± 14.5 a	14	94.60 ±16.4 ab
	Low	14	82.78 ± 10 b	16	83.07 ± 9.7 b	14	82.78 ± 10 b

Different letters in the same column indicate meaningful differences with a p-<0.05 value.

Letras distintas en una misma columna indican diferencias significativas con un p-valor<0,05.

In the case of P50b, NDVI and SAVI just detect meaningful differences between strata of high and low vigor, while EVI detects differences between strata of medium and low vigor and between high and low vigor.

Discussion

Only NDVI and SAVI agreed on the classification on a 95% and 100% of the pixels on each stratum. This result is consistent due to the fact that in the construction of the SAVI index the same spectral bands (R and NIR) as in NDVI are added, plus a specific calibration parameter (equation 2, page 170). This result agreed with previous studies although another kinds of vegetation covers were evaluated (3, 18, 20)

Regarding the difference in the EVI classification in relation to the other indexes, just the stratum of "high" vigor coincidence may be interpreted according to its construction (equation 3, page 170). Such index is more sensitive to the near-infrared band (NIR), allowing a better differentiation of the structural variations of canopy and plant physionomy (9).

This behavior may be considered similar to the previously observed during the assessment of different vegetation covers, in which EVI showed better class distinctions in densely vegetated categories (18).

In table 1, it can be observed the correspondence between the values of the production variables and the strata of high, medium and low vigor so that the areas of the highest vigor showed the highest production values. These results are similar to the ones found by other researchers when zoning the vineyards in order to obtain areas of different grape quality (12).

The information shown in MANOVA in table 1, allows to infer that the three calculated indexes detected meaningful differences for the production variables NRP and P50b between the areas of high and low vigor. This indicates that the use of any of them allows to clearly identifying strata with dissimilar vegetation vigor.

The behavior of the indexes is less clear in relation to the differentiation between the area of high and medium vigor or between the one of low and medium vigor, which is different for each production variable under consideration. This kind of analysis was not found in the reference bibliography, reason that encourages future studies.

Conclusions

The images given by the Sentinel-2 satellite offer a good alternative for intra-parcel zoning in grapevines for winemaking production because of its spatial and temporal resolution level, as well as its easy acquisition and free availability.

Since NDVI and SAVI indexes zoned basically in the same way, in the case of a pergola trellis system, the soil effect on the reflected radiation seems to be irrelevant, therefore, NDVI index would be enough to describe the vegetative expression behavior.

The three indexes allowed to clearly differentiate two vineyard areas regarding its very different vegetative growth and yield, elements that would require a different treatment regarding farming practices.

Consequently, these indexes may contribute to rationalize the farming practices adjusting the intensity of such practices to the same characteristics and needs of each one of these vineyard areas.

For the conditions of this study and the studied variety, the descriptive information of this study would suggest that EVI would not have the same usefulness to carry out an adequate zoning. This is because it would be less sensitive to differentiate areas of high vegetation vigor than the other two indexes, which in this sense would provide a very ambiguous description of the plot of land.

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Ánalisis de susceptibilidad de flujos de detritos en el Parque Provincial Aconcagua, Mendoza, Argentina

Debris flows susceptibility analysis in the Provincial Aconcagua Park, Mendoza, Argentina

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Resumen

Los flujos de detritos representan potenciales peligros geológicos que amenazan a la comunidad en el Parque Aconcagua. Estos procesos se generan en pendientes abruptas alcanzando grandes velocidades y volúmenes, siendo su principal causa histórica la saturación de los sedimentos o rocas a partir del deshielo en épocas estivales. En este trabajo se evalúa la susceptibilidad a la ocurrencia de flujos de detritos en dicha reserva en función de factores como litología, pendiente, altura, orientación de las laderas y presencia de vegetación. Estas variables se analizaron a través de superposición de mapas temáticos y un mapa inventario de flujos de detritos históricos en el entorno de un SIG. Se utilizó el modelo de base física SINMAP y métodos estadísticos de regresión logística y Wi, obteniendo diferentes mapas de susceptibilidad que fueron validados espacialmente con un mapa inventario de control. Todos los modelos presentaron buen desempeño, aunque SINMAP presentó diferencias en la zonificación con respecto a los modelos estadísticos, siendo la regresión logística la metodología más apropiada para la valoración de la susceptibilidad.

Palabras clave
peligrosidad espacial • factores condicionantes • flujos de detritos

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Abstract

Debris flows occurrence represents a potential geological hazard threatening community of the Aconcagua Park. These events are generated on steeper slopes reaching high velocities and great volume. Main historical cause of events is water saturation of sediments or rocks due to precipitation, snow thawing or permafrost degradation during warmer seasons. This paper focuses in the analysis of debris flow susceptibility considering conditioning factors such as lithology, slope, elevation, slope orientation and vegetation presence. These variables were analyzed through superposition of thematic maps and an inventory map of historical debris flows in a GIS environment. A physical model (SINMAP) and statistical methods of logistic regression and Wi were applied obtaining three different models spatially validated with inventory control map. The models showed good performance, even though the SINMAP shows differences in the zoning comparing with statistician ones, being logistical regression the more proper method for susceptibility assessment.

Keywords:
spatial hazard • conditioning factors • debris flows

Introducción

Un flujo de detritos es un movimiento en masa, inducido generalmente por la saturación de agua (5), que incluye varios fragmentos de rocas de distintos tamaños, sedimentos, agua y aire (11). En general, estos procesos se caracterizan por un desplazamiento rápido y presentan comportamiento de un líquido viscoso, capaz de transportar cualquier objeto que se encuentre en su camino (6).

Los flujos de detritos se originan en zonas de pendientes abruptas, pero una vez generados, pueden movilizarse en pendientes muy suaves. Generalmente se canalizan en hondonadas del terreno, sectores deprimidos y hasta cursos fluviales dando lugar a morfologías diferentes (4). Suelen generar albardones durante su recorrido depositándose en la parte inferior de las laderas dando lugar muchas veces a abanicos aluviales. Su característica principal es su gran poder de transporte, debido a las grandes velocidades que desarrollan, que hace a los flujos de detritos particularmente peligrosos. El material transportado puede desencadenar represamiento de cursos del río, los cuales pueden colapsar y producir serios daños aguas abajo.

Los impactos negativos que implican los flujos de detritos pueden ser reducidos mediante medidas de mitigación preventivas. La primera fase en la prevención es la identificación y caracterización del peligro (10).

El paso inicial para ello es la evaluación de la susceptibilidad a la ocurrencia de flujos de detritos, es decir, la probabilidad espacial de que ocurran flujos de detritos. El Parque Provincial Aconcagua forma parte del sistema de áreas Protegidas de la Provincia de Mendoza, Argentina (Ley 4807/83) recibiendo cada año una gran afluencia de visitantes. En la temporada 2007-2008 se registraron aproximadamente 7.000 visitantes (24).

En el área los flujos de detritos son muy activos y violentos, aunque en la actualidad se desconoce el comportamiento de estos procesos, su grado de actividad y el efecto negativo que podrían tener dentro de dicha reserva.

La principal causa de los flujos de detritos es la saturación de los materiales de las laderas durante el deshielo en la época estival. Rara vez estos flujos han sido reportados durante lluvias intensas de verano o asociados a sismos (16).

La peligrosidad asociada a estos eventos se ha visto concretada en hechos como arrastre de mulas, represamientos temporales de los ríos, etc. lo que resalta el gran riesgo de ubicar instalaciones vitales en los lugares más susceptibles a la ocurrencia de flujos de detritos (17).

En el presente trabajo se realiza un análisis regional de la susceptibilidad a flujos de detritos en el área del Parque Provincial Aconcagua con la hipótesis que dicha probabilidad espacial varía en función de factores condicionantes del terreno. Dicho análisis se realizó en el entorno de Sistemas de Información Geográfica comparando diferentes modelos estadísticos y uno de base física (SINMSP). Con ello, se generó una zonificación del área en función del grado de susceptibilidad a dichos procesos naturales siendo una herramienta de planificación y elaboración de estrategias para contribuir a evitar la ocurrencia de desastres.

Cabe destacar que se han considerado las zonas de arranque u origen de los flujos de detritos pero no se han contemplado sus trayectorias, variable difícil de evaluar mediante modelos estadísticos y que no constituye un parámetro a ser evaluado en el presente trabajo.

Área de estudio

El Parque Provincial Aconcagua se emplaza en el Departamento Las Heras, Mendoza, Argentina (figura 1, pág. 180), entre los 70°07' y 69°45' O y 32°35' y 32°48' S, en la provincia geológica de la Cordillera Principal en los Andes Centrales. Abarca una superficie aproximada de 71.000 ha. y alturas entre los 1.700 y los 6.900 m s. n. m., con pendientes de hasta 80°, predominan las laderas con orientaciones oeste y sureste.

La vegetación es escasa, dominando comunidades con fisonomía de matorrales, estepas, pastizales y praderas. La cobertura es baja excepto en zonas de vegas. Abarca dos cuencas principales, la cuenca del río Vacas y la del río Horcones. Durante la época estival, los cursos de estos ríos incrementan su caudal debido al aporte producto de la fusión de nieve y glaciares. Se observan grandes variaciones de los registros climáticos en cortos recorridos y periodos.

Según Koeppen (1931) el clima del área corresponde a Tundra entre los 2.700 y 4.100 m s. n. m., y a cotas superiores a clima Polar de hielos eternos donde la temperatura media mensual nunca supera los 0°C y el suelo permanece congelado todo el año. Los valores de precipitación media anual para la zona llega a 500 mm en los sectores más altos ocurriendo principalmente en el periodo invernal en forma de nieve (18).

El Parque Aconcagua pertenece a la región morfo-estructural de Cordillera Principal donde predominan las rocas de edad mesozoica representadas por sedimentitas marinas, depósitos epiclásticos y vulcanitas jurásicas-cretácicas (18). El complejo volcánico Aconcagua de edad terciaria aflora también en la región y las vulcanitas permotriásicas del Grupo Choiyoi (21). Estructuralmente la región pertenece a la faja plegada y corrida del Aconcagua con una serie de imbricamientos vergentes al oriente (22).

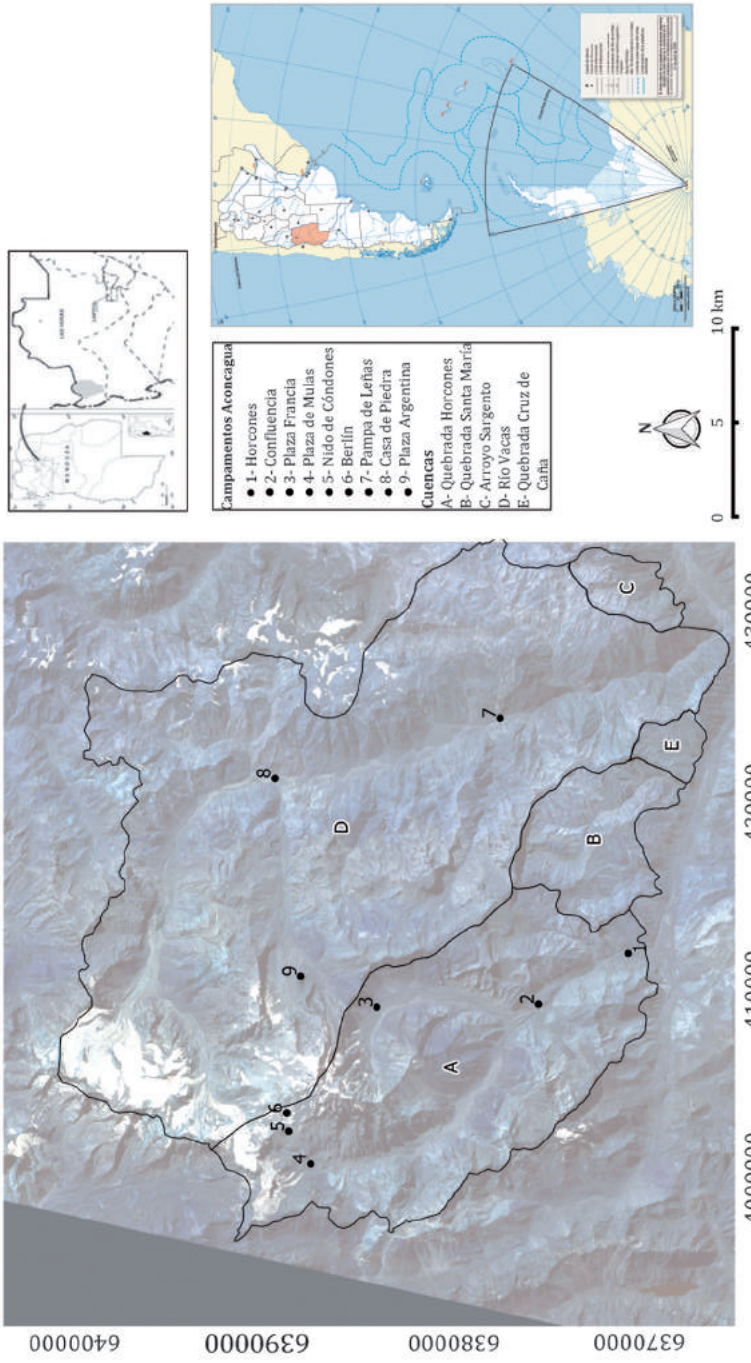

Figura 1. Ubicación del área de estudio, cuencas principales y campamentos del Parque Aconcagua.

Figure 1. Location of the study area, main basins and Aconcagua Park camps.

Materiales y métodos

Inventario de flujos de detritos y factores condicionantes

Se digitalizaron los puntos correspondientes a la zona de iniciación de cada flujo de detrito histórico del mapa inventario realizado previamente (23) y actualizando el mapa original (17).

Los puntos correspondientes a las zonas de iniciación de los flujos de detritos se digitalizaron a partir de una imagen ALOS georreferenciada del 2010 (GeoTIFF) con resolución espacial de 10 m y tiene un nivel de procesamiento de falso color compuesto. Así el mapa inventario a escala 1:250.000 muestra un total de 923 puntos de inicio. Aleatoriamente se seleccionaron 766 puntos para la ejecución de los modelos, separando el resto para el proceso de validación espacial de los resultados.

Posteriormente se seleccionaron las variables que se consideran condicionantes y determinantes de la susceptibilidad de los flujos de detritos en el área de estudio. Estas son: litología, elevaciones, pendientes, orientaciones de laderas y vegetación. El mapa litológico empleado se basó en el realizado previamente (18). El modelo digital de elevaciones (MDE) se descargó del sitio http://wist.echo.nasa.gov con 30 m de resolución (WGS 84/EGM 96). Las pendientes y orientaciones de ladera se derivaron a partir del procesamiento del MDE.

El mapa de vegetación empleado es el elaborado por Van Westen (1997).

El procesamiento de la información geográfica fue realizado mediante el empleo de Arc View 3X y de gvSIG 1.12. Para el análisis estadístico se utilizó el programa Infostat Profesional (8).

Modelos de evaluación de susceptibilidad a la ocurrencia de flujos de detritos

Para el análisis de la probabilidad espacial de ocurrencia de flujos de detritos se aplicaron el modelo de base física SINMAP y dos modelos de base estadística, Wi de Van Westen y regresión logística.

SINMAP (20) calcula y asigna un índice de estabilidad de taludes en base a información geográfica, principalmente MDE. Requiere como datos de entrada el mapa inventario de eventos y el MDE. A partir de este último se derivaron la pendiente y un índice de humedad topográfica.

Se utilizó una región de calibración única, es decir que los parámetros: fricción del suelo (phi), transmisividad (T), cohesión del suelo por el sistema radical (C) y recarga del agua (R) fueron utilizados por defecto aplicando los valores predefinidos por el modelo ya que no existe información detallada respecto de la variabilidad de estos parámetros en el área de estudio.

El modelo establece seis clases de estabilidad en función de los valores que tome el índice de estabilidad (tabla 1): estable, moderadamente estable, baja estabilidad, baja inestabilidad, inestabilidad media y alta inestabilidad. Estas categorías fueron utilizadas en los demás modelos para poder compararlos.

Tabla 1. Clases de estabilidad para el modelo SINMAP.

Table 1. Stability classes for the SINMAP model.

Condición	Clase	Predicción de estado
SI>1,5	1	Estable
1,5>SI>1,25	2	Moderadamente estable
1,25>SI>1,0	3	Baja estabilidad
1,0>SI>0,5	4	Baja inestabilidad
0,5>SI>0,0	5	Inestabilidad media
0,0>SI	6	Alta inestabilidad

El índice **Wi** (27), es igual al logaritmo natural de la densidad de flujos de detritos para una clase sobre la densidad de flujos de detritos para el área total de estudio (ecuación 1). De esta forma se obtuvo el peso asignado a cada una de las clases de las variables analizadas. Posteriormente se superpusieron los mapas temáticos con los pesos asignados a cada una de las clases y se obtuvo el índice de susceptibilidad a la ocurrencia de flujos de detritos (ISFD) mediante la sumatoria de estas capas (ecuación 2).

$$Wi = In\ (F1/F2) \quad (1)$$

F1 = Densidad de flujos para cada clase = N° de flujos para cada clase/ Área de cada clase

F2 = Densidad de flujos para el área de estudio = N° de flujos totales/ Área total analizada

$$ISFD = Litología_w + Altura_w + Pendiente_w + Orientación_w + Vegetación_w \quad (2)$$

donde:

$Litología_w$ = coeficiente *Wi* de la variable Litología

$Altura_w$ = coeficiente *Wi* de la variable altura

$Pendiente_w$ = coeficiente *Wi* de la variable pendiente

$Orientación_w$ = coeficiente *Wi* de la variable orientación

$Vegetación_w$ = coeficiente *Wi* de la variable vegetación

El análisis de regresión logística se aplicó para describir la relación entre la presencia o ausencia de flujos de detritos y el conjunto de variables independientes que se tuvieron en cuenta en este estudio.

Se realizó un muestreo de puntos de ausencia de flujo de detrito, a partir de la generación de un vector de puntos de 766 puntos de distribución aleatoria en la zona de ausencia de flujos. Se generó una base de datos con la información de alturas, pendientes, orientación, litología y vegetación para cada punto de ausencia y presencia de flujo de detrito. Para profundizar en la interpretación de los resultados se estimaron los odds-ratio o *coeficiente de probabilidades*.

Sea Y variable respuesta dicotómica tal que Y=1 representa presencia de flujo de detritos, Y=0 representa ausencia de flujo de detritos,

$$Y = logit(p) = ln(p/(1-p)) = C0 + C1X1 + C2X2 + ... + CnXn$$

donde:

p = probabilidad que la variable dependiente Y tome valor 1

p/(p-1) = cociente de chances

C0 = intercepto

Constantes C1, C2,....., Cn = miden la contribución de las variables independientes X1, X2, ..., Xn a la variación de Y.

El método está bien detallado en trabajos previos (26, 27).

Finalmente, los mapas de susceptibilidad obtenidos fueron validados espacialmente mediante la superposición de dichos mapas con el mapa inventario de flujos de detritos de control.

Resultados

Mapa inventario de flujos de detritos

En la figura 2 (pág. 183), se observa el mapa inventario de flujos de detritos para el área del Parque Provincial Aconcagua, digitalizado a partir del inventario de Randis (2012).

Se representan las zonas de iniciación de los flujos de detritos mediante puntos.

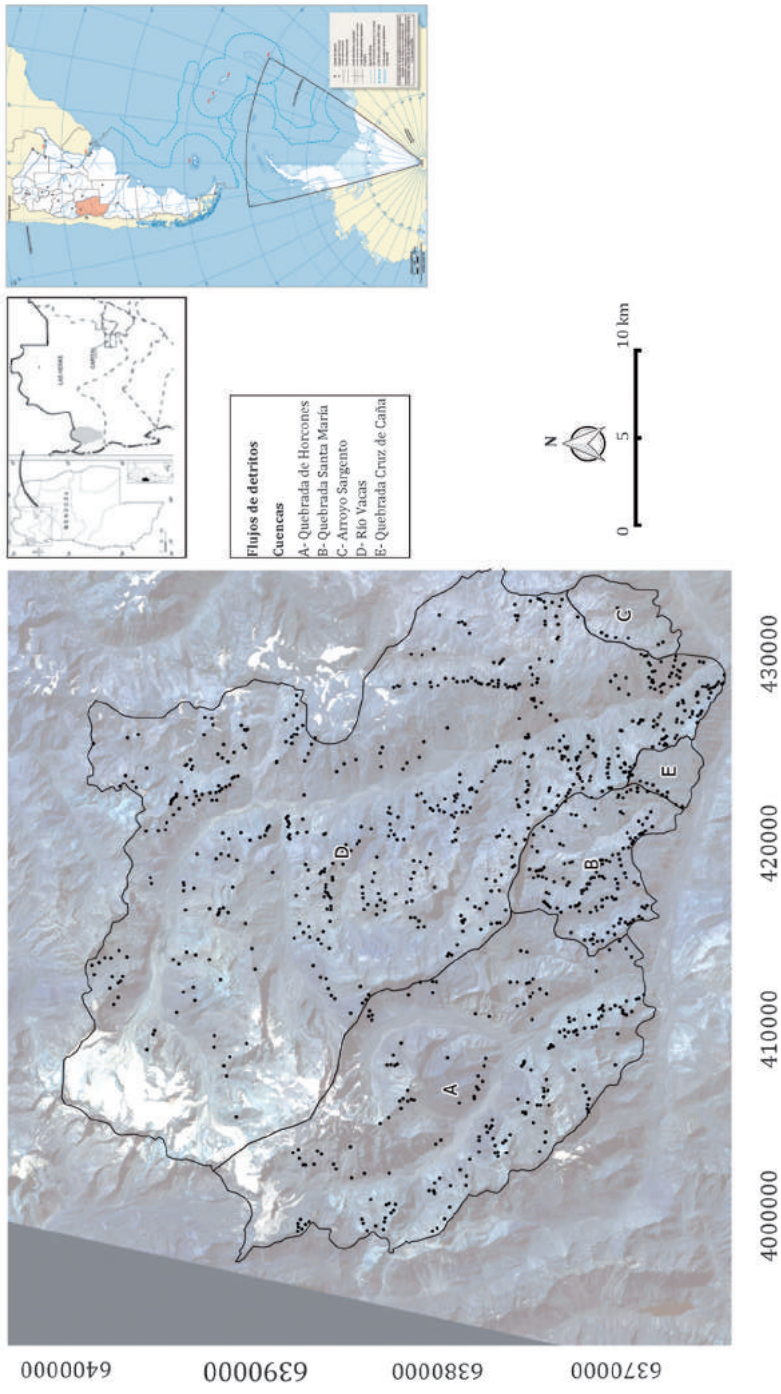

Figura 2. Inventario de flujos de detritos representados como puntos en sus zonas de iniciación.
Figure 2. Inventory of debris flows represented as points in their zones of initiation.

Evaluación de la susceptibilidad a la ocurrencia de flujos de detritos

Modelo SINMAP

El modelo clasificó el 51% del área como de baja inestabilidad, que corresponde a un escenario pesimista y con probabilidad de ocurrencia de flujos. Justamente la mayoría de los puntos del inventario se encuentran en esta categoría (65%). A su vez, el modelo clasifica un 8% del área como inestabilidad media y un 1% como inestabilidad alta. Las categorías de mayor estabilidad se encuentran representadas en el área de estudio en un 17% para estable, 8% para moderadamente estable y baja estabilidad en un 14,7%.

El mapa de susceptibilidad a la ocurrencia de flujos de detritos para el P.P. Aconcagua resultado de la aplicación del modelo SINMAP, y el histograma con los porcentajes de superficie para cada categoría de estabilidad se presentan en la figura 3A (pág. 185).

Modelo Wi de Van Westen

Según el índice Wi las litologías más susceptibles son las formaciones la Manga, Granito Cruz de Caña y Alto Tupungato; mientras que las menos susceptibles fueron Complejos Volcánico Aconcagua y los depósitos aluviales cuaternarios.

Las pendientes fuertes (16°-35°) y muy fuertes (35°-55°) son las más susceptibles a generar flujos. Esto puede deberse a que a pendientes mayores la cobertura detrítica es prácticamente inexistente y predominan otros procesos como caídas de rocas.

Con respecto a las orientaciones, las laderas N y NE, es decir, las que reciben más radiación solar, resultaron ser las más susceptibles, relacionado a que los flujos de detritos se inician generalmente por el rápido derretimiento de la nieve.

Las alturas más susceptibles son las que van desde los 2.900 a los 4.400 m s. n. m., coincidente con el dominio de los procesos periglaciales, disminuyendo a elevaciones superiores donde existen procesos glaciales. Por último la categoría ausencia de vegetación resultó ser la más susceptible a la ocurrencia de flujos de detritos.

El modelo clasificó el 36% del área de estudio como de baja inestabilidad, el 13% del área como inestabilidad media y un 2% como inestabilidad alta. Las categorías de mayor estabilidad se encuentran representadas en el área de estudio en un 2% para estable, 15% para moderadamente estable y baja estabilidad en un 32%. La figura 3B (pág. 185) muestra el mapa de susceptibilidad a la ocurrencia de flujos de resultado de la aplicación del índice Wi, y el histograma con los porcentajes de superficie para cada categoría de estabilidad.

Modelo con regresión logística

A partir de la aplicación de la regresión logística (tabla 2, pág. 186) se obtuvo la siguiente ecuación que explica la relación entre las variables consideradas que son significativas y la probabilidad de ocurrencia de flujos de detritos en el área de estudio:

Y = 2,43 - 0,00073 altura + 0,05 pendiente - 0,0026 orientación - 1,67 Complejo Volcánico Aconcagua - 1,8 depósitos aluviales - 0,51 vegetación

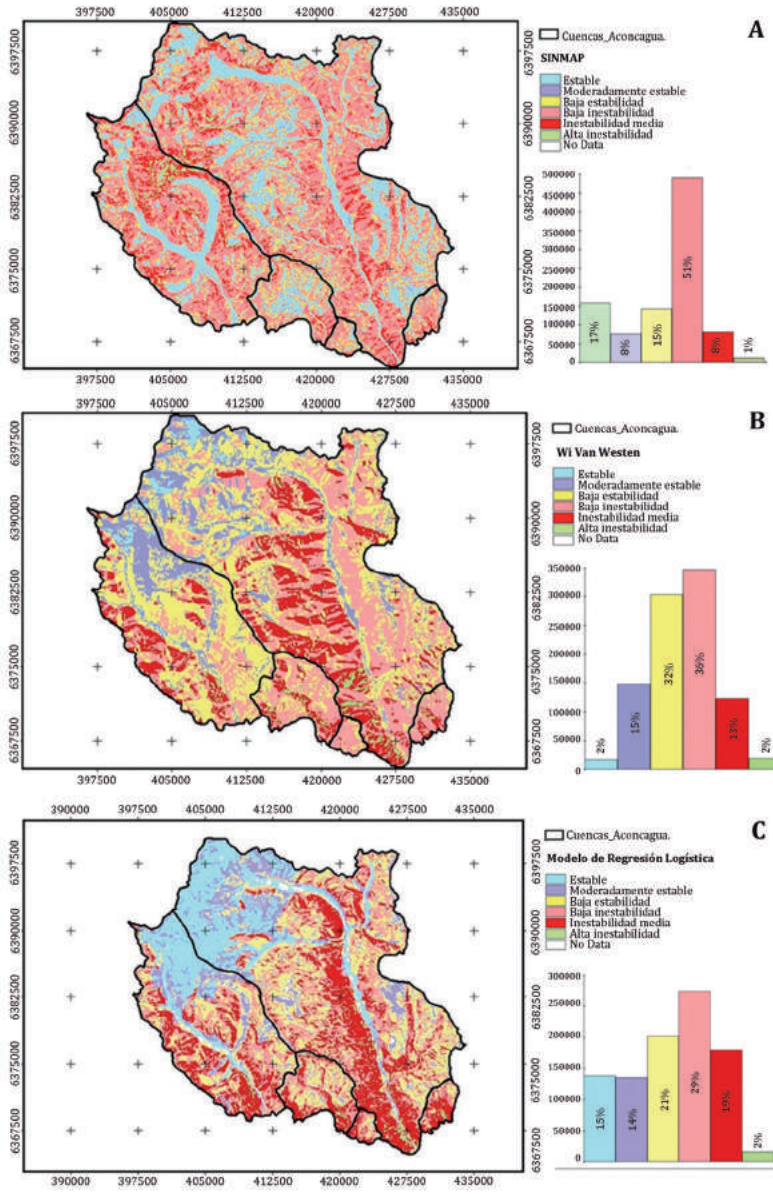

Figura 3. Mapas de susceptibilidad para el Parque Aconcagua obtenidos mediante la aplicación de los modelos: SINMAP (A), Wi (B) y Regresión logística (C) con los histogramas de los porcentajes de las áreas para cada categoría de estabilidad establecida por el modelo.

Figure 3. Susceptibility maps of the Aconcagua Park obtained by models SINMAP (A), Wi (B) and Logistic Regression(C) showing histograms with percentages of stability category areas classified by each model.

Tabla 2. Estimadores y su significación.

Table 2. Estimators and their significance.

Parámetros	Estimador	Error estándar	Odd-ratio	Wald LI (95%)	Wald LS (95%)	Wald Chi²	p-valor
Constante	2,43	0,54	11,34	3,94	32,64	20,28	<0,0001
Altura	-7,3E-04	1,3E-04	1,00	1,00	1,00	34,15	<0,0001
Pendiente	0,05	0,01	1,05	1,04	1,06	85,46	<0,0001
Orientación	-2,6E-03	5,7E-04	1,00	1,00	1,00	21,66	<0,0001
Complejo Volcánico Aconcagua	-1,67	0,20	0,19	0,13	0,28	71,07	<0,0001
Depósitos aluviales	-1,80	0,32	0,17	0,09	0,31	30,97	<0,0001
Vegetación	-0,51	0,18	0,60	0,43	0,85	8,37	0,0038

Los p-valores asociados a estos coeficientes son significativos, por lo tanto las variables pendiente, altura, orientación, vegetación, complejo volcánico Aconcagua y depósitos aluviales tendrán un efecto significativo a la hora de explicar la probabilidad de ocurrencia de flujos de detritos en la zona del Parque Provincial Aconcagua.

Los odd ratio asociados a las variables vegetación, litologías complejo volcánico Aconcagua y depósitos aluviales indican que la presencia de estas disminuye la probabilidad de ocurrencia de flujos de detritos.

El modelo clasificó el 29% del área como de baja inestabilidad, el 19% del área como inestabilidad media y un 2% como inestabilidad alta. Las categorías de mayor estabilidad se encuentran representadas en el área de estudio en un 15% para estable, 14% para moderadamente estable y baja estabilidad en un 21%.

La figura 3C (pág. 185) muestra el mapa de susceptibilidad a la ocurrencia de flujos de resultado de la aplicación del modelo de regresión logística, y el histograma con los porcentajes de superficie para cada categoría de estabilidad.

Validación espacial de los resultados

La capacidad de predección espacial de los modelos utilizados para el área de estudio es buena ya que entre el 76% y 81% de los flujos de detritos coincidieron con zonas de mayor inestabilidad en el proceso de validación (tabla 3).

Tabla 3. Porcentaje de flujos de detritos clasificados en las distintas categorías de estabilidad.

Table 3. Percentage of debris flows classified in the different stability categories.

Modelos	Clases de Susceptibilidad					
	Estable	Moderadamente estable	Baja estabilidad	Baja inestabilidad	Inestabilidad media	Alta inestabilidad
SINMAP	24%			76%		
Wi	20%			80%		
R. logística	19%			81%		

Discusión

La valoración de la susceptibilidad obtenida mediante los modelos utilizados es satisfactoria ya que predice entre el 76% al 81% de los flujos de detritos históricos en las zonas categorizadas con la mayor inestabilidad. Sin embargo, se observan discrepancias entre los modelos. El modelo SINMAP genera mayoritariamente valores de alta inestabilidad en el sector noroeste del área, mientras los modelos estadísticos indican una zona de menor susceptibilidad.

En el modelo físico la presencia del Complejo Volcánico Aconcagua, elevaciones superiores a 4400 m s. n. m. y fuertes pendientes resaltan el grado de inestabilidad, a pesar que a estas alturas se producen procesos glaciares que no dan lugar a la saturación del terreno para generar flujos de detritos, por lo cual las predicciones de los modelos estadísticos son más realistas.

El SINMAP ha sido aplicado por varios autores para evaluar susceptibilidad (7, 14, 25, 26). Aunque los modelos de base física pueden ser adecuados para el modelado de las condiciones hidrológicas que lleva a la iniciación de los flujos de detritos, tienen varias limitaciones cuando se aplica para predecir la distribución espacial de los mismos (2).

Con excepción de la morfología de la pendiente, de hecho, las variables físicas que controlan la distribución espacial de la zona de iniciación de los flujos dentro de estos modelos (es decir, parámetros físicos y mecánicos de la pendiente y el material) no pueden ser adquiridas en grandes áreas a un costo razonable. En el área de estudio se desconocen varios parámetros y existe una gran heterogeneidad en cuanto a las litologías y propiedades del terreno, por lo tanto es difícil conseguir la información para realizar las calibraciones del modelo. Se recomienda que el modelo SINMAP sea empleado en el área de estudio para analizar la inestabilidad en cuanto a parámetros topográficos únicamente (por ejemplo, pendiente, área de contribución), los cuales pueden ser obtenidos fácilmente a partir del DEM.

El análisis llevado a cabo a partir del Wi de Van Westen permite conocer las clases de cada variable más susceptible a ser afectada por flujos de detritos. Sin embargo, no determina la importancia relativa de cada variable en la valoración de la susceptibilidad.

La regresión logística basa el análisis en las variables que son significativas, excluyendo aquellas que no influyen significativamente en la probabilidad de ocurrencia de los eventos, proporcionando resultados más precisos. Es por ello, que la regresión logística resulta la más apropiada para la determinación de la susceptibilidad a flujos de detritos históricos por varios autores (1, 3, 9, 13, 19).

La literatura de los últimos 10 años muestra un claro aumento en el uso de análisis de regresión múltiple (1).

Asimismo, los análisis estadísticos muestran que varios factores ambientales contribuyen a la clasificación de las zonas con diferente susceptibilidad a la ocurrencia de flujos de detritos. Sin embargo, algunos de ellos ejercen mayor influencia, en este caso y según el análisis de regresión logística los factores que más afectan son: la pendiente, la orientación, la altura, la vegetación y las litologías "complejo volcánico Aconcagua" y "depósitos aluviales".

Los modelos Wi y logístico resaltan la gran susceptibilidad a la ocurrencia de flujos de detritos de la zona de ingreso

al Parque por la Quebrada del río Vacas. Los campamentos Pampa de Leñas y Casa de Piedra quedan clasificados en las categorías de mayor inestabilidad y el campamento Plaza Argentina coincide con zonas clasificadas en las categorías de mayor estabilidad a pesar que se encuentra afectada por grandes bloques de caídas de rocas (17, 18).

En la Quebrada del río Horcones, la zona de los Grises, aguas abajo del campamento Confluencia, queda clasificada en las categorías de mayor inestabilidad, coincidiendo con las observaciones de Moreiras (2008), quien la describe como una zona de conos aluviales muy activos donde se encauzan flujos de detritos con recurrencia anual.

Playa Ancha queda clasificada en las categorías de mayor estabilidad para generar flujos de detritos aunque dominada por procesos aluviales. La zona por donde se encuentra la senda hacia plaza Francia queda clasificada en las categorías de mayor inestabilidad.

Por último, la ubicación del campamento Horcones se encuentra en zonas de mayor inestabilidad, los alrededores de Confluencia y Plaza Francia quedan clasificados en las categorías de mayor inestabilidad.

Los flujos extraordinarios del sector de los Grises podrían represar el desagüe natural que existe entre el depósito de la morena y la ladera izquierda del valle produciendo anegamiento o inundación del campamento Confluencia (15).

Los campamentos Plaza de Mulas, Nido de Cóndores y Berlín se encuentran en zonas de menor inestabilidad según los resultados de la regresión logística. Estos resultados concuerdan con trabajos previos (15), donde se enfatiza que la localización actual del campamento de Plaza de Mulas es un sitio poco susceptible a ser afectado por flujos de detritos.

Con respecto a los campamentos a lo largo del valle del río de Las Vacas, Plaza Argentina queda clasificada en las categorías de mayor estabilidad, Casa de Piedra con mayor inestabilidad y por último los alrededores de Pampa de Leñas quedan clasificados en las categorías de mayor inestabilidad.

Los resultados hallados en este trabajo reflejan la susceptibilidad del terreno a generar flujos de detritos, pero no se contemplaron los sectores de trayectoria y depósitos de los flujos. Estos procesos suelen fluir pendiente abajo y converger en canales, cuando son originados en diferentes sectores de una cuenca, pueden combinarse en estos canales y aumentar su poder de destrucción.

Las áreas de mayor peligro se encuentran en las desembocaduras de gargantas, quebradas secas, abanicos aluviales e incluso ríos, ya que los flujos de detritos suelen encausarse en estos también (16). Es por esto que este trabajo debe complementarse con análisis de predicción de trayectorias de los eventos, ya que si bien suelen originarse en pendientes de 16 a 35°, luego de su iniciación, debido a la gran velocidad y al hecho de estar saturados de agua, pueden trasladarse hacia zonas de menor pendiente muy lejos de la zona de iniciación, y son capaces de erosionar los materiales a lo largo del recorrido e incrementar notablemente su volumen inicial. Por lo tanto, es fundamental realizar un análisis conjunto de los resultados aquí obtenidos con observaciones de campo y relatos de las características de eventos ocurridos en el pasado.

Debido a que los flujos de detritos no son los únicos procesos que representan un riesgo para la población, es necesario realizar estudios de susceptibilidad para todos los procesos de remoción en masa presentes en el área de estudio.

Con el fin de conocer la susceptibilidad total y realizar una zonificación regional en función de todas las amenazas presentes en el PPA.

Conclusión

El área de estudio tiene características favorables para la ocurrencia de flujos de detritos debido a sus fuertes pendientes representando una gran peligrosidad en la región.

La zonificación obtenida en este estudio permitió realizar un reconocimiento preliminar de las áreas con diferentes grados de susceptibilidad a generar flujos de detritos. Los modelos aplicados contribuyen a la predicción de futuros escenarios para el diseño de sistemas de prevención, aunque no contemplan los sectores de canalización y depositación del material una vez iniciado su movimiento.

El modelo SINMAP no es adecuado para analizar la susceptibilidad como única herramienta ya que no se dispone de información para realizar las calibraciones pertinentes para obtener resultados más fiables. Con respecto a la metodología Wi se puede decir que es útil para conocer las clases de las variables condicionantes más susceptibles a la ocurrencia de flujos. Sin embargo, el hecho de considerar en el análisis a factores que contribuyen en menor medida conlleva a una disminución de su rendimiento.

Por último la regresión logística se considera la metodología más apropiada por su facilidad de aplicación y poder de predicción. Se recomienda la utilización del mapa obtenido a través de este método para la planificación y el análisis relacionado con la gestión de riesgos.

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Fibrolytic activity of four *Trichoderma* strains grown on agro-industrial residues

Actividad fibrolíticas de cuatro cepas de *Trichoderma* crecidas en residuos agroindustriales

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Abstract

The aim of this study was to compare the cellulolytic and xylanolytic activities of two strains of *Trichoderma viride*, one of *Trichoderma reesei* and one of *Trichoderma harzianum* grown on four different substrates. Each substrate contained 20% wheat bran and 80% agro-industrial waste (corn stover (CS), sugarcane bagasse (SCB), *Yucca schidigera* fiber (YS), or compost elaborated from solid waste generated in the university cafeteria (CSW)). An interaction ($P < 0.01$) between the substrate and strain was detected for both cellulolytic and xylanolytic enzyme activities. The highest cellulolytic activity ($P < 0.01$) was obtained with *T. reesei* grown on YS, CS, and SBC, and the lowest was from the two *T. viride* strains grown on most of the substrates. The highest xylanolytic activities ($P < 0.01$) were detected for *T. harzianum* with YS and SCB and *T. reesei* with CSW and CS, while one *T. viride* strain exhibited intermediate and the other showed the lowest activity. In conclusion, *T. reesei* CDBB356 showed the highest fibrolytic activity for most of the tested substrates, a finding that suggests it has the highest potential for fibrolytic enzyme production. There is a potential application for *T. reesei* CDBB356 enzymes on ruminant feed supplements to improve forage digestibility.

Keywords
cellulolytic activity • xylanolytic activity • enzymes • agro-industrial waste • *Trichoderma*

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Resumen

El objetivo de este estudio fue comparar las actividades celulolíticas y xilanolíticas de dos cepas de *Trichoderma viride*, una de *Trichoderma reesei* y una de *Trichoderma harzianum* cultivadas en cuatro diferentes substratos. Cada substrato contenía 20% de salvado de trigo más 80% de residuos agroindustriales (rastrojo de maíz (CS), bagazo de caña de azúcar (SCB), fibra de *Yuca shidigera* (YS) o composta, elaborada a partir de residuos sólidos de la cafetería universitaria (CSW)). Se detectó una interacción (P <0,01) cepa por sustrato en las actividades enzimáticas celulolíticas y xilanolíticas. La mayor actividad celulolítica (P <0,01) se obtuvo con *T. reesei* en YS, CS y BC y la más baja con las dos cepas de *T. viride,* en la mayoría de los sustratos. Las actividades xilanolíticas más altas (P <0,01) se detectaron en *T. harzianum* con YS y SCB y *T. reesei* con CSW y CS, mientras que una cepa *de T. viride* fue intermedia y la otra tuvo las actividades más bajas. En conclusión, *T. reesei* CDBB356 mostró la actividad fibrolítica más alta en la mayoría de los sustratos, confirmando el mayor potencial para producir esas enzimas. Existe una aplicación potencial de las enzimas de *T. reesei* CDBB356 en suplementos alimenticios para rumiantes para mejorar la digestibilidad del forraje.

Palabras clave
actividad celulolítica • actividad xilanolítica • enzimas • residuo agroindustrial • *Trichoderma*

Introduction

Trichoderma reesei has important industrial applications because of its cellulolytic and hemicellulolytic enzymes (19). Among the cellulase-producing fungi, the genus *Trichoderma* shows a high capacity to produce both exoglucanases and endoglucanases, and media components along with the solid substrate used for culturing under solid-state fermentation help determine the type of enzyme(s) produced (9).

Trichoderma species are widely distributed and there is high biodiversity with a variety of biological activities in agricultural fields (16). In a study in east China, Jiang *et al.* (2016) identified 17 species, among which *Trichoderma harzianum* was dominant, whereas, in a similar study in central Europe, *Trichoderma viride* was the most abundant among 15 species found. One of the most studied strains of *T. reesei* was originally isolated on the Solomon Islands in the US; this strain was modified by metabolic engineering, and mutants of this strain are used in several industrial bioprocesses (6).

Fibrolytic enzymes can be used to improve digestibility, an important need with regards to feed utilization for ruminant production. Several fibrolytic enzymes have been developed and commercially used (20), most of them based on celullases and hemicellulases produced by *Trichoderma* and other fungi (5). The global market for feed enzymes was estimated at $899.19 million in 2014 and is expected to reach nearly $1.3 billion by 2020, a compound annual growth rate of 7.3% from 2015 to 2020 (28). In many

countries, commercial enzyme products have not been implemented as strategy to improve feed utilization because imported products are expensive and its inclusion is not profitable (20). A country that develops and produces its own fibrolytic enzymes represents an opportunity to improve forage digestibility at a low cost. Hence, identification of strains able to degrade agricultural lignocellulosic residues to develop new enzymatic products locally is important in underdeveloped countries. Thus, the objective of this study was to compare the cellulolytic and xylanolytic activities of two strains of *T*. *viride*, one of *T*. *reesei*, and one of *T*. *harzianum* grown on four agro-industrial wastes. Each culture was composed of 20% wheat bran and 80% corn stover (CS), sugarcane bagasse (SCB), *Yucca schidigera* fiber (YS), or compost elaborated from university cafeteria solid waste (CSW). This design produced four different substrates. Since the most extensively studied cellulose-secreting microorganism is the filamentous fungus *T*. *reesei*, and its fibrolytic activity has been characterized (28), the hypothesis was that *T*. *reesei* CDBB356 would exhibit the greatest potential to produce fibrolytic enzymes.

Materials and methods

Substrates and chemical composition

Composite representative samples of CS, SCB, and wheat bran were obtained from the experimental dairy farm at the University of Chapingo, Mexico; residual fiber from *Y*. *schidigera* (YS) from commercial production was obtained from Alltech de Mexico S.A. de C.V.; CSW was obtained from anaerobic and aerobic fermentation of organic residues from the Autonomous Metropolitan University cafeteria. All samples were oven dried at 45°C and milled to pass a 2 mm screen using a Wiley Mill (Standard model 4; Arthur H. Thomas Co., Philadelphia, PA). The dry matter and ashes in substrates were analyzed according to the AOAC (2), and neutral detergent fiber (NDF) and acid detergent fiber (ADF) content was determined; analyses were conducted according to Van Soest *et al.* (1991). Nitrogen content was determined with the Dumas procedure using a Leco FP-428® instrument.

Microorganisms and inoculum preparation

T. *viride* 1 (Culture Collection of CINVESTAV, México), *T*. *viride* 2 (Culture Collection of UAM-Xochimilco Phytopathology, México), *T*. *harzianum* Rifai (Genetic Resource Center strain collection at the Science Institute BUAP Center of Agroecology, México), and *T*. *reesei* CDBB356 (Culture Collection of CINVESTAV, México) were cultivated in Petri dishes that contained potato-dextrose agar (PDA) and incubated for 7 days at 27°C. The spores were harvested and counted as described by Escamilla-Alvarado *et al.* (2013).

Solid fermentation and enzymatic activity

Substrates were milled to pass a 2.38 mm screen. Subsequently, a mixture of 80% each substrate and 20% wheat bran was assembled (3). Culture media were prepared by placing 3 g of each substrate (the mix of agro-industrial waste and wheat bran) separately in 250 ml Erlenmeyer flasks and then adding 12 ml sterile water. All culture media were autoclaved (121°C, 15 psi, 25 min) and inoculated with 1 x 10^6 conidia/g dry substrate (gds) for each strain tested (26). Cultures were incubated for 7 days. To obtain the crude enzymatic extracts (CEE), each flask was placed in an ice bath on top of a stirrer.

The flask contents were resuspended and filtered with medical gauze, centrifuged (4°C, 7740 g, 15 min), and the supernatants (CEE) were stored at 4°C until its use for enzymatic determinations (4).

Xylanase and cellulase activities were determined by reducing sugars release with 3,5-dinitrosalicylic acid (DNS) (21), using Birchwood xylan (0.5%; Sigma-Aldrich) and 1% carboxymethylcellulose (Sigma-Aldrich) in sodium citrate buffer (0.5 M, pH 5.3).

The reactions were performed as described by Loera and Córdova (2003), using xylose and glucose for a standard curve; readings were taken with a Cary spectrophotometer at 540 nm.

An enzyme unit (U) was defined as the amount of enzymatic extract that released 1 µmol of reduced sugars per min per gds (15). Protein concentrations of the extracts were assayed according to the Bradford method (7), using bovine serum albumin as a standard. All determinations were made in triplicate.

Statistical design

Results were analyzed as a completely randomized design with a 4 x 4 factorial arrangement, where the factors were *Trichoderma* strains and substrates. Means were compared with the Scheffe test (30), and data was analyzed with JMP software (27).

Results and discussion

Table 1 presents the substrate chemical compositions. Lignocellulosic results showed a typical composition of high cell wall content and low protein from agricultural residues (1, 18), whereas CSW was similar to other restaurant wastes analyzed, with high water and protein content and usually low fiber content (14, 22).

An interaction between substrate and strain was detected for the enzyme activities (table 2, page 196; $P < 0.0001$). The highest cellulolytic activity was obtained with *T. reesei* grown in YS, CS, and SCB, while the lowest activity was for the two *T. viride* strains grown in most of the substrates. The cell wall composition in agro-industrial residues is usually high in cellulose and lignin (10, 33); lignin is the major factor in recalcitrance of cell walls to saccharification, particularly during enzymatic hydrolysis (10).

Table 1. Chemical composition (%) of the substrates used in solid-state fermentation.
Tabla 1. Composición química (%) de sustrato usado en fermentación sólida.

Item	CS	SCB	YS	CSW	Wheat bran
Dry matter	95.65 ± 0.15	97.66 ± 0.01	96.31 ± 0.39	26.8 ± 0.761	93.71 ± 1.22
Ash	7.12 ± 0.38	1.8 ± 0.025	2.35 ± 0.5	3.35 ± 0.160	5.38 ± 0.79
Crude protein	3.29 ± 0.20	------	0.75 ± 0.01	15.35 ± 1.72	15.9 ± 0.3
NDF	79.66 ± 1.62	86.41 ± 6.11	67.5 ± 3.17	82.73 ± 1.86	53.39 ± 2.78
Hemicellulose	25.91 ± 0.92	17.2 ± 2.46	10.35 ± 0.65	16.99 ± 0.43	33.49 ± 1.38
ADF	53.75 ± 2.05	69.21 ± 3.86	57.15 ± 2.85	65.74 ± 1.52	19.90 ± 2.87

NDF: neutral detergent fiber; ADF: acid detergent fiber.
NDF: fibra detergente neutro; ADF: fibra detergente ácido.

Table 2. Comparison of enzymatic activities and crude extract protein content of four *Trichoderma* strains after fermentation with four substrates.

Tabla 2. Comparación de las actividades enzimáticas y contenido de proteína del extracto crudo de cuatro cepas de *Trichoderma* después de la fermentación con cuatro sustratos.

Strain	Substrate	Activity		Protein, mg/ mL
		Cellulolytic, U/gds	Xylanolytic, U/gds	
T. harzianum	YS	36.09^{de}	385.01 ^{b}	0.263 ^{cde}
	SCB	71.49^{de}	358.56 ^{bc}	0.758 ^{e}
	CSW	48.81^{de}	306.03 ^{c}	0.384^{bcde}
	CS	94.95^{cd}	186.43 ^{de}	0.744 ^{ab}
T. viride 1	YS	33.18^{de}	132.53 ^{efg}	0.187 ^{e}
	SCB	39.10^{de}	65.83 ^{ghi}	0.156 ^{e}
	CSW	19.00 ^{e}	33.26 ^{i}	0.721 ^{abc}
	CS	71.50^{de}	329.67 ^{bc}	0.26 ^{de}
T. viride 2	YS	43.60^{de}	145.78 ^{def}	0.393^{bcde}
	SCB	63.35^{de}	91.56^{fghi}	0.278 ^{cde}
	CSW	31.79^{de}	43.05 ^{hi}	0.15 ^{e}
	CS	69.01^{de}	314.3 ^{bc}	1.12 ^{a}
T. reesei	YS	215.99 ^{a}	213.47 ^{d}	0.711^{abcd}
	SCB	191.74^{ab}	111.3 ^{fgh}	0.095 ^{e}
	CSW	134.78^{bc}	460.18 ^{a}	0.468^{bcde}
	CS	205.40 ^{a}	474.86 ^{a}	0.454^{bcde}
SEM		3.54	4.14	0.025
P-value interaction		0.0001	0.0001	0.0001
P-value strain		0.0001	0.0001	0.0008
P-value substrate		0.0001	0.0001	0.0001

Different letters within a row indicate significant differences (P < 0.01).
SEM: Standard error of the mean; gds: grams dry substrate; CSW: cafeteria solid waste.
Diferentes letras dentro de una fila indican diferencias significativas (P < 0,01).
SEM: error estándar de la media; gds: gramos de sustrato seco; CSW: residuos sólidos de cafetería.

The highest cellulolytic activity can be associated with cellulose content; for example, SCB contains at least 33% cellulose (33) and YS contains more than 40% (11). The cellulose content in CS is also high and varies from 35 to 42% (31).

The highest cellulolytic activities obtained from *T. reesei* (table 2) are similar to those reported by Vyas and Vyas (2005) for *T. viride* cultivated on groundnut shell waste, higher or similar to values reported with *Trichoderma asperellum* grown on agricultural straws (wheat straw, rice bran, wheat bran, and corn cob). Some of the values were greater than 145 U/gds (9), higher than cellulolytic activities found in commercial products used for ruminants (20, 25).

The highest xylanolytic activities were detected in *T. harzianum* grown on YS and SCB and *T. reesei* cultured with CSW and CS. Both *T. viride* strains showed low xylanolytic activity; strain 2 activity was slightly higher than strain 1. SCB and CS are characterized by high hemicellulose content (30, 32); however, xylanolytic activity was higher when the fungus was grown with YS, even though this substrate has a lower hemicellulose content (table 1, page 195).

The complex cell wall structure may induce different types of enzymes simultaneously. Indeed, studies with enzymes and treatments indicate that xylan and lignin removal enhances cellulose availability (5). *T. reesei* strains can modify their enzymatic activity according to the sugar(s) present as carbon sources, as demonstrated by Dondelinger *et al.* (2016) using different combinations of lactose, glucose, xylose, and a hemicellulosic hydrolysate.

Sipos *et al.* (2010) compared xylanolytic activities in *T. reesei* Rut C30 using different carbon sources (Solka Floc 200, lactose, and steam-pre-treated CS); some sources of lactose and Solka Floc 200 resulted in low specific activities, whereas CS promoted the highest activity.

In addition to substrates, there are differences in strains; an enzymatic extract obtained by solid-state fermentation of peach palm waste by *Trichoderma stromaticum* had an activity of 1440 U/g (8).

The highest xylanase activities in the present study were in the same range as commercial enzymes used for ruminants, values that have spanned from 134 to 222 UI/g (25).

The interaction between substrate and strain can be explained because the cell wall composition is different when grown in each substrate. Therefore, fungi are stimulated for different metabolic pathways. Dondelinger *et al.* (2016) used various carbon sources (lactose, glucose, xylose, and hemicellulosic hydrolysate) with two *T. reesei* strains, and the enzymatic activities were as different as those observed in this study. Raghuwanshi *et al.* (2014) also showed that a mutant *T. asperellum* strain duplicated enzymatic activities compared with the wild strain. Enzyme production can be optimized by changing pH, wheat bran level, protein sources, substrate concentration, and other factors (9). Ortiz Robledo, *et al.* (2017) concluded that the cutting age of the substrates also influences its fermentative characteristics. This results confirmed that the *Trichoderma* strains evaluated, especially *T. reesei* CDBB356, generate competitive levels of xylanolytic and cellulolytic activities compared to previous reports and even the enzymatic activities reported for commercial products. Moreover, since *Trichoderma* species grow fast enough to prevent contamination in solid-state cultures and produce enzymes that can break down the complex cell wall (35), the strains investigated in this work can be used as reference for further investigations that focus on elaborate enzymatic products as ruminant feed supplements. In special applications, the addition of supplementary enzymes such as α-xylosidase, endo-arabinase, and pectinases enhances the ability to degrade *Trichoderma* extract lingnocellulose, since these enzyme activities only occur at low levels in this fungus (6).

Conclusions

The *Trichoderma* strains used in this study showed versatility to produce fibrolytic enzymes in solid fermentation in all the evaluated substrates, with a marked variation in enzymatic profiles according to the combination of strain and type of substrate.

T. reesei CDBB356 stood out with the highest cellulolytic activity on CS and YS, while the highest xylanolytic activity was obtained on CSW and CS. Considering the relative low cost of the substrates used and the fact that *Trichoderma* species grow fast enough to prevent contamination in solid-state cultures, there is a potential application of *T. reesei* CDBB356 enzymes on ruminant feed supplements to improve food digestibility.

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Growth of *Stuckenia pectinata* under greenhouse and irrigation canal conditions in the lower valley of the Colorado River (Argentina)

Crecimiento de *Stuckenia pectinata* en condiciones de invernáculo y canales de riego del valle inferior del río Colorado (Argentina)

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Abstract

Stuckenia pectinata is an invasive submerged weed in the irrigation district in the lower valley of the Colorado River, Argentina. The objective of this study was to analyze the initial growth of *S. pectinata* from tubers, and its annual growth cycle in irrigation canals, in order to be efficient in adapting future control techniques. Tubers were planted in aquaria in order to evaluate the effect of their size, depth of burial and below zero temperatures on the initial growth. Under field conditions, samples of plants were collected from two irrigation canals, from October to March, in two complete growth cycles. Plant height and biomass of the leaves, stems and spikes were measured. The largest tubers were able to emerge from deep burial and generated larger plants than the smallest tubers. Frozen tubers did not germinate at any burial depths. Maximum biomass in the irrigation canal reached 1660 g DM m^{-2} with a peak at the beginning of summer. The elimination of biomass at the end of the irrigation season would result in small tubers that would die in the winter time. The information generated could lead to more appropriate and sustainable control.

Keywords
sago pondweed • tubers • freezing • burial depth • growth cycle • lower valley of Colorado River

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Resumen

Stuckenia pectinata es una maleza invasora sumergida de canales de riego del valle inferior del río Colorado, Argentina. El objetivo de este estudio fue analizar el crecimiento inicial desde tubérculos de *S. pectinata* y de su ciclo de crecimiento anual, para incrementar la eficiencia en la adaptación de futuras técnica de control. Los tubérculos se plantaron en acuarios, para evaluar el efecto de su tamaño, la profundidad de entierro y temperatura de congelación en el crecimiento inicial. En condiciones de campo, se recogieron muestras de plantas y biomasa de dos canales de riego desde octubre a marzo en dos ciclos completos de crecimiento. Se midieron la altura y el peso de las hojas, tallos y espigas de las plantas. Los tubérculos más grandes pudieron emerger de un entierro profundo y generar plantas más grandes que los tubérculos más pequeños. Los tubérculos congelados no germinaron a ninguna profundidad. La biomasa máxima en canales de riego alcanzó 1660 g de MS m^{-2} con un pico al comienzo de verano. La eliminación de la biomasa al final de la temporada de riego producirá pequeños tubérculos que morirán en el invierno. La información generada podría conducir a un manejo más apropiado y sostenible.

Palabras clave
lama • tubérculos • congelación • profundidad de entierro • ciclo de crecimiento • valle inferior del río Colorado

Introduction

The construction of the "Casa de Piedra" dam 700 km upstream from the irrigation network changed the ecological features of the water. The high load of suspended sediments that was carried down by the river, resulting in a very turbid-reddish water (giving the name "Colorado" to the river), was deposited in the dam (6). Therefore, increased light penetration in the irrigation canals facilitated the rapid development of aquatic weeds (8). One of the most important weeds is *Stuckenia pectinata* (Sago Pondweed), which significantly impedes the water flow due to the development of a large amount of biomass. Previous studies showed that in severe *S. pectinata* infestations the normal water flow can be reduced up to 60% in the irrigation canals, consequently, the water available for irrigated crops is critically reduced (2).

Stuckenia pectinata is a morphologically variable species due to its great phenotypic plasticity (19). It is a cosmopolitan, perennial, monocotyledon plant, with some branches on the stem (19). Plants are rooted in the sediment by a robust subterranean system containing roots, rhizomes and tubers (12). The stems, extending to 3 or 4 m in length, are branched with small alternate leaves (from 22 to 125 cm). Sexual reproduction is by drupelets which provide long-term dormancy and a broad dispersal, and by tubers for overwintering and short-distance dispersal (13). The irrigation canals of CORFO can have 260 tubers per m^{-2} with 54% of emergence (1). *Stuckenia pectinata* plants allocate up to 41.5% of dry biomass in the production of tubers which is considered as the most important

strategy for the re-establishment of a population (19). The threshold for controlling the submerged weed is 200 g of dry biomass per square meter to avoid blockage and the stopping of water flow in irrigation canals (4).

Unfortunately, the current weed management is carried out by hydraulic excavators with a cutting basket, which only operate efficiently with large sized plants during the irrigation season. The delay in the application of the hydraulic excavators promotes large tubers compared with other types of management (2). In fact, no weed management is undertaken in the last three months of the irrigation season which leads to the development of a large number of large tubers. This species survives the winter in the form of tubers. The principal infestation strategy is through tubers and seed is virtually not important according to Kantrud (1990). As the *S. pectinata* plant biomass increases in relation to the initial tuber size (18), the CORFO irrigation canals quickly become blocked, meaning that the current management practices may result in an increasing annual infestation.

Moreover, the dry irrigation canals are restructured and cleaned in winter time. Mechanical restructuring modifies the environment of the irrigation canals, burying the tubers to different depths. Tuber size and burial depth are significant factors in regulating *S. pectinata* growth (17). For this reason, knowledge of the effect of low temperatures, the size and burial depth of the tubers are essential for understanding the population dynamics.

In spite of the importance of the *S. pectinata* problem in irrigation canals, there is no useful information about its growth in the aquatic environment in the lower valley of the Colorado River. Determination of the growth cycle in the irrigation system would lead to treatment at the most feasible moment for obtaining the highest negative impact on the *S. pectinata* infestation. Considering that most of the irrigation canals (5,440 km) are infested in a short period of time, it is difficult to operate weed management techniques simultaneously. Hence, it is essential to have accurate information for scheduling the different control tasks as efficiently as possible. We hypothesized that by decreasing the tuber size, it is possible to delay biomass production and therefore, a lower effort is needed to control it during the irrigation season. Therefore, the objectives of the present research were to generate essential information on the effect of tuber biomass, as well as the interaction between the burial depth of tubers and freezing on the initial growth of *S. pectinata*, and to analyze the annual growth cycle in the irrigation canals of CORFO.

Material and methods

Site description

The irrigation district of CORFO (Development Corporation of the lower valley of the Colorado River) 39°30'9.77" S, 62°41'13.01" W located in the semiarid zone in the south of Buenos Aires province, which covers 140,000 ha of horticulture, pastures and cereal production including 5,441 km of canals (2). The landscape is characterized by a slight slope (0.002%) with Mollisol, Entisol and Aridisol soils (5). Rain decreases from north to south and the hydric deficiency is 400 mm year^{-1} so, irrigation is vital for crop production in the area. There are 42 days per year on average with temperatures below zero

degrees Celsius (6). The field study was carried out in 1995/96 and 1997/98 and the study under greenhouse conditions was conducted in 2000 and 2001.

The present research involved the evaluation of some factors of the initial growth of *S. pectinata* from tubers conducted under greenhouse conditions and the study of the annual growth cycle of the species under irrigation canal conditions.

Initial growth of *S. pectinata*

Effect of tuber biomass on initial growth

In the irrigation canals, sediment was passed through a mesh of 1 mm to separate tubers of diverse sizes. Different sized tubers were planted in black plastic 333 cm^3 pots filled with sandy loam soil in glass tanks under greenhouse conditions. Five categories of tubers were selected according to their biomass: 0-30 mg, 30-60 mg, 60-90 mg, 90-120 mg and higher than 120 mg. Four tubers of each category were planted at a depth of 2.5 ± 0.5 cm in each black plastic pot which was the optimal burial level according to our preliminary research (3) and similar to the research of Spencer (1986). These five treatments were replicated inside eight glass tanks filled with water up to the 40 cm level. The experiment was a complete randomized block designed with 8 replicates (tanks). Each block involved one fixed tank filled with water. In this way, the potential variation due to the aquarium position can be isolated from the natural variation of the principal parameter evaluated. The average temperature and turbidity of water were 20°C ± 2 and 9 NTU (Nephelometric Turbidity Unit), respectively. An air pump was incorporated in each tank. After 21 days from the beginning of the experiment (initial growth), all the plants were collected and the height, dry biomass of foliage, aboveground and belowground biomass were measured. Samples were placed in an oven at 60°C for 3 days.

Effect of freezing and burial depth of tubers on the initial growth

The tubers were categorized into three categories according to biomass: 0-60 mg, 60-120 mg, and higher than 120 mg. To analyze the reduction in the initial growth of *S. pectinata* caused by the effect of tuber biomass and the interaction between freezing temperatures and burial depth, four tubers without freezing and four tubers with 12 h of freezing at -3°C were sown at 7, 10, 15, 20, 25 cm in depth. The tubers were planted in silty clay loam soil in black plastic pots under greenhouse conditions. All 30 treatments were replicated four times in each of the five glass tanks filled with water up to the 40 cm level. The average temperature and turbidity of the water were 20°C ± 2 and 9 NTU, respectively. Air was incorporated with a pump. The percentage of emergence and aboveground plant biomass were recorded 32 days after the beginning of the treatments. A plant was considered as emerged when the first green part was seen on the sediment surface.

Annual growth cycle of *S. pectinata*

Research was conducted in two stretches of irrigation canals at CORFO which were identified as Canal 55.6 (hereafter Villalonga canal-39°51'55" S, 62°34'42" W) and 58 Sur (hereafter Buratovich canal-39°22'45" S, 62°26'56" W). The depth and water flow were 95 cm and 0.6 $m^3 s^{-1}$ in the Villalonga canal and 120 cm and 1 $m^3 s^{-1}$ in the Buratovich canal, respectively. Both canals were representative of the irrigation system and had a typical infestation of *S. pectinata*. The growth dynamic was

evaluated with randomized samples collected from October to May of the following year, during two growth cycles (1995/96 and 1997/98). Irrigation canals remain dry during winter time.

Data of the photoperiod (hours) were measured at the weather station located in Hilario Ascasubi (39°23'32" S, 62° 37'43" W). Specific conductance (water electrical conductivity) was recorded by a Hach conductivity meter CO150. Range of temperature was measured with a digital thermometer. The temperature of the water was 9 -12°C at the beginning of the observations and it increased to 25°C in the summer. Turbidity was measured using a turbidimeter Hach 2100P. The average water electrical conductivity was 1.03 ± 0.03 mS cm^{-1} and the pH ranged from 6.84 to 8.33. The average turbidity in the canal sections was 27.36 ± 17.72 UTN.

Biomass production

Ten biomass samples were collected by sampling date using an iron quadrat of 30 cm sides which was randomly placed in the sediment during the growth cycle. The non-subterranean biomass inside each quadrat was cut with scissors, put into a plastic bag, and taken to the Weed Ecophysiology Laboratory of CERZOS. Each sample date was carried out in different sections of the canal, thus, samples were independent of each other. Plant samples were washed and dried to a constant weight in an oven at 70°C.

Individual plant size

An individual plant was defined as a primary stem emerging from the sediment and all the parts included on it, such as secondary stems, leaves, and spikes. At each site, five individual plants were collected at random in the first growth cycle and 15 plants in the second cycle. All samples were washed and dried to constant weight in an oven at 70°C. The parameters measured included the plant height and dry biomass of stems, leaves and spikes.

Statistical analysis

The experiment was a complete randomized block designed with 8 replicates (aquarium), and its results were analyzed by ANOVA. Freezing and burial depth of tuber data on the initial growth were subjected to a 3-way ANOVA (fixed factors: tuber size, burial depth and freezing) with 5 replicates. With regard to the field research (2.2), plant height was transformed by natural logarithmic to assess variance homogeneity prior to statistical analysis (15). This parameter together with the total biomass and individual plant biomass, and spike biomass were analyzed by ANOVA. All the means were separated by a Fisher Least Significant Differences test (LSD. $P<0.05$). Analyses were conducted with INFOSTAT software (7).

Results and discussion

Initial growth of *S. pectinata*

Effect of tuber biomass on initial growth

The height and biomass of the plants were affected by the biomass of the original tuber. Even though there were no differences in plant height between tubers of 60-90 mg and 90-120 mg (figure 1a, page 206), these plants presented differences in biomass (figure 1b, page 206).

Analysis of the above and belowground biomass showed differences between the different categories of tubers (table 1, page 206).

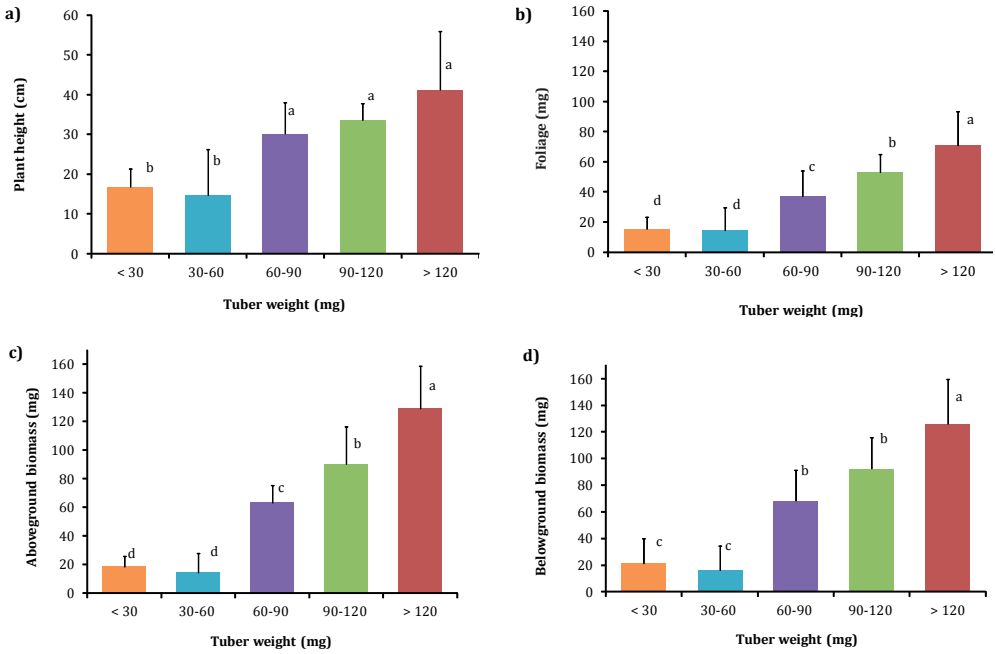

Figure 1. Effect of the tuber biomass (mg) on the initial growth (21 days) of *Stuckenia pectinata* growing in aquaria under lab conditions, a) plant height (cm), b) foliage (mg dry mass), c) aboveground biomass (mg dry mass), and d) belowground biomass (mg dry mass).

Figura 1. Efecto del peso de los tubérculos (mg) sobre el crecimiento inicial (21 días) de *Stuckenia pectinata* creciendo en peceras bajo condiciones de laboratorio, a) altura de plantas (cm), b) follaje (mg peso seco), c) biomasa no subterránea (mg peso seco), y d) biomasa subterránea (mg peso seco).

Table 1. Results of ANOVA for plant height, plant biomass, above and belowground biomass for different sizes of *Stuckenia pectinata* tubers under aquarium conditions.

Tabla 1. Resultados del análisis de varianza para la altura y peso de plantas, biomasa subterránea y no subterránea para diferentes tamaños de tubérculos de *Stuckenia pectinata* en ensayos de peceras

Source	Numerator DF	F	Pr > F
Plant height			
Treatment tuber size	4	13.2	<0.0001
Aquaria	7	1.8	0.1206
Plant biomass			
Treatment tuber size	4	35.2	<0.0001
Aquaria	7	5.1	0.0008
Aboveground biomass			
Treatment tuber size	4	62.8	<0.0001
Aquaria	7	2.5	0.0411
Belowground biomass			
Treatment tuber size	4	46.9	<0.0001
Aquaria	7	5.2	0.0007

Increments in the latter three categories of tuber biomass resulted in increases in the aboveground and belowground biomass (figure 1c and 1d, page 206).

The initial growth of plant biomass and height increased with the biomass of the tuber. The tubers heavier than 120 mg produced greater plant height and plant biomass than the other tuber categories. The same tendency was recorded for the above and belowground biomass (table 2). Spencer and Ksander (1995) determined that increasing tuber biomass resulted in increasing plant biomass, using a category of large tubers between 100-150 mg of biomass. Another experiment was conducted with four categories of tubers, the highest category being 91-100 mg which resulted in the highest plant biomass after 30 days of the trial (17).

Effect of freezing and depth of burial of tubers on the initial growth

Tubers exposed to freezing temperatures were not capable of sprouting. The smallest tubers (<60 mg) did not produce any plants at any of the burial depths. Moreover, there were no differences in plant biomass between the medium and large tubers at the same burial depth. Large tubers planted at 7 and 10 cm in depth generated more biomass than those buried at 20 and 25 cm; however, the biomass produced by large tubers planted at 15 cm showed no differences to those buried at 20 and 25 cm. Maximum emergence in large tubers was reduced by 80 % when planted at 25 cm.

The plant biomass of *S. pectinata* decreased with the burial depth of the tuber in agreement with Spencer and Ksander (1995). Similarly, Spencer (1987) also found that plant biomass increased with the initial tuber biomass and declined as the planting depth increased.

All the tubers that had been exposed to freezing temperatures for only 12 hours died. Similarly, overwintering tubers localized 2.54 cm under the snow that were exposed to 0°C or lower also died (21).

Table 2. Effect of the size and burial depth of tubers on percentage of germination (%) and the production of biomass (mg) of *Stuckenia pectinata* after 32 days under aquarium conditions*.

Tabla 2. Efecto del tamaño y profundidad de entierro de los tubérculos sobre el porcentaje de germinación (%) y la producción de biomasa de *Stuckenia pectinata* después de 32 días de crecimiento en peceras*.

Burial Depth	Percentage of germination (%)			Biomass (mg)		
	Tubers size			Tubers Size		
	Small (< 60 mg)	Medium (60-120 mg)	Large (>120 mg)	Small (< 60 mg)	Medium (60-120 mg)	Large (>120 mg)
7 cm	0	80	80	---	151.5 A	200.3 A
10 cm	0	0	100	---	---	254.7 A
15 cm	0	20	40	---	76.7 AB	77.8 AB
20 cm	0	0	40	---	---	63.95 B
25 cm	0	0	20	---	---	53.3 B

* Means with the same letter in the same column do not show differences according to the LSD Fisher test ($p < 0.05$).

* Medias con la misma letra en la misma columna no difieren estadísticamente acorde al test de diferencia mínima significativa de Fisher con una probabilidad < 0,05%.

In addition, year old tubers, or the oldest exposed to wet chilling, were not able to germinate (9). It would be advisable to perform mechanical tillage on the bottom of the canal to expose tubers to the normal freezing temperatures in winter, which would affect their subsequent infestation ability.

Annual growth cycle of *S. pectinata*

Biomass production

Stuckenia pectinata grows in the CORFO area as a spring-summer plant from October to February, when maximum radiation and water temperature facilitate the growth.

There were differences during the growing season in the biomass collected in different harvests (table 3).

Whereas in the first growth cycle the biomass showed two peaks, in the second growth cycle it only reached one peak at the end of the cycle (figure 2, page 209).

In the second growth cycle in the Villalonga canal, the maximum biomass accumulation was 1120 and 410 g DM m^{-2} in the first and second growth cycles, respectively (figure 2, page 209). In the Buratovich canal, the total biomass was significantly lower compared with the first cycle. Inverse behaviour was recorded in the Buratovich canal, where maximum biomass reached 800 g DM m^{-2} in the first growth cycle, and it was 1660 g DM m^{-2} in the second growth cycle (figure 2, page 209). The accumulation of biomass reached a maximum value of 1660 g DM m^{-2} at the end of February. In a monospecific stand of *S. pectinata* in The Netherlands, values of 1070 g DM m^{-2} were reported by Van Wijk (1988) without reaching the maximum levels. An infestation of 1900 g DM m^{-2} was reported in an estuary of South Africa, reaching the highest biomass level (10). Madsen and Adams (1988) cited biomass accumulation of 712 g DM m^{-2} in a Wisconsin stream.

Table 3. Results of ANOVA for biomass production, plant height and plant biomass, for *Stuckenia pectinata* during two growth cycles in Villalonga and Buratovich irrigation canals.

Tabla 3. Resultados del análisis de varianza para la producción de biomasa, altura y peso de plantas de *Stuckenia pectinata* durante dos ciclos de crecimiento en los canales de riego Villalonga y Buratovich.

Source	Canal	Cycle	Numerator DF	F	Pr > F
Biomass	Buratovich	First	10	31.09	<0.0001
		Second	8	49.2	<0.0001
	Villalonga	First	10	48.35	<0.0001
		Second	8	33.4	<0.0001
Plant height	Buratovich	First	12	19.8	<0.0001
		Second	8	114.8	<0.0001
	Villalonga	First	10	10.6	<0.0001
		Second	8	100.01	<0.0001
Plant biomass	Buratovich	First	12	29.4	<0.0001
		Second	8	21.8	<0.0001
	Villalonga	First	10	12.6	<0.0001
		Second	8	59.8	<0.0001

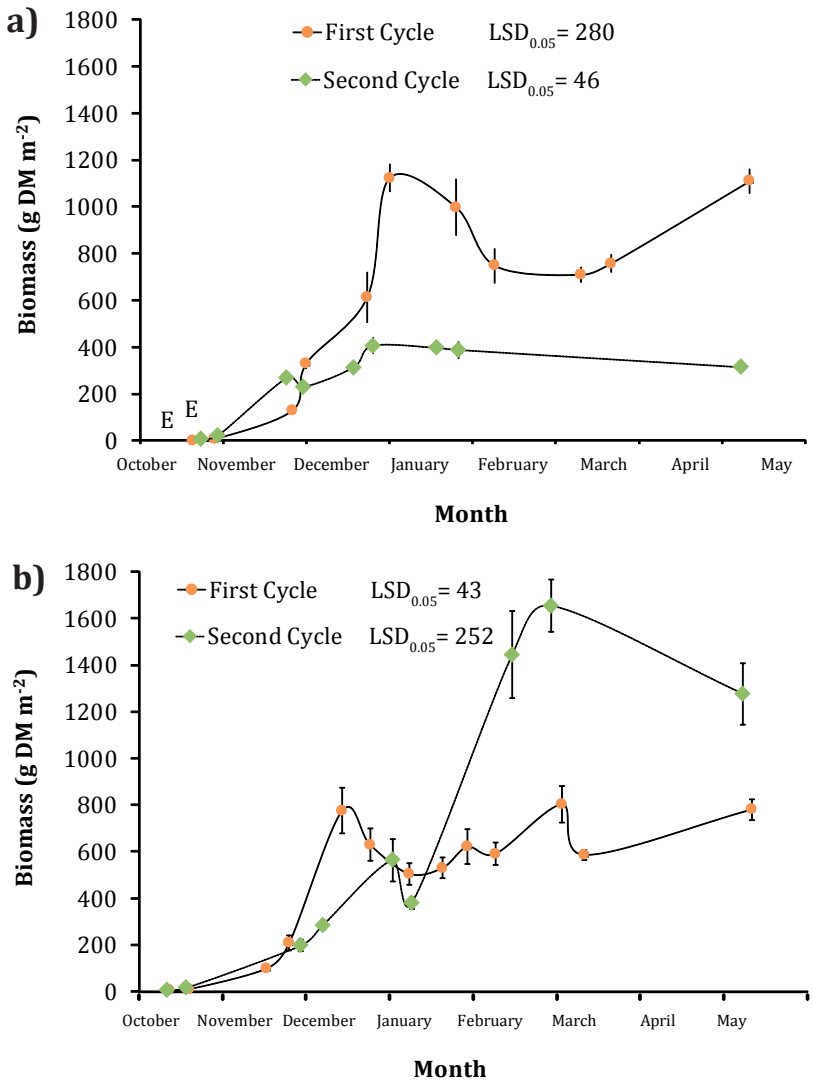

Figure 2. Dynamic of the aboveground biomass (g DW m^{-2}) of *Stuckenia pectinata* during two growth cycles in Villalonga (A) and Buratovich (B) irrigation canals.

Figura 2. Dinámica del crecimiento no subterráneo (g MS m^{-2}) de *Stuckenia pectinata* durante dos ciclos de crecimiento en los canales de riego de Villalonga (a) y Buratovich (b).

Individual plant size

Differences in plant height were recorded during the growing season (table 3, page 208). The maximum plant height recorded was 267 cm (figure 3). This value was greater than the value found in the North Baltic (208 cm) by Idestam-Almquisty and Kautsky (1995).

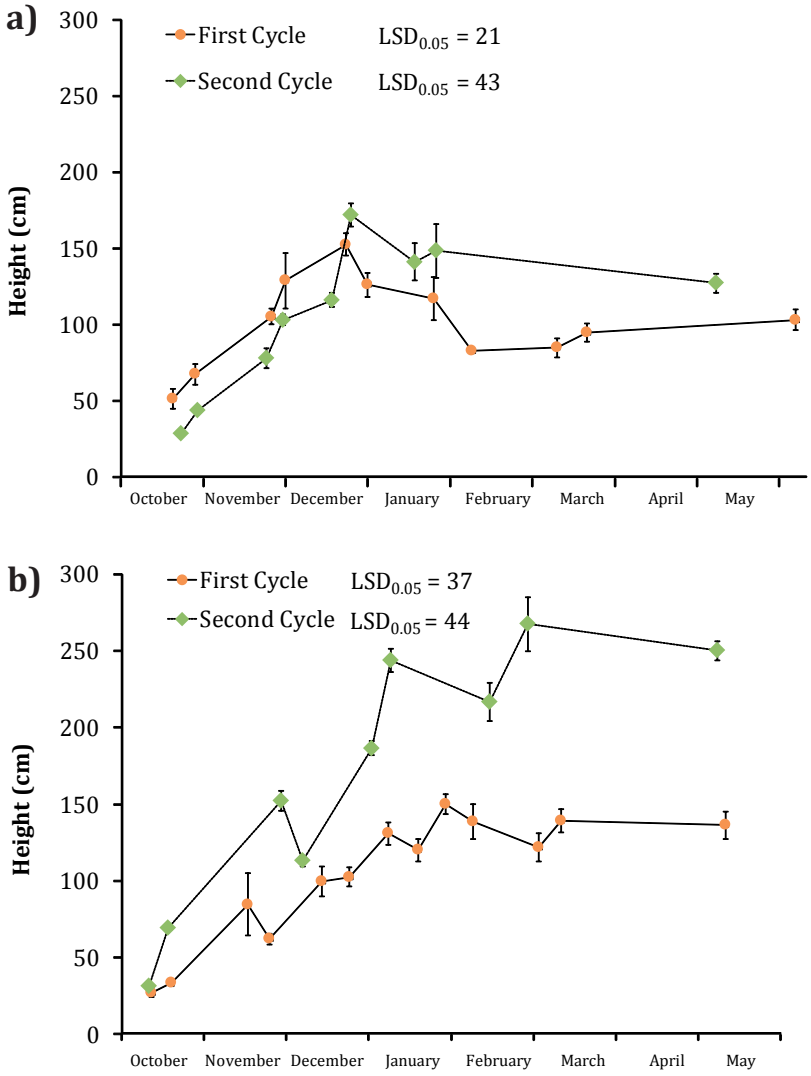

Figure 3. Plant height (cm) of *Stuckenia pectinata* during two growth cycles in Villalonga (A) and Buratovich (B) irrigation canals.

Figura 3. Altura de plantas (cm) de *Stuckenia pectinata* durante dos ciclos de crecimiento en el canal de riego Villanoga (a) y Buratovich (b).

The superior height of the plants that was recorded in plants harvested in the Buratovich canal compared with plants from the Villalonga canal can be attributed to the greater water depth in the first canal as it is known that plant height is associated with water depth (14). Moreover, the maximum plant height at the beginning of summer (late December/ early January) produced a blockage in the water flow and an urgent reduction in *S. pectinata* plants is needed.

There were differences in plant biomass. The maximum plant biomass in the Villalonga canal was 852 and 625 mg for the first and second growth cycles, respectively. In the Buratovich canal, maximum plant biomass was 1.263 and 2.230 mg for the same cycles (figure 4, page 212).

The maximum biomass of leaves and stems in each canal was recorded on the last sampling date in late autumn, whereas the maximum biomass of the spikes was recorded in late summer. In all cases, the highest values occurred in the first cycle in the Villalonga canal and in the second cycle in the Buratovich canal (figure 5, page 213-214). The maximum biomass of the leaves, stems and spikes in the Villalonga canal was 733.4, 317.9 and 30.0 mg, respectively. Whereas the maximum biomass of leaves, stems and spikes in the Buratovich canal was 1506.0, 785.4 and 87.1 mg, respectively (figure 5, page 213-214).Water flow, depth and canal orientation were variable in the irrigation system; consequently plant growth was different between the canals. Due to the fact that the rate of increasing biomass was different in each canal and year of study, the management should be adapted to each particular case. As the plant biomass reached the damage threshold of 200 g dry weight m^{-2} very quickly (4), the contact herbicide or mechanical control would have to be conducted early in the irrigation season (begining of October).

At the beginning of the growth period, plants of *S. pectinata* were only a few centimeters in height, and the aboveground biomass was comprised of 80 % of leaves and 20% of stem. Then, plants reached the water surface, by elongating the principal stem and without secondary stems, as cited by Vermaat and Hootsmans (1994). By late December, the dry matter of the leaves and stems tended to be similar. This is the moment when plants allocated a lower proportion of resources to the production of leaves. From that point onwards, the plants increased the proportion of leaves, reaching the end of the cycle with approximately double the dry biomass in leaves compared to the stems.

Fructification and seed production started in the middle of spring and continued throughout the rest of the growing season (figure 5, page 213-214). The same period of fructification was determined in Montana, USA by Yeo (1965). Spike production reached a high value of 187.6 mg of biomass in the second cycle at Buratovich. In running water, Gannie *et al.* (2016) only found 30 mg of spike biomass; however, no drupelets were produced because moving water inhibits pollination.

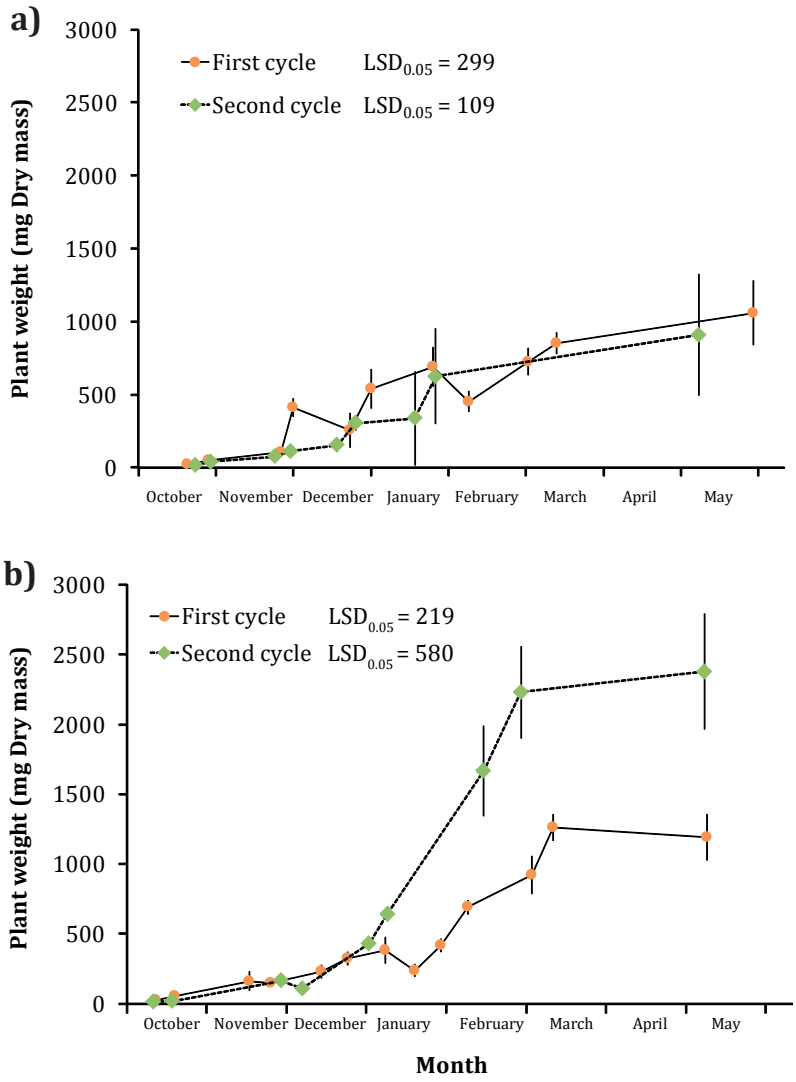

Figure 4. Plant biomass (mg) of *Stuckenia pectinata* during two growth cycles in Villalonga (A) and Buratovich (B) irrigation canals.

Figura 4. Biomasa de plantas (mg) de *Stuckenia pectinata* durante dos ciclos de crecimiento en el canal de riego Villanoga (a) y Buratovich (b).

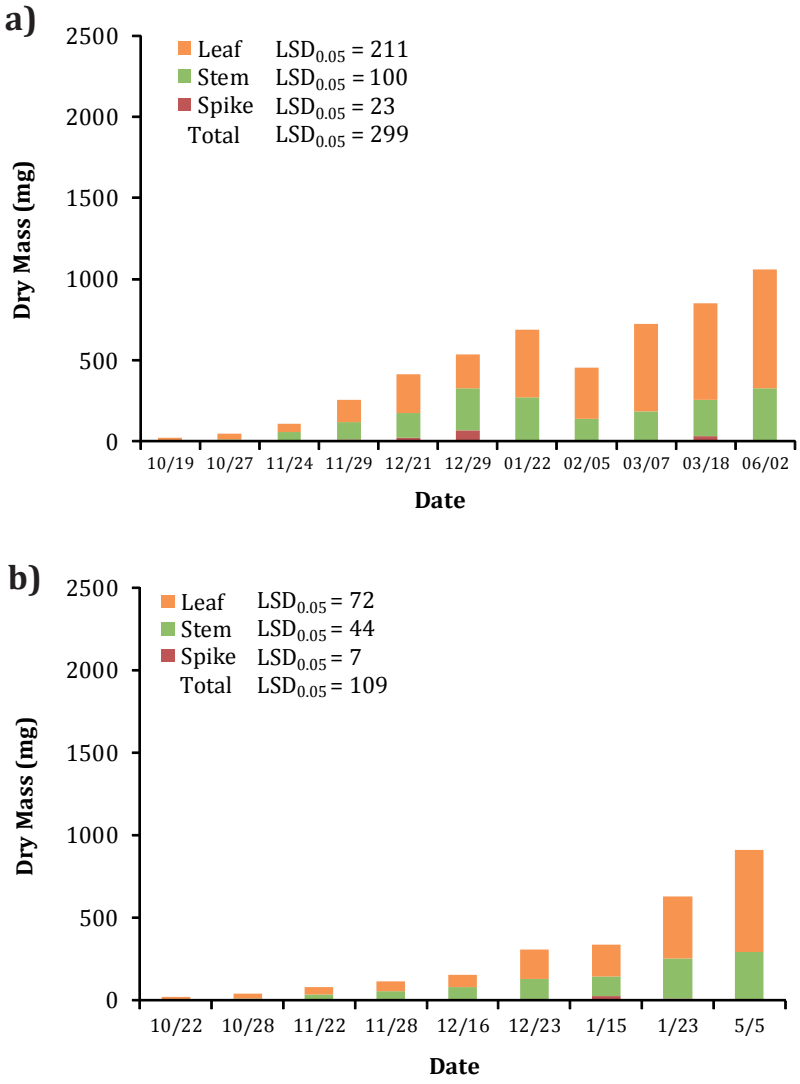

Figure 5. Leaf, stem and spike biomass of an individual plant of *Stuckenia pectinata* during two growth cycles (First A and C, Second B and D) in Villalonga (A, B) and Buratovich (C, D) irrigation canals.

Figura 5. Peso de hojas, tallos, y espigas de plantas individuales de *Stuckenia pectinata* durante dos ciclos de crecimiento (Primero A y C, Segundo B y D) en el canal del riego Villalonga (A,B) y Buratovich (C,D).

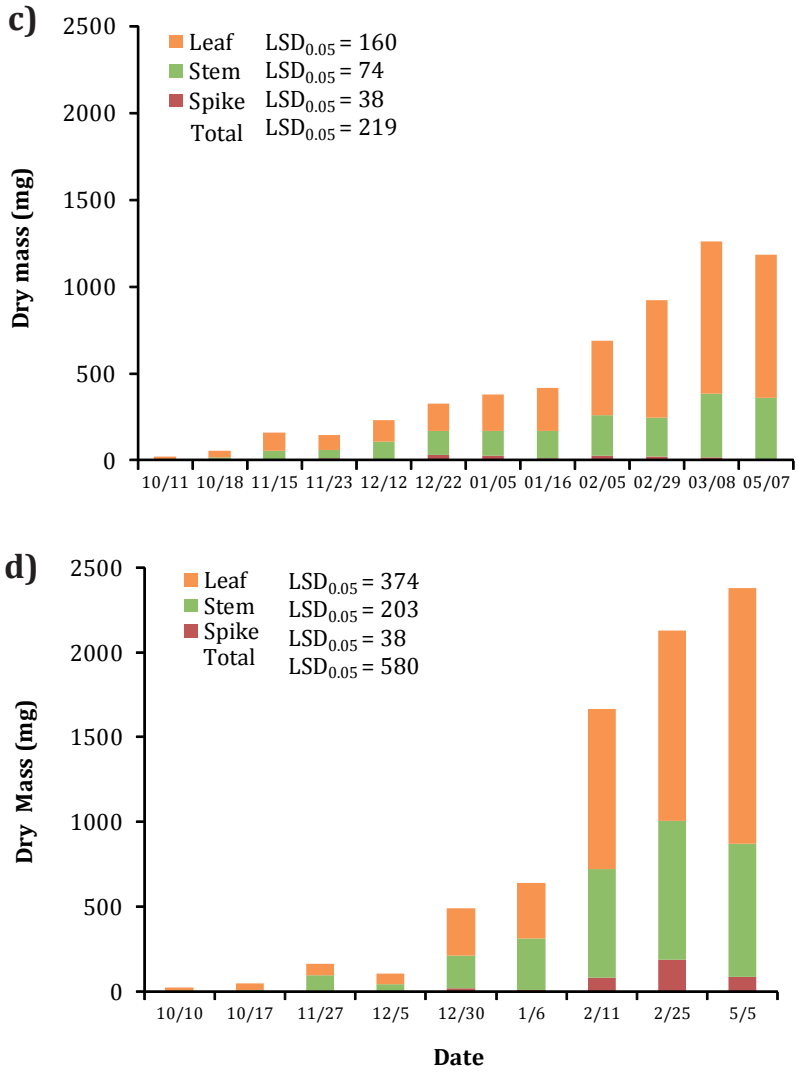

Figure 5. (cont.). Leaf, stem and spike biomass of an individual plant of *Stuckenia pectinata* during two growth cycles (First A and C, Second B and D) in Villalonga (A, B) and Buratovich (C, D) irrigation canals.

Figura 5 (cont.). Peso de hojas, tallos, y espigas de plantas individuales de *Stuckenia pectinata* durante dos ciclos de crecimiento (Primero A y C, Segundo B y D) en el canal del riego Villalonga (A,B) y Buratovich (C,D).

Conclusions

Freezing temperatures damaged tuber tissue and killed the tubers. Large tubers are capable of generating the largest plants and they emerge from up to 25 cm in depth; however, at the deepest sites they generate smaller plants than the superficial tubers. In order to reduce the number of future plants or to result in smallest size of plants, tubers have to be exposed to freezing temperature or be incorporated deep in the soil.

Changes in dry biomass of leaves, stems, spikes are produced during the growth season. At first, more leaves than stems are recorded. Finally, leaves increase until the end of the cycle. Spike weight is negligible in relation to the other plant constituents.

Contact herbicide, such as Acrolein, should be applied at the beginning of the growth season when plants have more leaves. When the stems increase on the plant, the amount of herbicide needed to control them should also be increased. Mechanical control is only effective with large plants, as most of the below ground biomass develops and new growth takes place rapidly.

Stuckenia pectinata reaches one of the highest values of biomass (1660 g DM m^{-2}) in the world. Also, the plant height reaches a high value of 2.67 m. High values of biomass and plant height lead to unavoidable canal blockage very quickly in the season.

According to the threshold of 200 g dry weight m^{-2} of Caffrey (1990) and the plant and biomass data of this research, several management procedures should be undertaken during the growing season to eliminate or reduce biomass to acceptable levels. Furthermore, many years of studies are needed to determine the real impact of these management techniques on biomass production and propagule depletion.

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Morphological variability of native maize (*Zea mays* L.) of the west highland of Puebla and east highland of Tlaxcala, Mexico

Variabilidad morfológica del maíz nativo (*Zea mays* L.) del altiplano poniente de Puebla y altiplano oriente de Tlaxcala, México

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Abstract

The objective of the present study was to analyze the morphological variability of native maize of the western highlands of Puebla and east of Tlaxcala, Mexico, in order to, in addition to defining it, relate it to races, commercial varieties and the altitude of the localities of collection. The genetic material evaluated were 134 populations collected in 34 localities, in addition to 10 witnesses. The experiments were established in three localities, using a 12 x 12 lattice. A total of 32 variables were evaluated, of which 27 presented highly significant differences, reflecting high variability at the level of morphological characters, many of them of agronomic interest. From the analysis of variance, 16 variables were selected for use in a cluster analysis using the Modified Localization Method, which concentrated the populations in six groups, most of them in group 1, with morphological characteristics of late-cycle varieties: tall plants, with more primary ramifications of the spike, ears of greater length and diameter and with greater length and thickness of grain. The conclusions indicate that the morphological variability of the populations is not associated with the altitude of the localities where they were collected and that these have a greater relationship with the Chalqueño race, little with the Cónico race, null with the Cónico Norteño and Palomero Toluqueño races and almost null with the commercial varieties.

Keywords
Zea mays L. • creole maize • plant genetic resources • germplasm • *in situ* conservation

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Resumen

El objetivo del presente estudio fue analizar la variabilidad morfológica de los maíces nativos del altiplano poniente de Puebla y oriente de Tlaxcala, México, para, además de definirla, relacionarla con razas, variedades comerciales y con la altitud de las localidades de colecta. El material genético evaluado fueron 134 materiales colectados en 34 localidades, además de 10 testigos. Los experimentos se establecieron en tres localidades, mediante un Látice12 x 12. Un total de 32 variables fueron evaluadas, de las cuales 27 presentaron diferencias altamente significativas, lo que refleja alta variabilidad a nivel de caracteres morfológicos, muchos de ellos de interés agronómico. Del análisis de varianza se seleccionaron 16 variables para utilizarse en un análisis de agrupamiento mediante el Método de Localización Modificado, el cual concentró a las poblaciones en seis grupos, la mayoría de ellas en el grupo 1, con características morfológicas de variedades de ciclo tardío: plantas altas, con más ramificaciones primarias de la espiga, mazorcas de mayor longitud y diámetro y con mayor longitud y grosor de grano. Las conclusiones indican que la variabilidad morfológica de las poblaciones no se asocia con la altitud de las localidades donde fueron colectadas y que estas tienen mayor relación con la raza Chalqueño, poca con la raza Cónico, nula con las razas Cónico Norteño y Palomero Toluqueño y casi nula con las variedades comerciales.

Palabras clave

Zea mays L. • maíz criollo • recursos fitogenéticos • germoplasma • conservación *in situ*

Introduction

The study of the genetic diversity of maize (*Zea mays* L. ssp. *mays*) in traditional agricultural production systems is important, since it is the primary source of food and nutrition for the rural population, based mainly on native varieties, both in Mexico and in other regions, such as southeastern Europe (41). Corn is not a typical result of natural selection, it was literally invented by the ethnic groups of Mexico (46). Over time, corn has evolved *in situ*, thanks to the joint intervention of men and women (26); the woman improves quality characters, while the man preserves the plant and increases its yield, especially in adverse conditions for the crop, such as drought.

Corn is the most important crop in Mexico, since, according to data from the Food and Agriculture Organization, in the period 2007-2017, an average of 23.4 million t was produced in an average annual area of 7.0 million hectares (8); In addition, it is the crop that involves a greater number of farmers (around 3.2 million out of a total of four million farmers in the country).

The state of Puebla participates with an approximate production of 10% of the national total, in an area that comprises 5% of the national total sown.

In the state, the Rural Development District of Libres is the one with the highest production, since in the 2007-2017 period it produced 28.9% of the state total, with average yields of 4.7 and 2.3 t ha^{-1} in irrigation and in rainfall conditions, respectively. However, the area sown in rainfall conditions in the same period represents 94% (approximately

102,788 ha), while in irrigation it is only 6% (approximately 6,221 ha) (40).

In the state of Puebla, research has been carried out that describes the morphological variability of maize populations: Mixteca Alta (2), part of the plateau (9, 17), Puebla Valley (16) and humid tropics (20), without an investigation to date indicating the morphological variability of the races planted in the Rural Development District of Libres, despite its importance in maize production.

The studies that could give an idea of the races are those of Wellhausen *et al*. (1951) and Cervantes and Mejía (1984), who mention that populations belonging to the Chalqueño and Cónico races are planted in the Valley of Puebla, adjacent to the District.

Despite the engrained tradition of the District of Libres as a producer of maize, a fluctuation in the area (ha) planted (123,298, 118,685, 127,841, 121,254, 118,910, 118,157, 116,678, 134,366, 122,413, 120,564 and 130,646 ha) has been observed (2007-2017 period), with a significant decrease in the years 2008, 2011, 2012 and 2013 (40), a situation that has serious implications from the point of view of the preservation of the native germplasm, in addition to the fact that the presence of generations with more field experience and knowledge about plant genetic resources is decreasing due to natural aging, without replacing new generations in agricultural activities, due to the marked process of emigration.

The inadequate preservation of germplasm can lead to genetic erosion, understood as the loss of a crop, of varieties within the crop or alleles within the variety (43). The importance of preventing such erosion lies in that the genetic diversity is a support for sustainable food production systems (6, 18) and for the generation of improved varieties that increase food production that will be demanded by the increasingly growing world population, estimated to exceed 9 billion in 2050 (14).

It is therefore urgent to document and evaluate the existing plant genetic resources (1, 3), in order to propose appropriate strategies for future use in the event of climate change and the incidence of biotic and abiotic adverse factors. In this context, the present study was conducted with the objective of analyzing the morphological variability of the native maize that originated in the corridor that includes the western highlands of Puebla (Libres and Mazapiltepec) and the eastern highlands of Tlaxcala (Huamantla), to know their main morphological characteristics and the relationship they still have with the maize races and commercial varieties grown in the region, as well as establishing some relationship of morphological variation with altitudinal patterns of the localities where the seed was collected.

Materials and methods

Description of the study area, the localities of collection and evaluation of localities

Three ecological niches were defined based on the criteria of Muñoz (1988): Libres (NE1), Mazapiltepec (NE2) and Huamantla (NE3), in the states of Puebla and Tlaxcala, respectively. In each ecological niche, a random stratified sampling was carried out (27) in order to select a representative number of municipalities and localities for the seed collection of maize populations, obtaining a total of 13 municipalities and 34 localities (table 1, page 220).

Table 1. List of localities of seed collection.
Tabla 1. Lista de las localidades de la colecta de semilla.

Ecological Niche	Locality	Key	Municipality	Altitude (m a. n. m.)
Libres	Buenavista de Guerrero	BdeG	Cuyoaco	2646
Libres	Emiliano Zapata	EZ	Cuyoaco	2490
Libres	Texcal	TEX	Cuyoaco	2493
Libres	Tepeyahualco	TEPE	Tepeyahualco	2337
Libres	Cuyoaco	CUY	Cuyoaco	2439
Libres	Temextla	TEM	Cuyoaco	2462
Libres	Ocotepec	OCO	Ocotepec	2445
Libres	Payuca	PAY	Cuyoaco	2382
Libres	Tehuatzingo	TEHUA	Libres	2412
Libres	El Fuerte	ELFUE	Tepeyahualco	2344
Libres	El Sabinal	ELSAB	Libres	2398
Libres	Juan Sarabia Pizarro	JSP	Tepeyahualco	2336
Libres	Miravalles	MIRA	Oriental	2395
Libres	Oriental	ORI	Oriental	2355
Libres	Pedernales	PED	Libres	2790
Libres	S. Antonio Virreyes	SAV	Oriental	2368
Mazapiltepec	S. José Ozumba	SJO	S. José Chiapa	2360
Mazapiltepec	Vicencio	VIC	S. José Chiapa	2376
Mazapiltepec	Nopalucan	NOP	Nopalucan	2456
Mazapiltepec	Sta. María Ixtiyucan	SMI	Nopalucan	2456
Mazapiltepec	Soltepec	SOL	Soltepec	2431
Mazapiltepec	Mazapiltepec	MAZA	Mazapiltepec	2353
Mazapiltepec	Álvaro Obregón	AO	Soltepec	2386
Mazapiltepec	Eréndira	ERÉN	Nopalucan	2379
Huamantla	Máximo Serdán	MS	Lara Grajales	2402
Huamantla	Col. Lázaro Cárdenas	CLC	Altzayanca	2704
Huamantla	Lomas de Junguito	LdeJ	Altzayanca	2495
Huamantla	S. Fco. Cuexcontzin	SFC	Cuapiaxtla	2433
Huamantla	S. José Xicoténcatl	SJX	Huamantla	2465
Huamantla	Benito Juárez	BJ	Huamantla	2476
Huamantla	Ignacio Zaragoza	IZ	Huamantla	2539
Huamantla	Los Pilares	PIL	Huamantla	2695
Huamantla	Zitlaltepec	ZITLA	Zitlaltepec	2588
Huamantla	Barrio San Lucas	BSL	Huamantla	2513

Altitude. Source Google Earth 7.1.2.2041.2013 / Altitud. Fuente Google Earth 7.1.2.2041. 2013.

Information on the localities where the experimental material was evaluated is presented below. Buenavista de Guerrero: NE1, 19°38'07" N L, 97°30'32" W L, altitude 2646 m a. s. l., rain 516 mm, average maximum and minimum annual temperature 23 and 4°C, planting April 14th; Máximo Serdán: NE3, 9°16'36" N L, 97°48'19" W L, altitude 2402 m.a.s.l., rain 598 mm, average annual temperature 21 and 5°C, planting April 21st; Mazapiltepec: NE2, 19°07'14" N L, 97°39'56" W L, altitude 2359 m a. s. l., rain 665 ml, average annual temperature 22 and 6°C, planting May 4th. The planting in the 3 localities was in the 2007 spring summer cycle.

Genetic material

134 native populations collected in the study area were evaluated, where the racial witnesses Cónico (Criollo del Mezquital), Cónico Norteño (Zac 58), Chalqueño Crema (7CSM), Chalqueño Palomo (Col-6583), Chalqueño del Valle de Toluca (Méx-158) and Palomero Toluqueño (Méx-5) were added.

Racial witnesses were used for the purpose of relating populations to any of them. Additionally, four commercial witnesses (Sintético Serdán, 32D06, Halcón and Z60) were used to see the agronomic potential of corn populations.

Design and experimental unit

The 144 materials were evaluated in a simple 12x12 lattice design at each experimental site. The experimental unit consisted of two rows of 5 m long and 0.8 m wide, where three seeds were sown every 50 cm to later leave two plants, generating a population density of 51,754 plants per hectare.

Each experiment was surrounded by 2 rows of plants on the sides and 10 m of plants in rows at the front and at the end of the experiment. The experimental units were separated by one meter in between them.

Crop management

The fertilization was carried out in two applications: during the first weeding all the phosphorus and 1/3 of the nitrogen were applied and during the second weeding the rest of the nitrogen. In Buenavista de Guerrero the fertilization dose was 100-30-00, in Máximo Serdán 100-40-00, and for Mazapiltepec 110-50-00. Weeding was carried out periodically and herbicide was applied once (2,4-D amine) at a rate of 1 L ha^{-1}.

Registered traits

The following traits were recorded: 1)- days to male flowering (DMF) and 2)- days to female flowering (DFF), counted from the day of planting until 50% of the plants presented dehiscent anthers and exposed stigmas, respectively, 3)- floral asynchrony (FAS), considered as the difference between DFF and DMF, recording these three traits at the experimental unit level; five random plants were subsequently selected to record the traits 4)- plant height (PH), from the stem base to the base of the spike and 5)- ear height (EH), from the base of the stem to the knot of insertion of the ear, both in cm, 6)- height index (HI), as the ratio EH/PH, 7)- number of leaves above the ear (LAE), 8)- number of primary ramifications of the spike (PRS), 9)- spike peduncle length (SPL) in cm, 10)- length of the branched spike section (LBSS) in cm, 11)- length of the spike's central branch (LPCB) in cm, 12)- total length of the spike (TLS) in cm, 13)- number of plants (NP), 14)- percentage of plants with more than one ear (PPM1E), 15)- percentage of sterile plants (PSP), 16)- plant-to-harvest aspect (PHA) in visual scale 1-5,

where 1 corresponds to plants with good appearance and 5 to plants with bad appearance, 17)- number of harvested ears (NHE), 18)- ear rating (ER) in scale 1-5, where 1 corresponds to a good aspect and 5 the opposite case, 19)- field weight (FW) in g, 20)- weight of five ears in g, 21)- grain yield per hectare (GYPH) in t ha^{-1}, using the formula:

$$\text{Yiel} = \frac{(\text{FW x \% humidity)x \%dry matter x SF}}{\text{Number of plants}} \text{ x } 45000/1000$$

Additionally, five-plant ear characters were recorded per experimental unit: 22)- ear diameter (ED) in cm, 23)- ear length (EL) in cm, 24)- number of rows (NR), 25)- grains per row (GPR), 26)- ear length/diameter ratio (ELDR). From each ear 10 grains were taken to measure 27)- width (W), 28)- length (L) and 29)- grain thickness (GT), all of them in mm, and 30)- grain length/width ratio (GLWR); In addition, the five ears were shelled and 31) - grain weight (GW) and 32)- cob weight (CW) in g were taken to calculate 33) - the shelling factor (SF) using the ratio GW/(GW + CW).

Statistical analysis

In order to assess the stability of the expression of the traits in the three ecological niches, a combined analysis of variance between environments was performed using the PROC GLM procedure of the SAS package (Statistical Analysis System) version 9.1 (38); The linear model used was as follows:

where:

Yijkl= the observation of the i-th collection in the j-th environment of the k-th repetition and the l-th block

μ= the general mean and constitutes a constant common to all observations

α_i= the effect of the I-th observation of the collection

γ_j= the effect of the j-th environment

δ_{ij}= the interaction effect of the i-th collection with the j-th environment

$B(L)_{l(kj)}$= the effect of the l-th block nested in the k-th repetition of the j-th environment

ε_{ijkl}= the experimental error associated with the experimental unit $Y_{ij(k)}$.

Subsequently, in order to detect and eliminate multicollinearity, defined as the linear dependence between traits and which implies singularity (the matrix is not full range) in the Mahalanobis variance-covariance matrix, in the multivariate post-analysis, a Pearson's simple correlation matrix was elaborated, using the PROC CORR procedure of SAS, eliminating highly correlated traits ($r \geq 0.7$); For example, between days of male and female flowering, which were highly correlated, only the one with the greatest biological significance was selected for later analysis. Traits were also debugged using the PROC STEPDISC procedure of SAS, applying the STEPWISE sequential method by which the discriminatory power of the traits involved in the analysis is analyzed.

$$Y_{ijkl} = \mu + \alpha_i + \gamma_j + \delta_{ij} + B(L)_{l(kj)} + \varepsilon_{ijkl}, \quad i = 1,2,.....,144, \quad j = 1,2,3, \quad k = 1,2, \quad l = 1,2,.....,12$$

STEPWISE is a multivariate technique that is applied when the traits have the property of having a normal multivariate distribution and have a common covariance matrix. This technique basically consists in the selection of a subset of informative traits or with greater discriminatory capacity to identify differences between the units of analysis.

However, this technique alone does not ensure a good selection of discriminant traits and should be complemented with other techniques and with the experience of the researcher to choose those traits that are as informative as possible of the existing variation (36, 37). Based on the F statistic, traits at 15% significance were selected.

The modified localization model or method (MLM) was used, which is a two-stage classification strategy (7, 10, 11, 12): in the initial stage, groups are defined using a hierarchical grouping method (UPGMA-unweighted pair group method with arithmetic mean), the location model is then applied to the groups formed, in which significant differences between and within groups will be verified using the Mahalanobis distance criterion, which estimates the distances and the parameters that define the groups in diversity studies (21).

The MLM has the advantage of combining all categorical traits into a single multinomial trait, which in turn can then be used in a single matrix, along with continuous traits; it is even possible to combine common grouping methods, such as Ward's method, with this technique (24).

Results and discussion

Variance analysis

In the analysis of combined variance FAS, SPL, PPM1E and PSP did not show significant differences for genotypes, while the remaining 28 traits were significant ($p \leq 0.01$), which is considered as an indicator of diversity (9, 17).

In most genetic diversity studies of native populations of maize races, high diversity is reported, mainly intrapopulation (19, 44, 45), although in some cases, such as in the Cacahuacintle race, it is minimal (13).

With respect to environments, PPM1E and PSP showed no statistical significance, FAS and HI were significant with $p \leq 0.05$, while the remaining 28 traits were significant with $p \leq 0.01$. As in this study, others have reported significant differences between environments in most of the traits evaluated in the native populations of maize races (2, 20).

Regarding the interaction genotypes × environments PH, EH, HI, LPCB, ED, GPR and GLWR did not show statistical significance (table 2, page 224), which implies that these traits respond in a similar way to changes in the environment between the various genotypes.

Trait selection

Multicollinearity is defined as the linear dependence between traits, its presence implies singularity (the matrix is not of full range) in the Mahalanobis variance-covariance matrix (exponential part of the multivariate normal distribution), which estimates the distances and parameters that form and define the groups in diversity studies.

One way to detect and eliminate multicollinearity is to perform a simple correlation analysis (ρ), followed by a method of trait selection.

Table 2. Mean squares, level of significance and coefficients of variation of morphological data of maize landraces tested in three localities of the Libres-Mazapiltepec-Huamantla región.

Tabla 2. Cuadrados medios, niveles de significancia y coeficientes de variación para datos morfológicos de maíces nativos evaluados en tres ambientes de la región Libres-Mazapiltepec-Huamantla, 2007.

Traits	Genotypes		Environments		Genotype × Environment		CV (%)
GYPH (t ha^{-1})	3414150.3	**	478346720	**	1981653.61	**	27
DMF (días)	200.84	**	127949	**	28.59	**	3.4
DFF (días)	195.07	**	145376	**	20.37	**	3.3
FAS (días)	10.72	NS	881	*	14.04	**	6.8
PH (cm)	1848.89	**	339529	**	74.41	NS	6.5
EH (cm)	1194.48	**	147327	**	55.5	NS	9.6
HI (cm)	0.01	**	0.04	*	0	NS	6
LAE	0.77	**	29.61	**	0.14	**	7.5
PRS (cm)	13.92	**	197	**	5.76	**	25.3
SPL (cm)	11.4	NS	9893	**	9.24	**	11.7
LBSS (cm)	18.76	**	10783	**	12.11	**	9.2
LPCB (cm)	3.84	**	152	**	1.48	NS	18.4
TLS (cm)	41.04	**	46124	**	24.31	**	6.8
ED (cm)	50.21	**	859	**	4.73	NS	4.6
EL (cm)	5.42	**	19	**	1.95	**	10.1
NR	9.17	**	35	**	1.31	**	7.1
GPR	20.91	**	1529	**	8.43	NS	11.4
ELDR	24.77	**	583	**	11.14	**	10.9
W (mm)	1.52	**	62	**	0.29	**	6
L (mm)	3.2	**	746	**	0.72	**	5.4
GT (mm)	0.32	**	3	**	0.11	**	6.8
GLWR	0.11	**	2.82	**	0.02	NS	7.2
GW (g)	23462.21	**	10303666	**	11658.35	**	20.1
CW (g)	841.66	**	33979	**	146.64	**	19.4
SH	0	**	0.29	**	0	**	2.2
NP	57.17	**	6829	**	41.5	**	12.3
PPM1E (%)	58.22	NS	2024	NS	44.3	**	12.8
PSP (%)	137.16	NS	10469	NS	123.26	**	14.2
PH (1-5)	1.04	**	16	**	0.31	**	17.4
NHE	61.05	**	18509	**	45.78	**	16.3
ER (1-5)	0.28	**	6	**	0.27	**	16.6
FW (g)	4381195.4	**	37898670	**	1178050.74	**	21

** Significance P≤0.001, * Significance P≤0.05, NS there is no significance.

** Significancia P≤0,001, * Significancia P≤0,05, NS No existencia de significancia.

Trait names are in Material and Methods.

Los nombres de las variables están en Materiales y Métodos.

According to Romero *et al*. (2002) and Mijangos-Cortés *et al*. (2007), of the pairs of traits that presented correlations greater than 0.75 in absolute value ($r < -0.75$ and $r > 0.75$), 9 traits with less agronomic importance were eliminated: DMF-DFF (0,98), PH-EH (0,94), DMF-TLS (-0,79), DFF-TLS (-0,78), DMF-SPL (-0,77), DFF-SPL (-0,78), SPL-TLS (0,92), LBSS-TLS (0,92) and EL-ELDR (0,79).

When applying the method of sequential selection of STEPWISE traits, the selected traits were HI, NR, ED, GLWR, DFF, PH, LAE, PRS, W, L, SH, LBSS, LPCB, EL, GT and PHA (table 3). Most of the selected traits are consistent with previous diversity studies (15, 19, 30, 31, 35).

Grouping analysis

The grouping analysis, using the Modified Localization Method, in its first classification strategy defined four groups. When applying the second classification strategy, the optimal number of groups that were formed by estimating the likelihood profile and obtaining the probabilities of belonging *a posteriori* to each of the 144 materials evaluated with maximum restricted likelihood was determined (39).

The likelihood test ran 14 iterations before converging on the value -1098,4327, after which it assembled 6 groups (figure 1, page 226).

When calculating the *a posteriori* probabilities for each of the 144 evaluated materials, they fluctuated between 0,88 and 1,00, which represented a good classification of each of the 144 materials under study (information not shown). It was observed that there are significant differences between groups and within groups using the distance criterion of Mahalanobis (21), which allowed to form 6 groups.

Additionally, and considering the large number of populations and the variability that the materials presented within Group 1, it was considered convenient to form subgroups within this group using the Modified Localization Method (MLM), thus integrating Subgroups 1a, 1b and 1c of figure 1 (page 226).

Table 3. Selected traits using the STEPWISE method.
Tabla 3. Variables seleccionadas utilizando el método STEPWISE.

CT	Partial R^2	Pr>F	CC	Pr> ASCC	CT	Partial R^2	Pr>F	CC	Pr> ASCC
HI	0.644	<.0001	0.005	<.0001	AG	0.306	<.0001	0.026	<.0001
NR	0.618	<.0001	0.009	<.0001	LG	0.642	<.0001	0.03	<.0001
ED	0.618	<.0001	0.013	<.0001	FD	0.311	<.0001	0.031	<.0001
GLWR	0.437	<.0001	0.016	<.0001	LM	0.293	<.0001	0.033	<.0001
DFF	0.452	<.0001	0.017	<.0001	LTRE	0.275	<.0001	0.035	<.0001
PH	0.378	<.0001	0.019	<.0001	GG	0.269	<.0001	0.037	<.0001
LAE	0.4	<.0001	0.021	<.0001	LRCE	0.25	<.0001	0.038	<.0001
PRS	0.358	<.0001	0.024	<.0001	ASF	0.207	<.0213	0.039	<.0001

VS: chosen trait, CC: Canonic correlation.
VS: Variable Seleccionada, CC: Correlación Canónica.
Trait names are in Material and Methods (page 219).
Los nombres de las variables están en Materiales y Métodos (pág. 219).

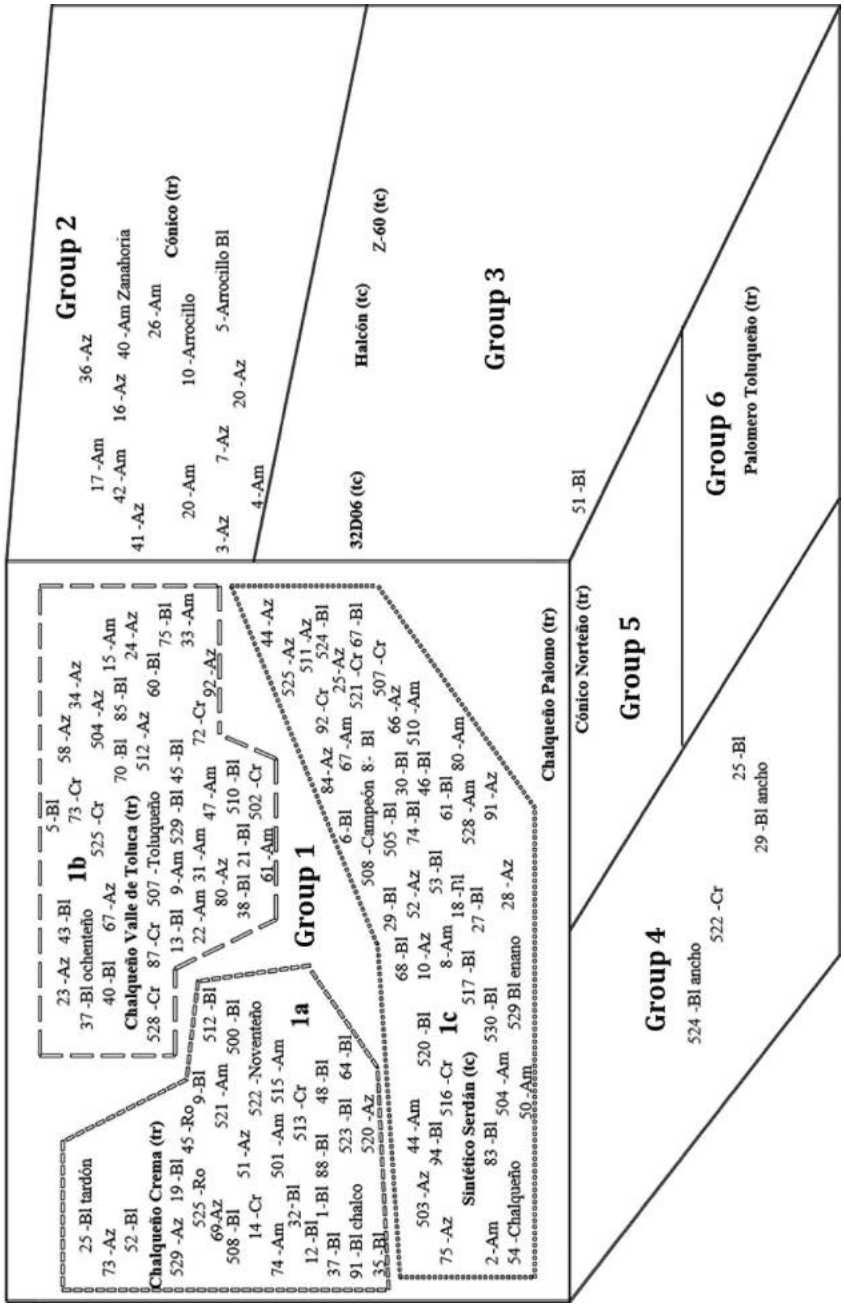

Figure 1. Groups and subgroups obtained by the Modified Localization Method.
Figura 1. Grupos y subgrupos ensamblados mediante el Método de Localización Modificado.

In table 4 (page 228), the morphological characteristics of each of the groups can be observed, as follows: Group 1 was made up of 119 materials, of which 116 are native populations, two racial witnesses belonging to the Chalqueño breed (Chalqueño Crema and Chalqueño del Valle de Toluca) and an improved variety (Sintético Serdán).

The materials of this group are late flowering, higher, greater number of PRS, occupies the third place in regards to NR, longer ears with larger diameter, longer and thicker grains, and it is the second group with wider grains. Within Group 1 is Subgroup 1a, consisting of 31 materials, of which 30 are native, plus the racial witness Chalqueño Crema. This subgroup presents the latest flowering varieties, the largest PH, and the highest number of PRS, longer ears with the largest diameter and less thick and less wide grains.

Subgroup 1b was integrated with 37 materials, 36 of which are native and one is the Chalqueño del Valle de Toluca racial witness. This subgroup is the earliest flowering of the three, the second with the highest PH, lowest number of PRS, highest NR, less wide grains and smaller thickness. Subgroup 1c was made up of 51 materials, 50 native and the witness Sintético Serdán; it is the second with the highest number of DFF and lowest PH, the second with the highest number of PRS and lowest NR, the second with L and with the highest W and with greater GT.

Group 2 was made up of 15 materials, 14 native and the Cónico racial witness. This group is the earliest flowering of all, the third with the highest PH, an average of 6,9 PRS, 15,2 rows per ear (NR). It ranks third place in terms of EL and ED, second in terms of W, third with respect to L, fourth in W, and third in GT. This group was characterized by grouping only yellow, blue and "arrocillo" grain materials, not including white color materials.

Group 3 was integrated with three commercial witnesses (Z-60, Halcón and 32D06) plus a native material, from Oriental, Puebla. These materials are late flowering, not very tall. They have a greater number of rows, longer ear length, and occupy the penultimate place in regards to PRS, second in ED, third in L, fourth in W, and last place in regards to GT.

Group 4 was made up of four native materials: 522 Cr and 524 Bl wide, from the localities of Los Pilares and Barrio San Lucas of the region of Huamantla, Tlaxcala and 25 Bl and 29 Bl wide, from Ocotepec and Payuca in the region of Libres, Puebla. These materials are of intermediate flowering and plant height. They are the ones with the lowest NR, and occupy second place in EL and fourth place in ED.

Group 5 was formed by the racial witness Cónico Norteño, which was the earliest flowering of all, the smallest in size, the penultimate in regards to NR, lower EL, the penultimate with respect to ED, lower L and second place with respect to W.

Group 6 was represented by a single material, the racial witness Palomero Toluqueño, which occupied the second place in EH, the penultimate in PH. It is the one who presented a higher NR, and occupied the penultimate place in EL, lower ED, narrower and thinner grains and had the penultimate place in L.

Table 4. Morphological data by group of 134 landraces, four control cultivars and six control maize races grouped by the modified localization method.

Tabla 4. Datos morfológicos por grupo de 134 poblaciones nativas, 4 testigos comerciales y 6 testigos raciales ensamblados mediante el Método de Localización Modificado.

Trait	Formed Groups																			
	Group 1		SUB1a		SUB1b		SUB1c		Group 2		Group 3		Group 4		Group 5		Group 6	
	n=119		31		37		51		15		4		4		1		1	
DFF (days)	114.8	a	116.6	a	113.6	b	114.5	b	104.6	b	114.4	b	104.6	b	90.8	d	97.8	c
FAS (days)	6.8	a	6.5	a	6.8	a	6.9	a	7.1	a	5.8	a	7	a	4.7	a	5.2	a
PH (cm)	179.4	a	187.2	a	177.3	b	176.1	b	153.6	b	130.3	c	153.6	b	96.9	d	130	c
HI	0.6	a	0.6	a	0.6	a	0.59	b	0.6	a	0.5	c	0.6	a	0.4	d	0.57	b
LAE	4.5	b	4.6	a	4.5	b	4.6	a	4.2	c	5.5	a	4	c	3.6	d	4.3	b
PRS	8.6	a	9	a	8.2	a	8.6	a	6.9	b	5.2	b	8	a	7.7	a	4.2	c
LBSS (cm)	29.6	a	30	a	29.6	a	29.3	a	27.7	a	29.2	a	28.2	a	24.7	c	26.1	b
LPCB (cm)	6.42	a	6.7	a	6.2	b	6.4	b	5.3	b	5.2	b	6.1	b	5.3	b	4.3	c
NR	14.6	b	14.5	b	14.9	a	14.3	b	15.2	a	15.8	a	9.9	d	12.1	c	19.6	a
EL (cm)	12.53	a	12.71	a	12.32	b	12.57	a	11.34	b	13.45	a	12.92	a	8.91	d	10.07	c
ED (mm)	47.4	a	47.8	a	47.1	a	47.3	a	43.5	b	46.8	a	40.1	c	36.1	d	34.5	d
L (mm)	14.5	a	14.8	a	14.4	b	14.4	b	13.3	b	12.4	c	13.2	b	11.4	d	11.7	c
W (mm)	7.9	b	7.9	b	7.7	c	8.1	a	7.2	c	7.7	c	9.5	a	7.8	b	4.7	d
GT (mm)	4.34	a	4.29	b	4.31	b	4.4	a	4.2	b	4.1	b	4.5	a	4.1	b	3	c
GLW	1.8	b	1.9	a	1.9	a	1.8	b	1.9	a	1.6	c	1.4	d	1.5	d	2.6	a
SH	0.88	b	0.9	a	0.9	a	0.9	a	0.89	b	0.83	d	0.89	b	0.86	c	0.92	a
GYPH (t/ha)	4850	a	4832	a	4892	a	4830	a	4064	b	5101	a	3827	c	1412	d	2003	d

Trait names are in Material and Methods. / Los nombres de las variables están en Materiales y Métodos.

Means with same letter by row are not different statistically. / Medias con la misma letra en cada fila son iguales estadísticamente.

Based on the above, the characteristics of the majority (86.6%) of the populations are related to the Chalqueño race, few (10.4%) with the Cónico race, none with the Cónico Norteño and Palomero Toluqueño races. It is worth mentioning that the Chalqueño race, which is distributed mainly in the eastern part of the state of Mexico (15) is not considered as one of the six main races of maize in Mexico, contrary to Cónico and Cónico Norteño races that are considered the second and fourth most common, respectively (28). Only 0.7% share characteristics with commercial varieties and 2.98% do not resemble any races or commercial varieties. Therefore, the majority of the populations are of late flowering cycle, of higher plants, with more primary ramifications of the spike, ears of greater length and diameter, and with greater grain length and thickness. Even though there is a high morphological variability in the populations, it is important to highlight the low relationship with maize races, since only a relationship was found with 2, despite having conducted the study in 34 locations of three ecological niches located in the state of Puebla and Tlaxcala.

In another study carried out in several locations in the central region of Mexico, the presence of up to 8 races of maize at the local level is reported (32), which can be explained based on the fact that this is considered one of the six regions of Mexico with the highest diversity of races (34).

The greater relationship of the populations with the Chalqueño race indicates that the adaptation area has been expanded, because the *in situ* conservation of the genetic diversity of the crops is dynamic (31), since the extension is due to the fact that each race has a new area of potential distribution, where proper management can favor the conservation of its diversity (42).

Regarding the relationship of the groups with grain color (white, cream, blue, yellow and red) of the populations, there is no direct relationship between the two, since all were present in Subgroup 1a, and, with the exception of red, in Subgroup 1b and Subgroup 1c, which grouped 83% of the populations. In the other groups, for having fewer populations and for being integrated by the commercial witnesses, white color prevailed.

The grouping of genetic diversity through multivariate statistical methods and the modified localization method (MLM) have been used in various studies on genetic diversity of maize races. Just to mention an example, Ortiz *et al*. (2008) formed eight groups with populations of 8 maize races from the High Valleys of Peru.

Relationship between locations and groups

In figure 2 (page 230), it can be seen that most of the seed collection localities were integrated in Group 1, associated with native materials genetically close to the racial witnesses of the Chalqueño race. When performing the breakdown in subgroups, it is observed that Subgroup 1a was associated with the localities of Álvaro Obregón (AO, 2386 m a. s. l.), Nopalucan (NOP, 2456 m a. s. l.), Sta. María Ixtiyucan (SMI, 2456 m a. s. l.), Zitlaltepec (ZITLA, 2588 m a. s. l.), Pedernales (PED, 2790 m a. s. l.), Ignacio Zaragoza (IZ, 2539 m a. s. l.) and Benito Juárez (BJ, 2476 m a. s. l.), indicating that these localities have a closer relationship with native materials associated with the Chalqueño Crema subrace. On the other hand, Subgroup 1b was associated with the localities of Temextla (TEM, 2462 m a. s. l.), Soltepec (SOL, 2431 m a. s. l.), S. Fco. Cuexcontzin (SFC, 2433 m a. s. l.), and S. Antonio Virreyes (SAV, 2368 m a. s. l.), which indicates that

these localities are mostly associated with native materials and the Chalqueño del Valle de Toluca witness; Subgroup 1c had a strong relationship with the localities of Buenavista de Guerrero (BdeG, 2619 m a. s. l.), Lomas de Junguito (LdeJ, 2495 m a. s. l.), Tehuatzingo (TEHUA, 2412 m a. s. l.), El Fuerte (ELFUE, 2344 m a. s. l.), Mazapiltepec (MAZA, 2413 m a. s. l.), Eréndira (ERÉN, 2379 m a. s. l.), S. José Xicoténcatl (SJX, 2465 m a. s. l.), Tepeyahualco (TEPE, 2337 m a. s. l.) and Col. Lázaro Cárdenas (CLC, 2704 m a. s. l.).

Group 2, which was strongly related to the Cónico racial witness, presented an association with the localities of Cuyoaco (CUY, 2439 m a. s. l.) and Emiliano Zapata (EZ, 2490 m a. s. l.).

On the other hand, Group 3 was strongly related to commercial witnesses and shared together with Group 5 the locality of Juan Sarabia Pizarro (JSP, 2336 m a. s. l.).

Group 4 was associated with the localities of Barrio San Lucas (BSL, 2520 m a. s. l.), Payuca (PAY, 2382 m a. s. l.), Los Pilares (PIL, 2695 m a. s. l.), Texcal (TEX, 2493 m a. s. l.) and Miravalles (MIRA, 2395 m a. s. l.). It should be clarified that the native materials of this group had no relationship to any racial witness.

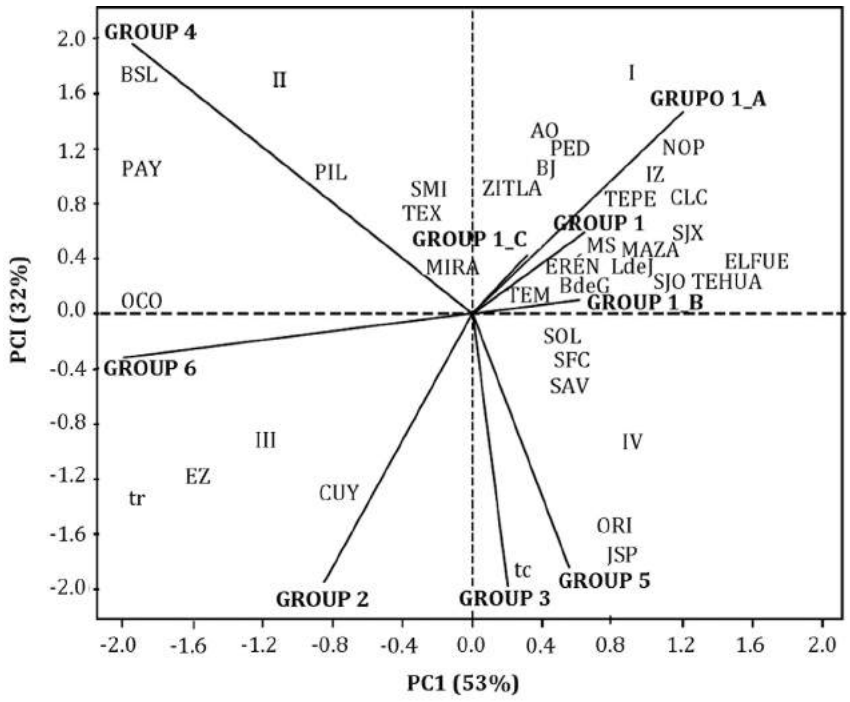

Figure 2. Relation among localities and the groups formed by the Modified Localization Method. The meaning of codes are in table 1 (page 220).

Figura 2. Relación entre las localidades y los grupos ensamblados mediante el Método de Localización Modificado. El significado de las claves está en la tabla 1 (pág. 220).

Group 5, which was related to the Cónico Norteño racial witness, but without native materials, was associated with the localities of Oriental (ORI, 2355 m a. s. l.) and part of Juan Sarabia Pizarro (JSP, 2336 m a. s. l.), while Group 6 was related to Ocotepec (OCO, 2445 m a. s. l.).

When relating the groups and subgroups to the altitude of the localities, it is observed that this ranges from 2386 to 2790 m in Subgroup 1a, from 2368 to 2462 m in Subgroup 1b, from 2337 to 2704 m in Subgroup 1c, from 2439 to 2490 m in Group 2, 2336 m in Group 3 and from 2382 to 2695 m in Group 4; in Group 5 and 6 there were no populations.

Therefore, the maximum altitudinal difference between the localities was 454 m, a difference that was not sufficient to associate the variation of the populations with an altitudinal pattern.

In other studies with a greater altitudinal difference, such as that of Diego-Flores *et al*. (2012) and that of Mercer *et al*. (2008), a relationship was found between the origin of the populations and the altitudinal pattern of evaluation.

Romero *et al*. (2002) mention that one of the effects of altitude on populations is associated with the temperature it generates and the effect on the regulation of genes responsible for flowering. The same authors, in a very large study on allelic diversity in flowering time in maize and its local adaptation, report 366 genes with significant association to altitude and 881 and 883 genes with significant association at days of female and male flowering, respectively.

Conclusions

There is a wide morphological variability among native maize grown in the study area, based on the statistical differences between vegetative, reproductive and yield traits, and in the 6 groups and the 3 subgroups of group 1 where the populations were located.

Most populations have vegetative, reproductive and yield characteristics typical of long or late flowering-cycle plants: tall plants, with more primary ramifications of the spike, ears of greater length and diameter, and with greater grain length and thickness, which is why they were mainly related to the Chalqueño race, to a lesser extent with the Cónico race, minimal or almost null with the commercial varieties and null with the Cónico Norteño and Palomero Toluqueño races.

The morphological variation of the populations is not associated with an altitudinal pattern, due to the little altitudinal difference between the localities.

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Germination temperatures and seed dormancy of two *Larrea* species (Zygophyllaceae) from the Monte Desert, Argentina

Temperaturas de germinación y dormición de semillas de dos especies de *Larrea* (Zygophyllaceae) del desierto del Monte, Argentina

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Abstract

The genus *Larrea* includes five species of desert shrubs distributed along the American Continent. These species produce dormant mature seeds, but the type of dormancy and the factors that produce it have been poorly assessed. The objective of this work was to determine the optimum germination temperatures of *L. cuneifolia* and *L. nitida*, to analyze the response to pre-germination treatments, and to evaluate the type of seed dormancy these species have. Seeds were incubated at five constant temperatures and were subjected to mechanical scarification and rinsed with running water to break dormancy. Seed coat permeability and the presence of water-soluble germination inhibitors were also assessed. The optimum germination temperature range was between 15-40°C for both species. A positive response to all pre-germination treatments was observed in *L. cuneifolia* (37-47%), while *L. nitida* showed higher germination percentage only with mechanical scarification (73%). Both species presented water-permeable seed coats, ruling out the occurrence of a physical dormancy. The inhibitory test of seed-coat extracts was positive for *L. cuneifolia*, suggesting the possible presence of a chemical dormancy. These results are valuable for conservation purposes and directly contribute to improving production of seedlings required for restoration projects.

Keywords
germination inhibitors • *Larrea cuneifolia* • *Larrea nitida* • optimum temperature • physical dormancy

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Resumen

El género Larrea incluye cinco especies de arbustos desérticos distribuidos a lo largo del Continente Americano. Estas especies producen semillas en estado de dormición, pero el tipo de latencia y los factores que la producen han sido poco estudiados. El objetivo de este trabajo fue determinar las temperaturas óptimas de germinación de dos especies de *Larrea*, analizar la respuesta a los tratamientos pre-germinativos y evaluar el tipo de dormición de las semillas. Las mismas se incubaron a cinco temperaturas, se sometieron a escarificación mecánica y se lavaron con agua corriente para romper dormición. También se evaluó permeabilidad de coberturas seminales y presencia de inhibidores de la germinación solubles en agua. El rango óptimo de temperaturas estuvo entre 15-40°C para ambas especies. Se observó una respuesta positiva a todos los tratamientos pre-germinativos en *L. cuneifolia* (37-47%), mientras que *L. nitida* mostró un mayor porcentaje de germinación solo con escarificación (73%). Ambas especies presentan semillas permeables al agua, lo que descartó la existencia de una dormición física. El test de inhibidores fue positivo para *L. cuneifolia*, sugiriendo la presencia de una dormición química. Estos resultados son valiosos para la conservación y contribuyen a mejorar la producción de plantines en proyectos de restauración.

Palabras clave
inhibidores de la germinación • *Larrea cuneifolia* • *Larrea nitida* • temperatura óptima • dormición física

Introduction

The genus *Larrea* is possibly one of the most widespread genera of desert shrubs on the American continent, including five species of xerophytic evergreen shrubs distributed in the different arid ecosystems (4, 27). One species (*Larrea tridentata*) inhabits in almost all the hot desert areas of North America (4). In South America, four other species (*Larrea ameghinoi* Speg.*, Larrea cuneifolia* Cav.*, Larrea divaricata* Cav. and *Larrea nitida* Cav.) are found along the Monte desert and the arid lands from Chaco and Patagonia, all of them being different biogeographic provinces of Argentina (14, 27). They are also found in arid areas of Chile, Bolivia, and Perú (14).

Germination studies on some *Larrea* species have demonstrated the presence of a high percentage of dormant mature seeds (8, 30). Arid zones are characterized by a high percentage (> 80%) of shrub species that produce dormant mature seeds, mainly with physiological and physical dormancy (6, 26). According to Nikolaeva's (1977) simplified version, dormancy can be classified in physiological (factors within the embryo, inhibit germination), morphological (underdeveloped embryo), morphophysiological (combination of both), physical (impermeable seed coat), chemical (inhibitors in seed coverings), mechanical (seed coverings restrict radicle growth) and combinational. Dormancy itself can be eliminated by a particular factor, or by the combination of some of them (*e.g.*, light, temperature, and/or specific compounds).

Some authors found out that *L. divaricata* seeds present a type of physical dormancy caused by an impermeable seed-coat. They found that an efficient way of breaking it is through mechanical scarification with fine sandpaper (8, 30). On the other hand, *L. tridentata* seeds apparently present water-soluble germination inhibitors on the seed-coat (19), and removal of the coat or rinsing with running water have proved to enhance germination (from 27% in the control to 35-44%) (3, 19, 29, 33). However, neither one of these authors assessed the factors that produce the type of dormancy present in these seeds.

In some cases, dormancy classification is poorly done given that the factors that cause it are not well evaluated. It has been observed that mechanical or chemical scarification may also promote germination of seeds with physiological dormancy. In such instances, dormancy disruption by scarification appears to be related to the weakening of the embryo covering layer, allowing the radicle to emerge from it (5). Hence, it turns necessary to assess water uptake, comparing imbibition in scarified *versus* non-scarified seeds, in order to evaluate if seeds have water-impermeable seed-coats and therefore, make a correct dormancy classification. On the other hand, the presence of water-soluble germination inhibitors in the seed coat is not proved by any mean in several cases (5, 40). Thus, testing their presence by making a seed extract (31, 40) and using it as a substratum on germination assays, could help determine the existence of a chemical dormancy.

For this study, we selected two species of the genus *Larrea* which inhabits contrasting zones of the Monte Desert, and with unstudied germination biology. On one hand, *L. cuneifolia*, which colonizes a widespread area, on the hotter and drier zones of the Monte Desert, and on the other hand, *L. nitida*, which is present in a more restricted range of colder zones, along watercourses, and linked with winter-type rains of Pacific origin (14, 38). Temperature is another critical abiotic factor that regulates seed germination and has important effects on dormancy and on the rate of germination of non-dormant seeds (7). Mean temperature expected for arid zone species is around 25°C (6, 34), and in general, germination temperature range is in accordance with the temperatures that are most favorable for seedling establishment and survival, but this could vary along their distribution area (12, 34, 39). According to the habitats where these two species grow, we could expect some differences in their germination temperature range and in the rate of the germination process.

The Monte biogeographic province is located in the western portion of Argentina, covering approximately 460.000 km^2. It is an arid region with water deficit almost all year and an average annual rainfall ranging from 30 to 350 mm. Its mean temperature ranges between 13 and 18°C (27, 37). These areas present moderate to severe degree of native ecosystem degradation (1), and restoration activities are challenging due to hard environmental conditions. *Larrea* species play a significant role in these ecosystems, besides being shelter and food for many small mammals and reptiles (10), they act as nurse plants for the establishment of other species (16, 36) and are keystone species of desert ecosystems (17, 23, 36). They consolidate the soil, given its wide and extensive radical system, forming extensive pure shrublands called "jarillales" (35). Currently, the lack of

information about seed germination and dormancy characteristics of these shrubs hampers to a large extent the production of saplings to restore degraded ecosystems in Argentina. Classifying seed dormancy of key species is a critical step in seedling production for restoration projects since it provides insights into suitable seed handling practices that promote germination (13, 20).

Considering their widespread distribution and their dominance in dry environments, to incorporate *Larrea* species into restoration programs of degraded areas, is a priority. Therefore, this study examined seed dormancy and germination requirements of two species of *Larrea* in order to efficiently produce a high amount of seedlings to restore arid regions of Argentina. Specifically, the hypotheses tested were

1- Seeds of *L. nitida* collected from cooler sites would have a greater germination rate at lower temperatures and a lower optimum temperature than *L. cuneifolia*.

2- According to the dormancy studies on other *Larrea* species, a physical dormancy imposed by their water-impermeable seed coat or a chemical dormancy due to the presence of water-soluble germination inhibitors in their seed coat may be present in these species. Based on this, we aim to determine the optimum germination temperatures of *L. cuneifolia* and *L. nitida* seeds, to analyze the response of two pre-germination treatments (scarification and rinsing with water), and to determine the type of seed dormancy these species have.

Material and Methods

Seed collection

Mature fruits of each species were collected from at least ten healthy adult shrubs: *L. cuneifolia* on March 2008 at the experimental field of IADIZA (Instituto Argentino de Investigaciones de las Zonas Áridas), Parque General San Martín, Mendoza (32°53'43" S; 68°52'32" W) and *L. nitida* in December 2007 in San Antonio Oeste, Río Negro (41°19'38" S; 65°22'8" W). They were stored in paper bags and kept at room temperature (20-25°C) until experiments were performed in July 2008. The spherical hairy fruits are formed by five mericarps, each one typically containing one seed. To obtain the seeds, fruits of both species were rubbed between two rubber sheets, and only well-formed seeds were used in the assays. The number of empty mericarps, number of well-formed seeds and weight of 1000 seeds are shown in table 1 (measure of four replicates of 50 fruits and seeds, respectively). Half of the mericarps collected were empty (sterile) and a high number of well-formed seeds were observed (table 1).

Germination experiment

Germination assays were performed in incubators (Precision G. C. A. Corporation, Scientific Model 818), and in laboratory ovens (Dalvo, Model M. C/I/2) using 9 cm diameter Petri dishes prepared with cotton and a filter paper disk.

Table 1. Percentage of well-formed seeds, sterile fruits and weight of 1000 seeds of *L. cuneifolia* and *L. nitida* used in the assays (S.E. between parentheses).

Tabla 1. Porcentaje de semillas bien formadas, frutos estériles y peso de 1000 semillas de *L. cuneifolia* y *L. nitida* utilizadas en los ensayos (E E. entre paréntesis).

Species	Sterile fruits (%)	Well-formed seeds (%)	Weight (g)
L. cuneifolia	55 (14.4)	96 (4.3)	4.09 (0.09)
L. nitida	49 (6.9)	95 (2)	3.32 (0.06)

This substratum was moistened to saturation with captan solution (commercial products) at 0.1% (w/v) to prevent fungal attack. The filter paper was remoistened with distilled water as necessary. In every test, four replicates of 25 seeds were randomly drawn from the total seed pool for each species and assigned to a treatment. Before germination treatments, seeds were surface sterilized in 15% sodium hypochlorite (36.8 g/l NaOCl) for 7 min, then rinsed three times with sterilized deionized water, and finally placed in Petri dishes. Experiments were conducted under constant (24 h) dark conditions.

Seeds were considered as germinated when the radicle reached more than 2 mm in length. The number of germinated seeds was registered daily and the germination capacity defined as the germination percentage cumulated over 15 days. Seeds viability was assessed with tetrazolium. In this test, imbibed seeds were cut in half and soaked in 0.5% tetrazolium chloride over 24 h at 30°C. Seeds with red embryos were considered viable and with white embryos, non-viable.

Temperatures

To determine the optimum germination temperatures of both species seeds were incubated in the dark, at constant temperatures of 10, 15, 25, 35 and 40°C. In preliminary assays, a high number of seeds under dormancy were observed, so a scarification treatment was done on both species to evaluate the effect of the different temperatures.

Besides the germination capacity, the Weighted Germination Percentage (WGP) was also measured in order to assess the rate of germination. This was calculated by giving maximum weight to the seeds that germinated first and progressively less weight to those that germinated subsequently (32), for a time of fifteen days: WGP= (15 x n1 + 14 x n2 + …+ 1 x n15)/ (15xN) x 100. Where: n1, n2…, n15 = is the number of seeds germinated on 1st, 2nd, and subsequent days until the 15th day, respectively; 15, 14 ... and 1 are the weights given to the seeds germinated on 1st, 2nd, and subsequent days until the 15th day, respectively. N is the total number of seeds placed for germination.

Pre-germination treatments

For both species, we applied two pre-germination treatments following successful treatments applied to other species from the same genus: mechanical scarification and rinsing with running water. For mechanical scarification (MS) seeds were placed on fine sandpaper (N° 150) and gently rubbed one by one. And for rinsing, seeds were placed in small bags of fine gauze under running water during 24, 48 and 72 h (W24, W48, W72 respectively). After these treatments, seeds were placed in Petri dishes at 25°C.

Inhibitory activity test

To analyze if there were some soluble inhibitors on the seed coat, we performed a test using "alelí" seeds (*Mathiola incana*) (Sivouri EM 1964, not published). To extract the possible inhibitors 3 g of *Larrea* entire seeds were placed in glass beakers with 30 ml of distilled water (1:10 relation), one for each species. It was kept at 30°C in a laboratory oven for 72 h and periodically stirred with a glass rod. They were drained on a sieve over a funnel to collect the extract in a suitable container. This extract was used to moist the substratum (cotton and a paper filter disk) on the Petri dishes where alelí seeds were placed. As the osmotic potential of the extract was low (-0,02 MPa), it was

not necessary to make dilutions. A control with distilled water was also prepared. Both treatments were put under 25°C.

The inhibitor activity was obtained by assigning to the number of alelí seeds that germinated in the control (after 15 days) the relative value of 100, expressing on this basis the germination in the treatments with the extract.

Imbibition of scarified seeds

Imbibition of mechanically scarified seeds was compared with that of non-scarified seeds, to evaluate permeability of *L. cuneifolia* and *L. nitida* seeds after scarification treatments. For each species, three replicates of 100 scarified and non-scarified seeds were weighed and submerged in distilled water in Petri dishes at 20°C. After 72 h of imbibition seeds were removed, patted dry with a paper towel to absorb surface moisture and reweighed. Percent water absorption was determined gravimetrically. The amount of water taken up was determined by the increase in seed weight:

$$W(\%) = (wi-wd)/wd*100$$

where:
wi and wd = weights of imbibed and dry seeds, respectively.

Statistical analysis

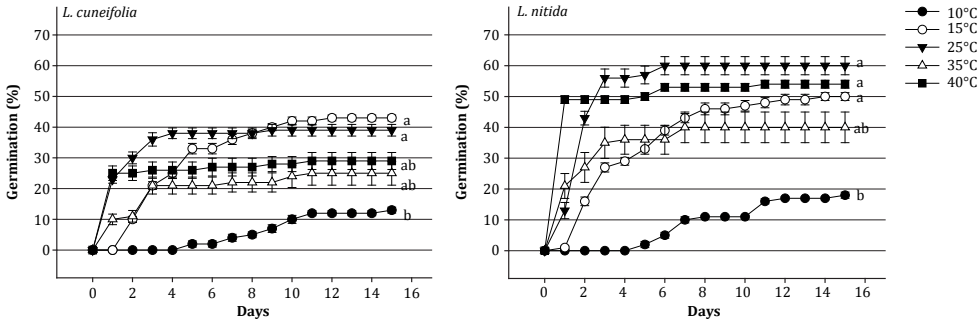
Germination data (germination percentage and WGP) was subjected to a two-way analysis of variance (ANOVA) with species and temperature as factors. Germination (in pre-germination treatments), germination of Alelí seeds and water imbibition were subjected to a one-way ANOVA with treatments, incubation medium and scarification treatments as factors, respectively. Germination percentages were transformed (arcsine square root) before the analysis in order to meet analysis assumptions. If significant differences were detected by ANOVA, Tukey's test was used for means comparisons. Statistical analysis was performed with Infostat 2013. Untransformed data appears in all figures and tables.

Results

L. nitida seeds exhibited a higher germination capacity than did those of *L. cuneifolia,* and both species germinated in a wide range of temperatures (figure1, page241).In*L.nitida*,theaveragemaximum germination capacity was 60% at 25°C, but it did not differ significantly from percentages reached at 15, 35, and 40°C (F = 9.53, P = 0.0005, figure 1, page 241). In *L. cuneifolia*, the maximum was 42% at 15°C and neither did it differ significantly from 25, 35 and 40 °C (F = 6.13, P = 0.004, figure 1, page 241).

At 10°C, seeds of both species attained lower germination percentages, and the process started later (5-6 days after the initiation of the assays) than over the other temperatures (1-2 days). Also, they reached two-thirds of the final germination percentage, over a wide range of temperatures (15-40°C), on the second day (figure 1, page 241). The interaction between species and temperatures was not significant (F=1.15; P= 0.352).

Weight germination percentage index (WGP) of both species at 10°C (6.1 and 9 for *L. cuneifolia* and *L. nitida*, respectively), was significantly lower than for the other temperatures (F = 9.9, p = 0.0004 for *L. cuneifolia* and F = 14.8, p < 0,0001 for *L. nitida*), so the germination process was slower. At the other temperatures WGP differences were not statistically significant.



Plots followed by the same letter are not significantly different at $p < 0.05$.

Las líneas seguidas por la misma letra no son significativamente diferentes para $p < 0,05$.

Figure 1. Time course of germination (mean ± SE) for *L. cuneifolia* and *L. nitida* seeds at different temperatures.

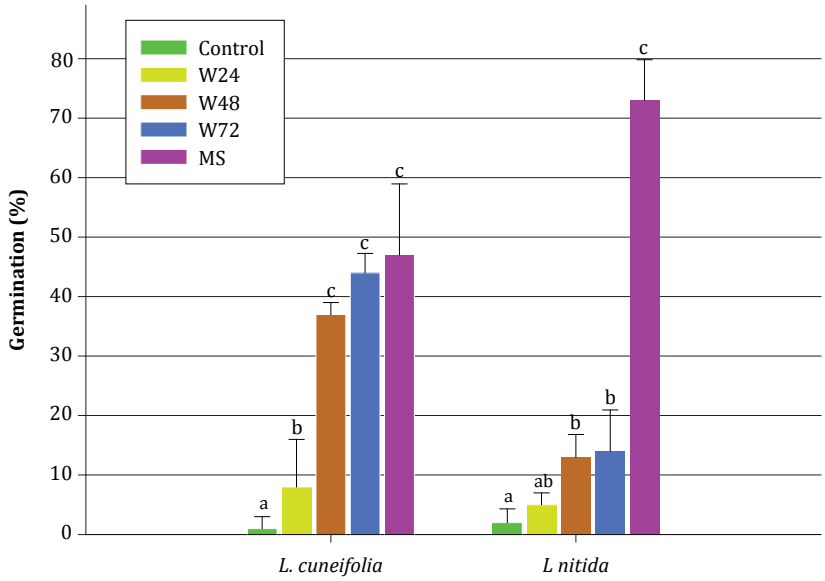
Figura 1. Evolución temporal de la germinación (media ± EE) de semillas de *L. cuneifolia* y *L. nitida* a diferentes temperaturas.

With respect to the pre-germination treatment, seeds of both species under control conditions germinated less than 2% (figure 2, page 242). Since seed viability was high (99% and 100% for *L. cuneifolia* and *L. nitida* respectively), almost all seeds were under dormancy. Dormancy release treatment by mechanical scarification enhanced final germination percentage of both species (figure 2, page 242), and higher values were obtained on *L. nitida* (figure 2, page 242). In *L. cuneifolia* seeds, mechanical scarification and rinsing for 48 and 72 h had the same effect on germination improvement (figure 2, page 242). Besides, with W48 and W72 germination processes was completed faster than with MS (1 day), since seeds were already imbibed in water and almost all of them (approximately 90%) germinated during the rinsing.

The inhibitory activity of *L. cuneifolia* seeds extract significantly reduced the germination percentage of alelí seeds with respect to the control (table 2, page 242). Whereas with *L. nitida* extract this difference was not significant. Scarified seeds absorbed similar water percentages than non-scarified seeds in both species (table 3, page 243), and *L. nitida* absorbed less water than *L. cuneifolia*.

Discussion

Since *Larrea* species are well adapted to arid environments, dormancy mechanisms allow a distribution of germination in time and space, ensuring that the environmental conditions are the most suitable for it to be completed (13, 15, 26). *L. cuneifolia* and *L. nitida* seeds presented dormancy mechanisms since almost all seeds did not germinate under control conditions, as mentioned for other species from the genus *Larrea* (3, 8, 30). Responses of both species to pre-germination treatments were different between them. Scarification promoted germination in both cases and rinsing with running water (W48 and W72) only promoted germination in *L. cuneifolia*.



Dissimilar letters for each species indicate significant differences (p<0.05) among treatments.

Letras distintas para cada especie indican diferencias significativas (p<0,05) entre los tratamientos.

Figure 2. Germination (mean ± SE) for seeds of *L. cuneifolia* and *L. nitida* subjected to mechanical scarification (MS), rinsing with running water for 24, 48 and 72 h (W24, W48 and W72 respectively) and under control conditions (Control) at the end of the assay (15 days).

Figura 2. Germinación (media ± EE) de semillas de *L. cuneifolia* y *L. nitida* sometidas a escarificación mecánica (MS), lavado con agua corriente durante 24, 48 y 72 h (W24, W48 y W72 respectivamente) y en condiciones control (Control) al final del ensayo (15 días).

Table 2. Mean germination of Alelí seeds (incubated at 25°C) under distilled water (control) and *L. cuneifolia* and *L. nitida* seeds extract at the end of the assay (15 days) and the ANOVA results.

Tabla 2. Germinación media de semillas de Alelí en agua destilada (control) y en extracto de semillas de *L. cuneifolia* y *L. nitida* al final del ensayo (15 días) y los resultados del ANOVA.

Species	Incubation Medium	Alelí seeds germination (%)	ANOVA results
L. cuneifolia	Control	81 (9.5) a	F=12.23 p=0.0027
	Seeds extract	39 (18.3) b	
L. nitida	Control	81 (9.5) a	F=3.94 p=0.0589
	Seeds extract	66 (9.5) a	

Dissimilar letters indicate significant differences (p<0.05) among incubation medium within each species (SE between parentheses).

Letras distintas indican diferencias significativas (p<0,05) entre los medios de incubación dentro de cada especie (EE entre paréntesis).

Table 3. Water imbibition (W) in scarified and non-scarified seeds of both species for 72 h, and their ANOVA results.

Tabla 3. Absorción de agua (W %) de semillas escarificadas y no escarificadas de ambas especies durante 72 h, y los resultados del ANOVA correspondiente.

Species	Scarification treatment	W (%)	ANOVA results
L. cuneifolia	Non-scarified seeds	59 (5.61) a	F=1.38 p=0.3048
	Scarified seeds	64 (4.75) a	
L. nitida	Non-scarified seeds	34 (3.68) a	F=4.59 p=0.0989
	Scarified seeds	39 (1.41) a	

Dissimilar letters indicate significant differences ($p<0.05$) among scarification treatments within each species (SE between parentheses).

Letras distintas indican diferencias significativas ($p<0,05$) entre los tratamientos de escarificado dentro de cada especie (EE entre paréntesis).

Both species presented water-permeable seed coats, ruling out the occurrence of a physical dormancy. On the other hand, *L. cuneifolia* may have a chemical dormancy induced by the presence of water-soluble germination inhibitors. Besides, both species presented a wide range of germination temperatures, and there were no major differences between them in their optimum temperatures, as expected.

Temperature requirements of these two *Larrea* spp. were estimated through the different tested temperatures, where high percentages were observed around 25°C, a mean temperature expected for arid zones species (6, 34). The temperature at which seeds reach the higher germination percentage in the shortest time is called optimum germination temperature (12). In this case, both species presented a range of optimum temperatures between 15-40°C (27-42% for *L. cuneifolia* and 40-60% for *L. nitida*). Seed dispersal of native shrubs from the Monte Desert occurs mainly in summer, and seedlings emergence in the next spring-summer, when rainfall season starts (24, 25), which is in accordance with the temperatures found for these *Larrea* species. Ostler *et al*. (2002), found in *L. tridentata* a greater germination percentage with soil temperatures of 23 °C during April, and its germination limits were between 10 and 40°C (3).

Since low germination percentages (2%) were observed in these species under control conditions, seeds were mechanically scarified before incubation at different temperatures. Consequently, the environmental range of conditions for the seeds to germinate becomes wider (2, 7) providing a possible explanation of the broad set of optimum temperatures observed in these species.

On the other hand, as *L. nitida* colonizes the colder parts of the Andes, in a more restricted range than *L. cuneifolia*, it was expected that a lower optimum temperature or a narrower temperature range than the ones found here, could be observed. These outcomes differ from the ones previously found on some *Prosopis* species that inhabit the Monte Desert, in which a higher germination capacity at low temperatures was observed in the species that present a more southerly distribution (12, 39).

A positive response to all dormancy-break treatments was observed in *L. cuneifolia*. *Larrea tridentata* from North America also presents similar responses to these treatments (3, 29, 33). The inhibitory test was positive since half of the alelí seeds did not germinate under *L. cuneifolia* seed extract, and the water uptake analysis showed that this species does not have water-impermeable seed coat. Therefore, it may be concluded that this species may have a type of chemical dormancy induced by water-soluble inhibitors present on the seed coat. The arid climates are characterized by scarce unpredictable rainfall, generally concentrated. After such rains, massive germination of plants may occur. This mechanism, observed in arid zones species, is the product of water-soluble germination inhibitors present in the seed coat. Gentle rains do not remove these inhibitors, so germination does not occur until the rains are more intense (22). Xylem-ring counts in *L. tridentata* seedlings showed that past germination and establishment occurred in response to heavy late-summer rainfall (9).

Despite these observations, chemical dormancy is not considered as such in many cases since there is not much evidence stating that seed dormancy, in nature, is regulated by the presence of inorganic compounds/ions in seed covering layers (5). Looking at the studies that test inhibitor activity of extracts (40), there is no possibility of knowing if the inhibitor came from the embryo and/or seed parts, nor if it would prevent embryo growth of non-dormant seeds of the species from which it was extracted. Besides, in many cases, species that present germination inhibitors also present a physiological dormancy. It has been observed that after this dormancy is broken, the embryo becomes insensitive to the inhibitors (6, 18). Therefore, the chemical dormancy is being considered part of the physiological dormancy in the newest classification system (6). Seeds of *L. tridentata* under rinsing treatments showed similar germination percentages (3, 29, 33), but when leachates from their fruit coats were tested on their own seeds, it did not inhibit their germination as it did in other species (21).

On the other hand, *L. nitida* showed a higher response to mechanical scarification, but no significant differences were found in the water uptake measurements between scarified and non-scarified seeds. Therefore, this species does not present a physical dormancy caused by an impermeable seed coat, nor a chemical dormancy produced by germination inhibitors. The positive response to scarification could be related to a mechanical restriction of the seed coats, but this type of dormancy is actually under discussion since in many cases seeds also present physiological dormancy and after dormancy releases, the embryo has enough vigor to break seed covering layers (6). Another possibility may be a non-deep physiological dormancy, since as mentioned before, many species with this type of dormancy may increase germination with scarification, despite not having impermeable seed coats (5). Besides, physiological dormancy is one of the most common types in species from arid zones (6, 11). A study in *L. divaricata* detected higher germination percentages in seeds with mechanical scarification (88%), while soaking in gibberellic acid (GA_3) displayed similar results. The combination of both treatments resulted the most effective (30). This could also be the case of *L. nitida*. These species may have a physiological dormancy rather than a physical type. Therefore, it is necessary to continue studying in order to clearly define the type of dormancy of this species.

Conclusions

Larrea cuneifolia and *L. nitda* seeds presented dormancy mechanisms and scarification treatment promoted germination in both species, while rinsing with running water only promoted in *L. cuneifolia*. Both species presented water-permeable seed coats, ruling out the occurrence of a physical dormancy. On the other hand, *L. cuneifolia* may have a chemical dormancy induced by the presence of water-soluble germination inhibitors. Besides, both species presented a wide range of germination temperatures, and there were no major differences between them in their optimum temperatures, as expected.

The production of seedlings of *L. cuneifolia* and *L. nitida* for restoration programs implies a great effort and the knowledge of harvest times, quantity of sterile fruits, quality of seeds, dormancy level and pre-germination treatments. Our results allow us to better understand seed germination biology of this two *Larrea* species. Although we could not define the type of seed dormancy, and more research should be done in the future, this information is valuable and directly contributes to improving the efficiency of seed use and the production of a high amount of seedlings required for restoration projects.

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Long-term fertilization with dairy cattle slurry in intensive production systems: effects on soil porosity and pore morphology

Fertilización a largo plazo con purín de vaca lechera: efectos sobre la porosidad del suelo y la morfología de poros

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Abstract

In Mediterranean environments, livestock effluents might improve soil physical properties. The study was located in an intensive crop production system of northwest Spain. After nine consecutive years of dairy cattle slurry (DCS) use as fertilizer, the aim of the experiment was to evaluate the impacts of DCS on soil porosity and pore shape. Soil texture was loam. The applied DCS rates were equivalent to 170 and 250 kg N ha^{-1} (170DCS and 250DCS, respectively) and they were complemented with mineral N up to 450 kg N ha^{-1} (two crops). A nonfertilized control was included. Digital binary images were obtained from soil thin sections. Pores with an apparent diameter (AD) >30 μm were analysed. The 250DCS treatment improved soil porosity (>30 μm): it doubled in comparison with the 170DCS and the control. The application of DCS favored the presence of pores with an AD >400 μm, the roughness for AD >100 μm and the elongation in the AD interval of 100-200 μm. From the study, the 250DCS treatment is recommended as it increases macroporosity (compaction reduction) and produces more elongated and tortuous pores, which will be a constraint for fast drainage but it will be advantageous in coarse textured soils.

Keywords
double-cropping • organic fertilizer • soil pore shapes • micromorphology of pores

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Resumen

En zonas Mediterráneas del noroeste español bajo producción intensiva, la aplicación de deyecciones ganaderas podría mejorar las propiedades físicas del suelo. Tras nueve años de fertilización con purín de vacas lecheras (DCS), se planteó evaluar los efectos del DCS sobre la porosidad edáfica y la morfología de los poros. La clase de familia textural era franca gruesa. Las dos dosis de DCS evaluadas equivalían a 170 y 250 kg N ha^{-1} (170DCS y 250DCS, respectivamente) y se complementaban con N mineral hasta 450 kg N ha^{-1} (dos cultivos). Se incluyó un control, sin fertilización. A partir de láminas delgadas de suelo se obtuvieron imágenes digitales binarizadas. Se analizaron los poros de diámetro aparente (AD) >30 µm. El tratamiento 250DCS duplicó la porosidad (>30 µm) respecto a 170DCS y el control. La aplicación de DCS favoreció la presencia de AD >400 µm, la rugosidad para AD >100 µm y la elongación de poros en el rango 100–200 µm. La dosis de 250DCS es recomendable al incrementar la macroporosidad (implica una disminución de la compactación) y favorecer unas formas de poros más alargadas, con mayor tortuosidad, que dificultarían el drenaje rápido, lo cual es beneficioso en suelos con contenido importante de arena.

Palabras clave
doble cultivo • fertilizantes orgánicos • forma de poros edáficos • micromorfología de poros

Introduction

The intensification of forage production by means of double-annual cropping, is linked with a dynamic agricultural system that enhances the circular economy, since the plant biomass harvested becomes livestock feed, and the animal excrements are used to cover the nutrient demands of the forage crops (14, 16, 34).

In the European Union areas which have been designated as vulnerable to contamination of subsurface waters by nitrates, the application of N from organic sources is limited to 170 kg N ha^{-1} $year^{-1}$ (Directive 91/676/EEC) (9), although the rates applied can be complemented with mineral N. This regulation covers the N demand of many annual crops. However, in a double-annual cropping system, the application of 250 kg N ha^{-1} $year^{-1}$ from livestock excrements (manures and slurries) is an alternative to maintain high forage yields while saving expenses on additional mineral N (3, 32, 33).

Furthermore, organic fertilizers introduce organic matter (OM) into the soil, which is especially needed in Mediterranean climates where soils are characterized by their low levels of OM (17).

In the European Union, the Common Agricultural Policy includes "conditionality" in order to maintain the potential productivity of land resources and prevent soil degradation (10).

Conditionally is a set of legally defined good agricultural and environmental practices that farmers, who receive direct (or for rural development) subsidies, are obliged to implement. These

requirements include the maintenance of soil OM, an objective which is significantly covered by the use of livestock waste in fertilization schedules.

The addition of OM affects soil physical properties such as porosity (23). In addition, porosity affects soil ecosystem services (1). Thus, porosity becomes an indicator of the sustainability of a specific agricultural management method.

The characteristics of the soil porous system have a direct relationship: (i) with the retention and movement of water and gases (18), (ii) with biological and chemical properties (31), (iii) with erosion risks and sediment transport, including contaminants (36), and (iv) with the development of the plant root system (28). In addition, the soil porous system is sensitive, mainly through macroporosity (30, 45), to changes induced by agricultural activities such as tillage, irrigation or fertilization. Nevertheless, the assessment of impacts on porosity requires knowledge of how it is distributed (range of apparent diameters (AD) and their shape).

The use of micromorphological techniques allows the study of macroporosity (>60 μm) in soil thin sections (19) and even the mesoporosity which, in this study, is associated with AD between 30 and 60 μm.

The double-annual forage cropping system increases machinery traffic over soil in comparison with a single annual crop system. Harvesting implies the carrying off of a substantial weight of fresh plant material, which is maximized when two cuts per crop are done (*e.g.* ryegrass).

The traffic over the soil surface can cause compaction (6, 35) and a decrease of soil porosity. In these intensive Mediterranean systems, there is only limited information available about the effect of organic fertilizers on macroporosity, since research has been mainly focused on cereal agricultural systems (5, 44) or on annual rotations (8) where pig slurry and cattle manure are used as fertilizers.

The hypothesis of this research is that the application of dairy cattle slurry (DCS) influences soil physical fertility. The study is carried out in the framework of a long-term experiment (2008-2016), under a dry Mediterranean climate. The main objective was to assess the effects of DCS, applied at different rates, on soil porosity and pore morphology.

Materials and methods

A fertilization experiment was established in 2008 in the Tallada d'Empordà, Girona, NE of Spain (altitude 18 m a. s. l., 42°03'02" N, 03°03'37" E) and was maintained for nine years (until 2016). The current study was conducted at the end of the 2016 cropping season.

Soil and climatic characteristics

The soil was classified as Oxiaquic Xerofluvent (42) and the family particle-size class was coarse-loamy. The surface soil horizon (0-0.30 m) had a loam texture (485 g sand kg^{-1}, 405 g silt kg^{-1} and 110 g clay kg^{-1}), a pH (potentiometry, water 1:2,5; w:v) of 8.2, an electrical conductivity (1:5, 25°C, w:v) of 0.18 dS m^{-1}, an organic matter content (Walkley-Black method) of 18 g kg^{-1} and 140 g kg^{-1} of equivalent calcium carbonate (Bernard's calcimeter method).

The area has a dry Mediterranean climate according to Papadakis classification (20). During the 2008-2016 period, the average annual temperature was 14.8°C, with a maximum daily average of 23.4°C registered in July, and a minimum daily average of 7.9°C registered in January.

The average annual precipitation and evapotranspiration (2) in the period 2008-2016 were 608 and 986 mm, respectively.

Experimental context

During the 2008-2013 period, a double-annual crop forage rotation: maize (*Zea mays* L.)-ryegrass (*Lolium multiflorum* L.) was established. Forage maize was sown in spring (May) and it was harvested in autumn (September) at the doughy grain stage. Ryegrass was sown in autumn (September) and it was harvested at maximum biomass before coming into ears, first harvest was done in March and last harvest in May (38).

In the 2013-2016 period, after the maize harvest, the rotation was modified and it included: rapeseed (*Brassica napus* L.) -grain maize (short cycle)-grain maize (long cycle)-rapeseed, which means four crops in three years. Rapeseed was sown in autumn (September) and it was harvested in June. Grain maize was sown in spring: (June, short cycle; April, long cycle) and it was harvested in October. Maize was irrigated (sprinkler system) during the spring-summer period and the rest of the crops were not irrigated. Stubble of rapeseed and maize (grain) was incorporated into the soil but the rest (stalks) was removed. Main tillage before sowing was done with a mouldboard or a disc harrow (~0.25 m deep).

The fertilization trial was designed as a randomized block, with three treatments and three repetitions per treatment (blocks). The plot surface area was 40 m^2 (5 m x 8 m).

The treatments were: one control (without fertilizer) and two rates of DCS. The rates (± standard deviation) of DCS were 52 (± 10) and 77 (± 14) m^3 ha^{-1}. These rates contributed to 170 and 250 kg N ha^{-1}, and they were distributed between two successive crops, prior to spring and autumn sowings (170DCS: 100 and 70 kg N ha^{-1}; 250DCS: 150 and 100 kg N ha^{-1}, respectively). The treatments with DCM were complemented in each rotation of two crops, with mineral N as topdressing (calcium ammonium nitrate, 27%) up to a total of 450 kg N ha^{-1} for spring and autumn sowings (170DCS: 200 and 80 kg N ha^{-1}; 250DCS: 150 and 50 kg N ha^{-1}, respectively).

The exception was the 2014-2015 cropping season where a summer crop (maize) was followed by a summer crop (maize) but the fertilization schedule for summer crops was maintained.

Previous to each application, a DCS sample was analysed. For the 2003-2016 period, the mean values (n = 15) of the DCS evaluated parameters were: pH of 8.4 (± 0.5) by potentiometry (1:5, soil:water), electrical conductivity (1: 5, soil: water, at 25°C) 4.6 (± 2.7) dS m^{-1}, dry matter of 8.7% (± 2.9) over fresh matter (by gravimetry at 105°C) and 75.7% (± 5.0) of OM over dry matter (by ignition at 550°C). Thus, during the 2008-2016 period, prior soil samplings, the 170DCS and 250 DCS plots have received an average amount of 25.4 Mg OM (dry) ha^{-1} and 37.6 Mg OM (dry) ha^{-1}, respectively.

There were no significant differences between yields associated with the evaluated DCS treatments (data not shown) and yields in the control were 36% lower (as an average). In DCS treatments, the average forage yield as ryegrass (two cuts/cycle) and maize were 10 and 21 Mg dry matter ha^{-1} (drying at 65°C), respectively; the grain yield average of rapeseed was of 2 Mg ha^{-1} (9% moisture content) and the grain yield average of maize was 10 and 15 Mg ha^{-1} (14% moisture content) for the short and long cycles, respectively.

Soil sampling and soil porosity analysis: pore-size distribution and shape

In June 2016 (~ eight months after the last DCS application), after the winter crop (rapeseed) harvest, rectangular blocks (depth 0.06 m, 0.09 m wide and 0.20 m long) of undisturbed soil samples (n = 9) were obtained from each treatment.

The blocks were dried at room temperature and impregnated with polyester resin with fluorescent dye (Uvitex ©). One vertical thin section (0.05 m wide, 0.13 m long) was made from each block according to the procedures described by Benyarku and Stoops (2005). From each thin section, two images (42 mm long x 31.5 mm wide) were obtained under two light scenarios: parallel polarized and crossed polarized. Cross polarized images were processed with the Olympus Stream program (26) to obtain digital binary images. These images were used to analyse total porosity, which included pores with an apparent diameter (AD) >30 µm (the minimum threshold for the established procedure). From each image, the analysis of pore-size distribution was based on an open mathematical algorithm: the Quantim4 library program (43).

The area occupied by pores was divided into five intervals according to the pores' AD: 30-60 µm, 60-100 µm, 100-200 µm, 200-400 µm and >400 µm. The pore shape, when its AD was >60 µm, was analysed by means of four descriptors according to Ferreira and Rasband (2012): circularity, aspect ratio (AR), roundness (R) and solidity (S).

The circularity [4π*(pore area)/(pore perimeter)^2] indicates a perfect circle when it reaches the value of 1. The AR or the ratio of the ellipse adjusted to the pore (major axis/minor axis) is an index that describes the pore elongation, so that at higher values, the pores are more elongated. The roundness [4*(pore area)/(π*(major axis)^2)] indicates whether the pore has rounded (leading to 1) or angled (leading to 0) edges. The solidity (area of the pore/convex area of the pore) assesses the roughness, irregularity and tortuosity of the pore walls (high values correspond to smooth pores and low ones to rough pores).

Statistical analysis

Statistical analyses were performed using the SAS program (33). The analysis of variance (ANOVA) was carried out after verifying the assumptions of normality and homogeneity of variances. Porosity percentages between 200-400 µm and >400 µm, were normalized by the square root transformation and for the range 30-60 µm they could not be normalized.

The solidity data in the 60-100 µm range could also not to be normalized. When the analysis detected significant differences, the means were compared by the Least Significant Difference (LSD) test, at the 0.05 probability level.

Results

The different fertilizer treatments had a significant influence on the porous area (pores with AD >30 µm) and on their distribution in the different ranges (table 1, page 253). The porosity in 250DCS (25.6%) significantly increased (nearly doubled) when comparing with the control and 170DCS (table 2, page 253). Differences were maintained in three different AD ranges of pores: 60-100 µm, 100-200 µm and 200-400 µm (table 2, page 253).

Table 1. Analysis of variance (DF= degrees of freedom, MS= mean squares, *P*= probability value) of the porosity (>30 µm) and their pore apparent diameter (AD) distribution (%) according to the fertilization treatments (TR).

Tabla 1. Análisis de varianza (DF= grados de libertad, MS= cuadrados medios, *P*= valor de probabilidad) de la porosidad (>30 µm) y distribución (%) del diámetro aparente (AD) de poros[a] en distintos rangos para los tratamientos (TR) de fertilización.

Pore-size (AD)[a]		**>30 µm**		**60-100 µm**		**100-200 µm**	
Source	DF	MS	*P*	MS	*P*	MS	*P*
Between TR	2	311.91	<0.0001	5.22	0.0003	35.31	0.0002
Between blocks	2	55.77	0.002	0.28	0.37	5.66	0.07
Between samples of TR	3	20.85	0.03	0.25	0.43	1.11	0.59
Within samples (residual)	10	4.64		0.25		1.64	

Pore-size (AD)[a]		**200-400 µm**		**>400 µm**	
Source	DF	MS	*P*	MS	*P*
Between TR	2	1.88	<0.0001	2.25	<0.0001
Between blocks	2	0.56	0.004	0.36	0.04
Between samples of TR	3	0.06	0.44	0.43	0.02
Within samples (residual)	10	0.06		0.08	

[a] The percentage pores with an AD between 30-60 µm could not be normalized.

[a] No se pudo normalizar el porcentaje poros de AD entre 30-60 µm.

Table 2. Average porosity (>30 µm) values (%) [a] (n=6) and its distribution in different pore apparent diameter ranges [b] for each fertilization treatment [c].

Tabla 2. Valores medios [a] (n=6) de porosidad (>30 µm) y de su distribución (%) en diferentes rangos de diámetros aparentes de poros [b] para cada tratamiento de fertilización [c].

Treatments	**Porosity (%)**	**Porosity distribution (%)**				
	>30 µm	**30-60 µm**	**60-100 µm**	**100-200 µm**	**200-400 µm**	**>400 µm**
Control	12.2 B	1.67	3.29 B	4.65 B	2.14 (1.40)B	0.44 (0.60)C
170DCS	14.1 B	2.29	3.65 B	4.06 B	2.26 (1.47)B	2.16 (1.42)B
250DCS	25.6 A	2.78	5.05 A	8.53 A	5.85 (2.40)A	3.43 (1.80)A

[a] Numbers in brackets are $x^{0.5}$ transformed values. Mean values in columns followed by different letters are significantly different according to the LSD test at 0.05 probability level.

[b] The 30-60 µm diameter interval does not fit the normal assumption for ANOVA.

[c] Treatments: control (without N) and dairy cattle slurry fertilization (DCS) where numbers indicate the N applied rates of 170 or 250 kg ha^{-1} $year^{-1}$.

[a] Los números entre paréntesis son los valores medios transformados mediante la raíz cuadrada. Los valores medios en las columnas seguidos de diferentes letras son significativamente diferentes en base a la mínima diferencia significativa.

[b] No se pudo normalizar el porcentaje de los poros con diámetro aparente entre 30-60 µm.

[c] Tratamientos: control (sin aporte de N) y fertilización con purín bovino de leche (DCS) donde los números asociados indican las dosis de N aplicado 170 o 250 kg N ha^{-1} $año^{-1}$.

The control and 170DCS only significantly differed in the range of AD of pores greater than 400 µm, where 170DCS had a higher percentage (table 2, page 253). These porosity differences between treatments were also qualitatively observed in the images from thin soil sections (figure 1, page 255).

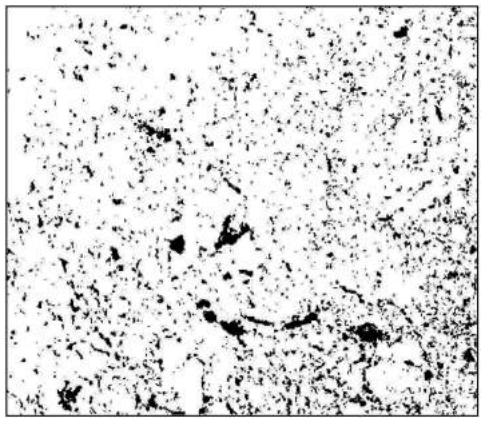
The evaluated pore shape descriptors showed significant differences (table 3).

Table 3. Analysis of variance (DF= degrees of freedom, MS= mean square, *P*= probability value) of different shape descriptors for each fertilization treatment (TR) and for the different ranges of pore apparent diameters (AD).
Tabla 3. Análisis de varianza (DF= grados de libertad. MS= cuadrados medios. *P*= valor de probabilidad) para los diferentes descriptores de forma de los poros para los diferentes rangos de diámetro aparente (AD) de poros y tratamiento de fertilización (TR).

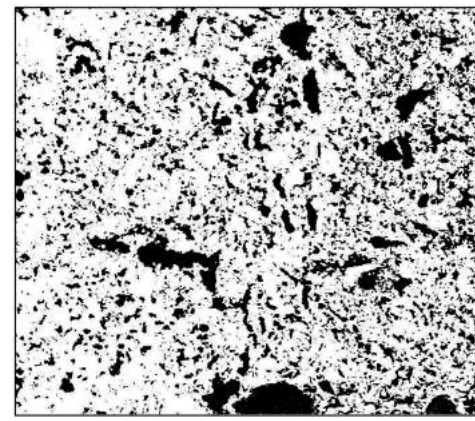
Pore-size (AD)	Shape pore descriptors		Circularity		Solidity[a]		Aspect ratio		Roundness	
	Source	DF	MS	*P*	MS	*P*	MS	*P*	MS	*P*
60-100 µm	Between TR	2	0.001	0.07	-	-	0.026	0.05	0.004	0.04
	Between blocks	2	$1.2E^{-4}$	0.75	-	-	0.002	0.77	$2.4E^{-4}$	0.79
	Between samples of TR	3	$4.0E^{-4}$	0.42	-	-	0.004	0.62	$5.4E^{-4}$	0.66
	Within samples (residual)	10	$4.0E^{-4}$		-		0.007		$9.9E^{-4}$	
100-200 µm	Between TR	2	0.004	0.05	$5.4E^{-4}$	0.02	0.032	0.01	0.003	0.02
	Between blocks	2	$1.0E^{-4}$	0.89	$5.6E^{-6}$	0.94	$5.1E^{-4}$	0.90	$5.6E^{-6}$	0.99
	Between samples of TR	3	$4.1E^{-4}$	0.70	$9.4E^{-5}$	0.42	0.004	0.50	$3.0E^{-4}$	0.61
	Within samples (residual)	10	$8.7E^{-4}$		$9.2E^{-5}$		0.005		$4.7E^{-4}$	
200-400 µm	Between TR	2	0.007	0.006	0.003	0.008	0.013	0.21	$5.4E^{-4}$	0.22
	Between blocks	2	$1.4E^{-4}$	0.84	$7.2E^{-5}$	0.81	0.009	0.34	$2.7E^{-4}$	0.44
	Between samples of TR	3	$7.7E^{-4}$	0.45	$2.3E^{-4}$	0.59	0.002	0.86	$7.2E^{-5}$	0.87
	Within samples (residual)	10	$8.1E^{-4}$		3.4^{-4}		0.007		$3.1E^{-4}$	
>400 µm	Between TR	2	0.005	0.002	0.004	0.003	0.029	0.07	$6.5E^{-4}$	0.07
	Between blocks	2	$7.1E^{-4}$	0.26	$9.3E^{-4}$	0.12	0.013	0.26	$4.2E^{-4}$	0.16
	Between samples of TR	3	$2.7E^{-4}$	0.63	$1.4E^{-4}$	0.76	0.002	0.86	$1.9E^{-4}$	0.44
	Within samples (residual)	10	$4.6E^{-4}$		$3.5E^{-4}$		0.009		$1.9E^{-4}$	

[a] The solidity values for pores with an AD between 60-100 µm have not been normalized.
[a] No se pudo normalizar los valores de solidez de los poros de AD entre 60-100 µm.

Control



250DCS



170DCS

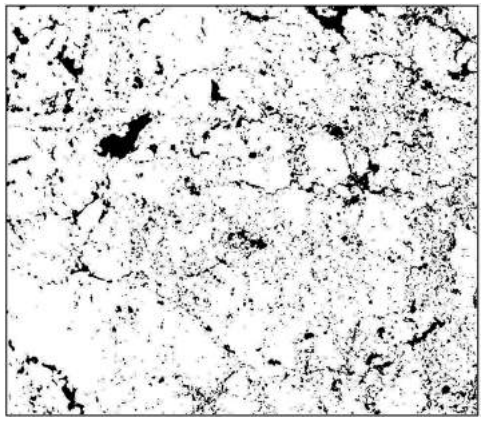


Figure 1. Porosity images (black color) from thin sections, vertically orientated from the soil surface, and for the different fertilizer treatments: Control (without slurry addition), dairy cattle slurry at a rate of 170 kg N ha^{-1} (170DCS) or 250 kg N ha^{-1} (250DCS). Image size was 42.0 mm long and 31.5 mm width.

Figura 1. Imágenes de porosidad del suelo (en negro) obtenidas a partir de láminas orientadas verticalmente desde la superficie del mismo y asociadas a diferentes tratamientos de fertilización: control (sin aporte de purín) y con aporte de purín de vaca lechera (DCS) aplicado a dosis de 170 kg N ha^{-1} (170DCS) o 250 kg N ha^{-1} (250DCS). El tamaño de cada imagen es de 42,0 mm de largo y 31,5 mm de ancho.

The application of DCS led to decreases in the solidity in pores with an AD greater than 100 µm; the circularity was also reduced but only above AD of 200 µm, while the roundness decreased in the pore range from 60 to 200 µm (table 4, page 256).

Finally, when DCS was applied, the aspect ratio (AR) increased in the AD pore range of 100-200 µm (table 4, page 256).

Table 4. Average values [a] of the pore shape descriptors for each fertilization treatment [b] and for the different ranges of apparent pore diameters.

Tabla 4. Medias [a] de los descriptores de la forma de los poros para los diferentes rangos de diámetro aparente (AD) de poros y para cada tratamiento de fertilización [b].

Pore-size (AD)/ Treatments	Shape pore descriptors			
60-100 µm	**Circularity**	**Solidity**	**Aspect ratio**	**Roundness**
Control	0.938	0.875	1.440	0.742A
170DCS	0.910	0.865	1.565	0.690B
250DCS	0.917	0.871	1.540	0.702AB
100-200 µm				
Control	0.797	0.860A	1.807B	0.618A
170DCS	0.752	0.842B	1.945A	0.577B
250DCS	0.758	0.847B	1.915A	0.587B
200-400 µm				
Control	0.595A	0.793A	2.053	0.548
170DCS	0.530B	0.751B	2.014	0.530
250DCS	0.542B	0.762B	2.072	0.543
>400 µm				
Control	0.348A	0.662A	2.243	0.512
170DCS	0.300B	0.617B	2.221	0.517
250DCS	0.293B	0.620B	2.113	0.532

[a] Mean values in columns followed by different letters are significantly different according to the LSD test at 0.05 probability level.

[b] Treatments: control (without N) and dairy cattle slurry fertilization (DCS); where numbers indicate the N applied rates of 170 or 250 kg N ha^{-1} $year^{-1}$.

[a] Valores medios en las columnas seguidos de diferentes letras son significativamente diferentes a un nivel de probabilidad de 0,05 con base a la prueba de mínima diferencia significativa.

[b] Tratamientos: control (sin aporte de N) y fertilización con purín bovino de leche (DCS) donde los números asociados indican las dosis de N aplicado, 170 o 250 kg N ha^{-1} $año^{-1}$.

Discussion

The significant increase of the porous area (>30 µm) in the 250DCS treatment (49% higher than 170DCS and the control, table 2, page 253) agree with the findings of Pagliai and Antisari (1993) about the increase in soil porosity percentage associated with high doses of pig slurry applications (300 m^3 ha^{-1}) in a maize crop. Mellek *et al.* (2010) when applying DCS (180 m^3 ha^{-1} $year^{-1}$) in a double-annual cropping system, attributed these porosity changes to the direct effects of the OM incorporation, among which were the improvement of the aggregation and the decrease of the soil bulk density, and their indirect effects among which were a higher root density that, after decomposition, increased the porous space.

The OM soil addition from slurries is low when compared with other solid organic wastes (8, 21), so the 170DCS did not supply enough OM to make an

improvement in soil porosity detectable. However, a porosity increase (>30 μm) was detected at the higher dose of 250DCS (table 2, page 253). This increase is advantageous for the soil-water-plant relationship, and for the maintenance of a good soil structure. In addition, in soil superficial layers (first 0.05 m), it could reduce superficial compaction (27, 37) and assure the different soil services such as gaseous exchange (1).

Our results emphasize that the pore range between 60 and 400 μm is sensitive to the fertilizer type and rate applied, and therefore this range could be introduced as an indicator of physical soil quality.

In addition, at the 250DCS rate, the porosity increase was distributed among different ranges, which enhances the different functionality of pores (41), both in water storage (30-60 and 60-100 μm, table 2, page 253), and in the water and air fluxes, as a guarantee of their improved supply to roots and microorganisms (12, 41).

Finally, the increment in the pore range between 100 and 400 μm (table 2, page 253) is important to avoid any constraint in root development and growth (25, 41), since the root hairs usually have diameters between 0.1 and 0.3 mm.

The porosity linked to AD >400 μm increases gradually, from the control, with the rate of DCS applied (table 2, page 253).

In a broad sense, these pores usually result from soil fauna activity, mainly earthworm activity (40, 41).

The incorporation of organic fertilizers such as DCS stimulates microbial activity (7, 15, 45) and increases macrofauna which changes the soil surface layer, increasing the presence of large pores (11, 18). Thus, it results in structural improvement. In contrast, its decrease, as shown in the control (>400 μm, table 2, page 253), can be related to compaction problems (18) and lower soil faunal activity. The large pores (>400 μm) in the 250DCS treatment do not correspond to cracks, which are commonly associated with low levels of soil OM contributions (5).

Changes in pore shapes were observed, regardless of the DCS applied rate (table 4, page 256). Dairy cattle slurry increased the elongation (AR, 100-200 μm, Circularity, 200-400 μm and >400 μm, table 4, page 256), angularity and roughness (R, 100-200 μm; S, 200-400 μm and >400 μm, table 4, page 256) of pores. These changes could result in an increase in soil water holding capacity, leading to better water management efficiency, as well as facilitating the soil penetration by roots (15, 22, 27, 44). This fact is of particular relevance in intensive systems, with a substantial machinery traffic because that annually doubles or triples when compared with a single annual crop. Also, when the soil is wet, the elongated pores with rough and irregular walls are more difficult to seal than the circular and smooth ones. Thus, in the face of activities favouring disaggregation (transit of machinery, impact of raindrops or irrigation), they would not be totally closed, *i.e.* the circulation of fluids would not be compromised (24, 29).

Our results support the option of considering, in intensive systems, the application of 250 kg N ha^{-1} (split into winter and summer crops) of organic origin (through contributions of DCS) to improve soil physical fertility. Our findings reinforce the positive evaluation of this rate, from the agronomic point of view, reported by other authors (32, 33) in similar agricultural systems.

Conclusions

In a coarse loam soil, when an intensive production system is introduced, the DCS rate equivalent to 250 kg N ha^{-1} (split into winter and summer crops) increased porosity (AD greater than 30 µm) up to 26%. This figure almost doubled with respect to the one (14%) found at the lower rate of 170 kg N ha^{-1}; the latter did not differ from the control. Slurries, independently of the rate and compared with the control, mainly increased the presence of pores with an AD greater than 400 µm. This fact acts as a warning of the potential existence of compaction problems when OM is not applied. In addition, changes in the pore shape by DCS (roughness and elongation increases) would favour water retention, which is important in winter rainfed crops. These results confirm that in a double cropping programme, the application of DCS at an equivalent rate of to 250 kg N ha^{-1} could prevent compaction (increases macroporosity) despite the intensity of machinery traffic, while it helps to slow down the potential drainage of infiltrated water by increasing the tortuosity of the pores.

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Performance of grape marc and organic residues compost as substrate in lettuce (*Lactuca sativa*) seedlings

Eficiencia del compost de orujo de uva y residuos orgánicos como sustrato en plantines de lechuga (*Lactuca sativa*)

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Abstract

Composting is an aerobic process used to treat organic residues, which results in a high quality product, able to be adopted as plant substrate or soil amendment. In the present study, the performance of compost on the germination and biomass of *Lactuca sativa* var Grand rapids seedlings, with and without fertilization, was evaluated. The two types of composts used were prepared from two different raw materials: grape marc and a mixture of grape marc, goat manure, leaves and alfalfa. The experiment was carried out in seedling trays, in a split plot design with two factors (fertilization and substrate) and four repetitions. Sand were used as control and a commercial substrate as traditional treatment. Results indicated that fertilization had not significant effect on germination, but increased seedling biomass. Both compost also increased lettuce biomass, with the highest values obtained in mixture compost treatments. Only sand produced the lowest germination values, while no differences were detected among the other substrates. Compost mixture showed the highest seedling biomass, suggesting a higher quality as a plant substrate. It is necessary to perform further analyses and studies with different organic residues in order to determine physico-chemical and biological properties to evaluate the quality of the product obtained.

Keywords
composting • goat manure • split plot • germination index

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Resumen

El compostaje permite reducir y reciclar residuos orgánicos, generando un producto apto para ser utilizado como sustrato para el crecimiento de plantines florales y hortícolas o como enmienda de suelos. En el presente trabajo, se evaluó el efecto del compost elaborado a partir de orujo de uva agotado y de una mezcla de orujo, guano de cabra, hojas y alfalfa, sobre la germinación y biomasa de plantines de *Lactuca sativa* var Grand rapids, con y sin fertilización. Se trabajó en bandejas de siembra con un diseño de parcelas divididas con dos factores (sustrato y fertilización), utilizando, arena como control y un sustrato comercial como tratamiento tradicional. Los resultados indicaron que la fertilización no tuvo efecto significativo sobre la germinación, obteniéndose los menores valores con la arena, sin encontrarse diferencias entre el resto de los sustratos. Por otro lado, ambos tipos de compost produjeron mayor biomasa que el sustrato comercial y la arena, efecto que se incrementó con la fertilización. El compost mezcla fue el sustrato que produjo el mayor crecimiento de plantines de lechuga, lo que indicaría una mayor calidad para ser utilizado como sustrato. Es necesario continuar con estudios para determinar las propiedades fisicoquímicas y biológicas del compost que permitan evaluar la calidad de producto obtenido.

Palabras clave
compostaje • guano de cabra • parcelas divididas • índice de germinación

Introduction

In central western Argentina, viticulture is one of the most important agricultural activities, with a production of 11.8 million hL of wine in 2017 in Cuyo region (www.inv.gov.ar). However, as a result of this activity, around 500 t of solid effluent are generated every 23,000 hL year^{-1} of wine produced, consisting mainly of stalks, marc, flock and sludge (12).

The composting process makes it possible to reduce and stabilize organic waste and it is even possible to degrade some contaminants such as pesticides, herbicides and chemicals into less toxic substances (16), while eliminating pathogens in the thermophilic stage (14). It is an aerobic biological process by which biodegradable organic waste is transformed into a homogeneous rich in nutrients material, which can be used as plant substrate and soil amendment (6, 9, 11). There are different large-scale processing systems but the most commonly used at the farm level are those of piles or rows, static or aerated. In the latter case performing periodic turning manually or by means of machinery, obtaining a stabilized product with suitable physicochemical and biological properties make it useful as a soil amendment and as a substrate.

Composting is an interesting and useful proposal for recycling a large volume of organic waste generated by the distillery activity. Carmona *et al.* (2012) indicated that compost obtained from this waste can be used, under a correct irrigation and fertilization management, for plant seedling productions. The objective of this work was to evaluate the

performance of the compost obtained from spent grape marc and a mixture of spent grape marc, goat manure, leaves and alfalfa, with and without fertilization, as substrate for lettuce germination and seedling production.

Materials and methods

Compost was made in aerated piles of approximately 3 m^3 (2 m length, 1.8 m width, and 0.8 high), with no cover, periodically turned every 30 days.

Two raw materials were used: spent grape marc (skins, seeds, stalks, stems and stalks from grape), and a mixture of spent grape marc, goat manure, leaves and alfalfa, in a volume proportion 1:1:3:0.5 respectively.

The piles were located in Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (EEA Mendoza-INTA, 33°00'38" S, 68° 50'59" W at 921 m a. s. l.).

Under greenhouse condition (T≈ 22°C), 55 x 28 cm sowing trays, with 171 speedling cells, one *Lactuca sativa* var Grand rapids seed was placed in each cell at a depth of 2 mm and they were periodically irrigated every 48 h.

Eight treatments with four replicates were performed in a randomly distributed split plots design.

Two factors were evaluated: fertilization as a major plot, with two levels: fertilized (+F) and no fertilized (-F), and substrate as a minor plot, with four levels: sand (S), commercial substrate (K, Kekkilä® DSM 1, Professional, Finland,), grape marc compost (GMC), and mixture compost (MC, table 1). Sand was used as a control substrate and the commercial substrate as a traditional treatment.

In the +F treatments, 5 mL per plot of substrate of a 0.25% nutrient solution (KSC® 2 NPK 23-5-5-5, Timac Agro USA) were applied weekly by spraying. In each plot, 28 and 10 units of measurement were utilized to evaluate germination and seedling production respectively.

Table 1. Physico-chemical properties of compost (1:5 v/v water suspension) and commercial substrate (Kekkilä®) used in germination and seedling production assay.

Tabla 1. Propiedades fisicoquímicas del compost (suspensión en agua 1:5 v/v) y del sustrato comercial (Kekkilä®) utilizado para los ensayos de germinación y producción de plantines.

Compost	pH	EC	C:N	NH_4^+	NO_3^-	ρ_{app}	PA
		(dS m^{-1})		(mg kg^{-1})		kg L^{-1}	%
Kekkilä®	5.9	0.2	-	-	-	0.09	90
GMC	6.50 ± 0.16	1.16 ± 0.33	17.91 ± 0.16	34,89 ± 11.67	5.22 ± 1.74	0.36 ± 0.01	29.41 ± 2.35
MC	7.79 ± 0.10	1.23 ± 0.03	14.42 ± 0.82	52.36 ± 42.67	9.71 ± 2.73	0.38 ± 0.00	31.72 ± 0.64

Physico-chemical properties of compost (1:5 v/v water suspension) and commercial substrate (Kekkilä®) used in germination and seedling production assay. EC: electrical conductivity; ρ_{app}: apparent density; PA: porous with air; GMC: grape marc compost; MC: mixture compost.

Propiedades fisicoquímicas del compost (suspensión en agua 1:5 v/v) y del sustrato comercial (Kekkilä®) utilizado para los ensayos de germinación y producción de plantines. EC: conductividad eléctrica; ρ_{app}: densidad aparente; PA: poros con aire; GMC: Compost de orujo de uva; MC: compost mezcla.

Germination was evaluated determining Total Germination (TG), Medium Time Germination (MTG, 3) and Emergency Rate Index (13):

$$MTG = \frac{\sum D * n}{\sum n}$$

where:
n = number of germinated seeds on a day D
D = number of days recorded since the beginning of germination

$$ERI = \sum \mathrm{Xn\ (c\text{-}n)} / \mathrm{N}$$

where:
Xn = number of germinated seeds counted on day n
c = number of days from sowing to emergency end
n = day on which the emergency begins expressed as number of days after sowing
N = total number of germinated seeds.

To evaluate seedling biomass production in each treatment, total dry weight (DW, at 70°C for 48 hours) of 60 days old plants was determined for each treatment.

The effect of the factors on germination seedling biomass was evaluated by ANOVA with split plot model and LSD Fisher mean comparison test ($p \leq 0.01$). In order to determine differences among treatments, beside standard error for media estimation, a single standard error of ANOVA (SE_{ANOVA}) for each variable analyzed was considered. For this purpose, InfoStat software version 2015 was used (InfoStat Group, FCA, National University of Córdoba, Argentina).

Results

Effect of compost on germination

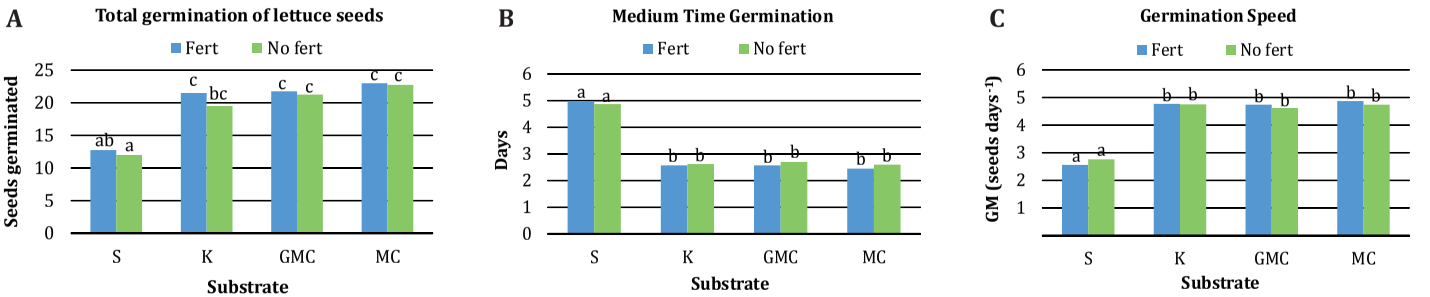
Compost had a positive effect on the germination of lettuce plants, In both GMC and MC, TG of seeds was significantly higher than S, and no different from K (figure 1A, page 265). MC reached the highest values of 23.00 ± 0.91 and 22.75 ± 0.95, GMC of 21.75 ± 0.48 and 21.25 ±2.17, K of 21.50 ± 2.10 and 19.50 ± 0.50, and S of 12.75 ±1.89 and 12.00 ±2.35 germinated seeds, respectively for +F and -F (SE_{ANOVA}: ± 2.25).

Although with +F treatments a greater quantity of germinated seeds was obtained, there were no significant differences with respect to those -F. These results suggest that the factor that most influenced the germination was the substrate, with no significant effect of fertilization ($p \leq 0.01$).

The MTG was determined mainly by the substrate, with no interactions and no significant effect of fertilization (figure 1B, page 265).

Compost and commercial substrate treatments (MC: 2.57 ± 0.06 and 2.70 ± 0.11; GMC: 2.45 ± 0.19 and 2.60 ± 0.07; K: 2.57 ± 0.15 and 2.62 ± 0.07 days, respectively for + F and -F) showed significantly lower values than S (4.98 ± 0.27 and 4.87 ± 0.58 days, SE_{ANOVA}: ± 0.29).

In ERI, results were opposite to those observed in the germination time. MC, GMC and K (4.74 ± 0.36 and 4.62 ± 0.50; 4.87 ± 0.19 and 4.74 ± 0.10; 4.77 ± 0.18 and 4.75 ± 0.09 seeds germinated per day, respectively for +F and -F) presented significantly higher values with respect to S (2.56 ±0.36 and 2.76 ± 0.50 seeds germinated per day, SE_{ANOVA}: ± 0.31, figure 1C, page 265).



Graph A: Total germination of lettuce seeds; graph B: Medium time germination expressed as days from sowing based on Bewley and Black (1986); graph C: Emergency Rate Index, expressed as number of seeds germinated per day (Shmueli and Goldberg 1971). Split plot ANOVA was performed, with LSD Fischer comparison ($p<0.01$).

A: Germinación total de semillas de lechuga; B: Tiempo Medio de Germinación, expresados en días desde la siembra (Bewley and Black 1986); C: índice de la Tasa de Emergencia, expresado como número de semillas germinadas por día (Shmueli and Goldberg 1971). Se realizó un ANOVA con un modelo de parcelas divididas y comparación LSD Fischer ($p<0,01$).

Figure 1. Germination and plant emergency of *Lactuca sativa* var Grand rapids seeds in different substrates: Sand (S), Commercial substrate (K), Grape Marc Compost (GMC), Mixture Compost (MC: grape marc, goat manure, leaves and alfalfa).

Figura 1. Germinación de semillas de *Lactuca sativa* var Grand rapids en diferentes sustratos: Arena (S), Sustrato comercial (K), Compost de orujo de uva (GMC), Compost mezcla de residuos (MC: orujo de uva, guano de cabra, hojas y alfalfa).

Effect of compost on plant biomass

The dry weight of lettuce seedlings was significantly affected by the interaction substrate*fertilization.

The MC+F treatment (101.93 ± 2.81 mg) was significantly superior to the rest of the treatments, followed by GMC + F and MC-F (88.80 ± 2.16 and 82.83 ± 2.50 mg respectively), which were not differentiated from each other, and by GMC-F and K+F that showed similar values (68.58 ± 2.86 and 61.58 ± 2.86 mg respectively, SE_{ANOVA}: ± 2.1).

In general and in each substrate, the average values were higher in + F than - F treatments, being the seedlings belonging to both types of compost the ones that presented higher biomass (figure 2).

Discussion

The results indicated that the compost could be used as a substrate for growing lettuce seedlings in sowing trays. Both compost exhibited higher germination values than sand and similar to commercial substrate, and lettuce biomass was also higher in compost treatments, with higher values in mixture.

According to Beltrán Santoyo *et al.* (2017), Bernal *et al.* (2009) and Moral *et al.* (2009), the use of different types of waste, especially manures, would enrich and improve the biological and physicochemical properties of compost, obtaining a higher quality product to be used as a substrate or soil amendment.

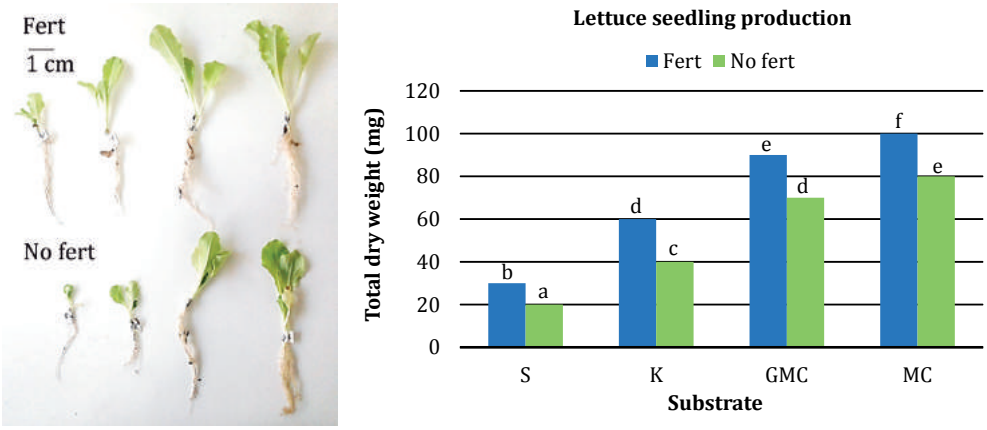


Figure 2. *Lactuca sativa* var Grand rapids seedlings production in different substrates: Sand (S), Commercial substrate (K), Grape Marc Compost (GMC), Mixture Compost (MC: grape marc, goat manure, leaves and alfalfa). Split plot ANOVA was performed, with LSD Fischer comparison ($p<0.01$).

Figura 2. Producción de plantines de *Lactuca sativa* var Grand rapids en diferentes sustratos: Arena (S), Sustrato comercial (K), Compost de orujo de uva (GMC), Compost mezcla de residuos (MC: orujo de uva, guano de cabra, hojas, y alfalfa). Se realizó un ANOVA con un modelo de parcelas divididas y comparación LSD Fischer ($p<0,01$).

In general, fertilization produced an increase in plant biomass but had no effect on the germination of lettuce plants. This suggested that the use of fertilizers is not necessary during germination, where the seed would mainly use its own reserves, but would have a positive effect on growth and development stages.

However, it is important to note that both unfertilized compost had higher biomass values than commercial substrate and sand with fertilization, and the compost mix was significantly higher. This could be given by the nutritional contribution of the compost from the different raw materials, being sufficient for an optimal plant development.

Baran *et al.* (2001) indicated that grape marc compost should be blended with other substrate for optimal results, and in agree with that it was founded that composting with other residues produce a higher quality product.

Composting process allows treating and reducing large volumes of organic waste.

In the case of the wine industry, a stable, nutritious and sanitized product can be obtained from spent grape marc, with excellent properties to be used as a substrate for potted plants (7, 17).

The application of this methodology on a large scale would generate environmental and economic benefits for the industry, through waste treatment and emission reduction, and for producers and nurseries, by accessing a product with superior properties in comparison with commercial, more expensive substrates and fertilizers (13).

Based on the residues used, to evaluate physicochemical and biological characteristics of compost, becomes necessary studying composting process alternatives, determining the content and availability of micro and macronutrients, and the microflora that benefits plant growth.

Finally, and according to Luo *et al.* (2018), it is important to determine the quality of compost that would be used as a commercial substrate or soil amendment, evaluating different proportions, with different plant species and production systems.

Conclusion

Composting is a waste treatment alternative that generates a suitable product to be used as substrate for germination and growth of lettuce plants.

Mixture of different organic waste, especially goat manure, would generate a product of higher nutritional quality that would not require additional fertilization for crop production.

Both, GMC and MC, presented adequate characteristic for lettuce plants growing, surpassing the commercial substrate, offering the producer a cheaper and more efficient alternative for the production of different vegetable seedlings.

Composting is an adequate alternative to reduce and treat industrial organic residues.

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Effect of traffic with a light-weight tractor on physical properties of an Aridisol soil in Almeria, Spain

Efecto del tráfico con un tractor liviano sobre las propiedades físicas de un suelo Aridisol en Almería, España

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Abstract

The objective of this work was to evaluate the effect of repeated traffic with a light-weight tractor on the physical/mechanical properties of an Aridisol soil from eastern Almería (Spain). The soil has been used for almond (*Prunus amigdalus* L.) production for the past 29 years. A light modal tractor (≈15 kN overall load) and different traffic frequencies or treatments; namely, 0 (control, no traffic), and 1, 5, 7, and 10 passes, respectively, were used. The following variables were measured: cone Index (CI); bulk density (BD); total soil porosity (TSP); water infiltration into soil (I), and ruth depth (RD). The results showed that, only treatments 7 and 10 led to significant increases in CI and BD throughout the soil profile (0-450 mm). Changes in TSP in those treatments were consistent with changes in soil bulk density. No significant differences in RD were found when the tractor passed 1 or 5 times. All traffic treatments resulted in significant compaction in the topsoil layer (0-150 mm) and soil physical conditions that would be regarded as unsuitable for establishment of most arable crops.

Keywords
soil carrying capacity • cone index • axle load

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Resumen

Nuestro principal objetivo fue evaluar los efectos sobre las propiedades físicas del suelo causados por el tráfico de un tractor que es de uso común en el este de la provincia de Almería, España. El trabajo se realizó sobre suelo Aridisol, en el distrito de Vélez Blanco, en el sureste de España. El suelo se ha utilizado para la producción de almendras (*Prunus amigdalus* L.) durante los últimos 29 años. Se utilizó un tractor modal con diferentes frecuencias o tratamientos de tráfico: 0 (sin tráfico), 1, 5, 7 y 10 pasadas, respectivamente. Las variables medidas fueron las siguientes: (1) índice de cono (CI); (2) densidad aparente (BD); (3) porosidad total del suelo (TSP); (4) infiltración del suelo (I) y profundidad de huella (RD). Los resultados mostraron que, solo 7 y 10 pasadas del tractor produjeron aumentos significativos en los valores de CI y BD en el perfil medido del suelo (0 - 450 mm). Además, este trabajo ha demostrado que la compactación del suelo por el tráfico de un tractor liviano (≈15 kN) disminuye la TSP en todo el perfil del suelo estudiado. No se encontraron diferencias significativas en RD cuando el tractor pasó 1 o 5 veces. Todos los tratamientos de tráfico compactaron el horizonte superficial del suelo (0-150 mm) generando condiciones físicas o mecánicas que no serían adecuadas para el establecimiento del cultivo.

Palabras clave
capacidad portante del suelo • índice de cono • peso en el eje

Introduction

Traffic with heavy equipment can cause severe soil compaction, particularly, when field operations are repeatedly conducted on the same track (8, 9). Botta *et al.* (2002) identified two aspects of compaction that require investigation: (i) topsoil compaction within the cultivated (Ap) horizon, and (ii) subsoil compaction. Subsoil compaction includes any plough pan that develops in the upper part of the subsoil as a result of tillage, that is, at the interface of the Ap horizon. Such compaction, may be only alleviated by subsoiling, and in some climates or soil types may be also assisted by natural processes such as drying-wetting, shrinking-swelling, and freezing-thawing cycles, and also by macrofauna activity, and root growth. However, these processes may not be effective in some cropping systems subject to relatively high field traffic intensities.

Traffic-induced compaction in the subsoil tends to be cumulative as standard tillage operations are rarely performed at depths greater than about 25-30 cm. Only subsoiling operations are capable of solving this problem, but in some soils the effects of deep tillage are transient. Maximum subsoiling depth attainable by conventional equipment is approximately 600 mm; however, mechanical loosening of soil to alleviate deep compaction can prove difficult, expensive and therefore not practical at depths greater than about 40 cm (12).

For most crops, root growth and development are severily affected by compaction; thereby, affecting water and nutrient uptake. A study in the eastern Pampas region of Argentina (4) showed

that high clay content soils compacted to CI higher than about 2200 kPa and bulk densities of 1.7 Mg/m^3 or higher at a depth of 400-600 mm reduced peach (*Prunus persica* L.) yields by about 30%. Threadgill (1982) notes that soils with a CI >2000 kPa reduced crop yields, and at >1500 kPa, there was reduced root growth in groves. Iancu *et al.* (1996) demonstrated that bulk density increased by 1.7%, soil penetration resistance increased by 15% and saturated hydraulic conductivity (HC) decreased by 31% with four different treatments with the crop grown along a contour and on lower, middle and upper terraces for two types of soil (brown colluvial and slightly eroded) in apple groves at a soil depth of 0-1.0 m. Nuñez-Moreno and Valdez-Gascon (1994) showed that soil conditions affect yields and growing conditions in citric groves in a semiarid climate in northwestern Mexico. The mean orange yield was ca.162 kg/tree in the best area and 48 kg/tree in the worst. Comparison of soil data in the best and worst areas showed that in the worst soil, compaction was greater by 1500 kPa, the infiltration rate was lower, and there was an increase in silt content which reduced plant growth.

Tree cover crops in the Mediterranean basin (*e.g.*, olives, almonds, nuts, and grapevine) are grown in over 9 million hectares. These crops are important part of the Mediterranean diet and require relatively simple agricultural practices for their production (10). However, between 3 and 7 tractor passes per year are required for tillage and weed control, and such repeated traffic on same track can lead to severe subsoil compaction.

Objectives and hypothesis

Quantify the change in soil parameters of Aridisol soil due to light tractor traffic.

Enhance knowledge about the effects of agricultural tractor traffic on soil during common labours on same tracks in eastern Almería, Spain.

Our hypothesis was that: Topsoil and subsoil compaction produced by traffic with a light-weight tractor (≈15 kN) with low ground pressure tyres depends of the number of tractor passes.

Materials and methods

The site and crop operations

The experiment was conducted in the Vélez Blanco District of the Province of Almería in southeast Spain (37°41' N, 2° 5'W) at an altitude 828 m a. s. l. (semiarid climate). The soil is an Aridisol (18). The soil physical and mechanical properties are given in table 1 (page 273).

Treatments

Experiments were performed in a 29-year-old Marcona almond (*Prunus amigdalus* L.) orchard and on soil with cereal crops production. Almond plantation density: 6 x 6 m, 4 and 5 m tall with a trunk that is about 20 cm in diameter.

Five tractor traffics frequencies or treatments were imposed on 200 m long by 4 m wide (800 m^2) plots, where the experimental variable was 0 (control plot), 1, 5, 7 and 10 tractor passes, respectively, over the same track in three replications in completely randomized plots (7). The inter-row passes were made by one 2WD tractor equipped with single rear tyres. Description of the tractor used in the study and technical specifications are given in table 2 (page 273). Tractor speed during the experiment was 4.6 km/h with no hitch load. Before the traffic treatments were applied, plots were plowed once with a rotary tiller (3). This treatment represents a tillage system commonly used in the region.

Table 1. Soil profile characteristics of the Aridisol soil.
Tabla 1. Perfil típico del suelo Aridisol.

Depth (mm)	0 - 180	180 - 280	280 - 450	450 - 650	+ 650
	A1	B21t	B22t	B3	C
Soil Organic carbon (g kg^{-1})	8.1 ± 0.20	3.2 ± 0.2	4.0 ± 0.51	1.7 ± 0.50	1.8± 0.42
Total nitrogen (g kg^{-1})	1.28 ± 0.04	0.8 ± 0.02	0.9 ± 0.10	0.4 ± 0.02	0.5 ± 0.01
C/N ratio	6.32	4.00	4.44	4.25	3.60
Clay (<2 m) g kg^{-1}	143 ± 2.34	251 ± 2.36	237 ± 2.68	173 ± 1.87	135± 2.64
Silt (20-50m) g kg^{-1}	521 ± 3.30	525 ± 2.98	582 ± 3.12	691 ± 2.87	566± 1.91
Sand g kg^{-1}	336 ± 1.39	224 ± 1.87	181 ± 1.88	136 ± 0.96	299 ± 1.92
pH in H_20 (1: 2.5)	7.9 ± 0.03	8.1 ± 0.03	8.0± 0.02	8.3 ± 0.01	8.3 ± 0.05

Table 2. Description of the tractor and technical specifications.
Tabla 2. Descripción del tractor y especificaciones técnicas.

Tractor	2WD Tractor Design
Engine power (CV/kW)	47/34.4
Front tyres	650 - 16
Rear tyres	12.4 - 28
Inflation pressure, front tyre (kPa)	160
Inflation pressure, rear tyre (kPa)	95
Overall weight (kN)	15
Front weight (kN)	4.5
Rear weight (kN)	10.5
Mean ground pressure per for front tyre (kPa)	20.1
Mean ground pressure per rear tyre (kPa)	32.3

The tyre inflation pressure was within the range advised by the manufacturer for load and speed (Goodyear Agricultural Tyre Division, 2018, https://www.goodyear.com.au/tyres/tractor-and-agricultural).

La presión de inflado de los neumáticos estaba dentro del rango recomendado por el fabricante para la carga y la velocidad (Goodyear Agricultural Tire Division, 2018, https://www.goodyear.com.au/tyres/tractor-and-agricultural).

Statistical analyses were performed by the Statgraf program 7.1. An analysis of variance (ANOVA) was carried out (17), and means were analyzed by Duncan's multiple range test.

Parameters monitored

Cone index (CI), Bulk Density (BD), soil water content (SWC), Total porosity of soil (TSP), soil Infiltration (I), and rut depth (RD) were measured on the same day as the traffic treatments were applied. The parameters (CI, DB, SWC, TSP and I) were measured along the wheel tracks on the bottom of the RD in the trafficked plots (which was taken into account at data analysis) and were taken across the entire plot for the untrafficked control. The CI was measured with a mechanic penetrometer (2). Each datum is the average of 20 samples for each plot at the depth range of 0-450 mm. The procedure used to obtain the BD and SWC values, is described in Botta (2000). Total topsoil

porosity was calculated from BD using soil particle density. Infiltration (I) was determined using the ring infiltrometer method. Rings were 0.25 m in diameter and 0.4 m height and were inserted 0.20 m deep in the soil to prevent lateral seepage loss. The average infiltration was determined from 20 locations per plot. This value was computed only for the topsoil (0-200 mm) because crop root development and nutrient uptake are concentrated there. Rut depth (RD): A description of the procedure used to determine RD is included in Botta *et al.* (2018).

Results and discussion

Soil water content, Cone index and Bulk density

The SWC as determined on the day the traffic treatment were imposed was 14.7% (w/w) in the topsoil (0-150 mm), 15.5% (w/w) at 150-300 mm, and 30.1% (w/w) at 300-450 mm, and there was no significant difference in the SWC between the different depth intervals.

Therefore, variations in CI at depth were not due to SWC, which suggested that cone index was a reliable indicator of the degree of soil compaction as a function of the traffic treatment.

The value of CI for the control increased with an increase in soil depth, because of the natural increase in soil resistance originated from the weight of soil above the measured depth (table 3). Lateral forces on the penetrometer cone increase with increasing depth, therefore a higher force is needed for the cone to displace through the soil. Resistance can also increase with depth because of changes in soil texture, gravel content, structure and historic traffic compaction.

Table 3. Cone Index (kPa) measured at the tyre centerline, after 0, 1, 5, 7 and 10 passes of a tractor, respectively.

Tabla 3. Índice de Cono (kPa) medido en el centro de la huella después de 1, 5, 7 y 10 pasadas de tractor, respectivamente.

Depth (mm)	Control plot	1 pass	5 passes	7 passes	10 passes
Topsoil (0-150 mm)					
0	360 a	1355 b	1400 b	2012 c	2100 c
50	465 a	1378 b	1423 b	2020 c	2122 c
100	577 a	1412 b	1490 b	2091 c	2133 c
150	770 a	1500 b	1523 b	2100 c	2290 b
Subsoil (>150 mm)					
200	1522 a	1566 a	1600 a	2287 b	2333 b
250	1694 a	1700 a	1721 a	2442 b	2500 b
300	1710 a	1767 a	1777 a	2600 b	2689 b
350	1756 a	1789 a	1800 a	2850 b	2945 b
400	1787 a	1800 a	1816 a	3010 b	3100 b
450	1774 a	1810 a	1831 a	3201 b	3290 b

Values with different letters (horizontally) are significantly different at each depth ($P < 0.01$) Duncan's multiple range test.

Los valores con letras diferentes (horizontalmente) son significativamente diferentes para cada profundidad ($P < 0,01$ Prueba de rango múltiple de Duncan).

Without traffic, increases in CI (>1700 kPa) between 300 and 450 mm deep, are likely due to high clay content at that depth interval and historic machinery traffic. An effect of traffic at this depth was also observed in the 7 and 10 passes treatments where CI values were significantly greater than that of the control.

Traffic frequencies or treatments of 1 and 5 passes had greater CI than the control only in the topsoil (0-150 mm), as shown in table 3 (page 274). By contrast, for 7 and 10 passes treatments, this study shows that compaction caused significantly greater changes in all the soil properties measured compared with the control. Such changes in soil physical properties were observed in the entire soil profile (0-450 mm depth interval).

For these treatments (7 and 10 passes), CI values were higher than 2000 KPa and 3000 KPa in the topsoil and subsoil, respectively, which denote over-compaction. Also for these treatments, such CI values exceeded critical values of soil strength above which root growth and expansion are significantly affected (*e.g.*, 6, 8, 13, 15). For the 7 and 10 passes treatments, peak CI values were found in the subsoil (400 to 450 mm).

Soil bulk density data was consistent with observations of CI only for the 7 and 10 passes treatments (table 4), showing that BD increased significantly after traffic and also with an increase in soil depth. The before traffic condition (control) also showed relatively high bulk density values. Without traffic, bulk density at the surface exceeded the 1.2 Mg/m^3 threshold recommended by Ressia *et al.* (1998) at 200 mm depth. Bulk density was higher than 1.5 Mg/m^3 either with or without traffic between 300 and 450 mm.

Bulk density and CI always increased with the number of passes, but BD tended to be less responsive than CI, probably because of the relatively high pre-traffic density. Despite this, there was an overall increase in BD of about 33% on average relative to the control, and differences between the control and the 1 or 5 passes treatments were not significant ($P < 0.01$). The corresponding increase in CI was 197% on average. Differences between the control and the 7 and 10 passes treatments were significant at the 0-200 mm depth interval.

Table 4. Bulk density (Mg/m^3) values at three depth intervals under different degrees of tractor traffic (0, 1, 5, 7 and 10 passes of a light-weight 2WD tractor).

Tabla 4. Valores de densidad aparente (Mg/m^3) en tres rangos de profundidad bajo diferentes grados de tráfico del tractor (0, 1, 5, 7 y 10 pasadas de un tractor 2WD liviano).

Depth (mm)	Control plot	1 pass	5 passes	7 passes	10 passes
0 - 150	1.24 a	1.26 a	1.29 a	1.44 b	1.65 b
150 - 300	1.30 a	1.33 a	1.43 a	1.63 b	1.67 b
300 - 450	1.52 a	1.53 a	1.54 a	1.64 b	1.66 b

Values with different letters (horizontally) are significantly different at each depth ($P < 0.01$ Duncan's multiple range test).

Los valores con letras diferentes (horizontalmente) son significativamente diferentes para cada profundidad ($P < 0,01$ Prueba de rango múltiple de Duncan).

Rut depth (RD)

Examination of the soil response to traffic at relatively shallow depths revealed that rut depth increased as the tractor traffic increased (table 5). The topsoil is the layer most vulnerable to both soil compression and soil displacement from the passage of tractors. The greatest values of rut depth were measured when the tractor passed 7 and 10 times. The results of Duncan's multiple range test ($P<0.01$) showed significant differences between the rut depth for the four traffic treatments (table 5).

Soil disturbance near the surface increased with the number of tractor passes, but RD was never deeper than 100 mm in none of the treatments, however this was consistenly deeper for the 10 passes treatment. In addition, for the 10 and 7 passes treatments, there was a significant correlation between RD and soil compaction (R^2 values were between 0.89 and 0.93 for CI, and between 0.85 and 0.94 for BD, respectively, $P<0.01$) deeper in the profile (200-600 mm depth interval). For the 1 and 5 passes treatments, this correlation was not significant (R^2 values were between 0.003 and 0.05 for CI, and between 0.008 and 0.215 for BD, respectively, $P > 0.01$).

Water infiltration into soil

As shown in figure 1 (page 277), only the 7 and 10 passes treatments caused a statistically significant reduction in water infiltration into soil over the 0 to 200 mm depth profile compared with the control plot. This result agrees with those reported in earlier sobtained by numerous researchers (*e.g.*, 5, 7).

Total soil porosity of soil

Total porosity of soil (TSP) is an important indicator of compaction (table 6, page 277) as it relates to density properties. Differences in TSP between the 7 and 10 traffic treatments for the 0-450 mm depth interval were statistically significant ($P < 0.01$) compared with the control. The 10 passes treatment caused the greatest reduction in TSP, consistent with the high traffic intensity applied through that treatment. This response was more significant in the subsoil (from 300 to 450 mm deep). Total porosity values for this treatment were lower than about 40%, which is considered to be the limit TSP above which crop yield can be significantly affected. These results are in accord with those of Håkansson and Reader (1994) who showed that by light vehicles can cause significant subsoil compaction after repeated passes.

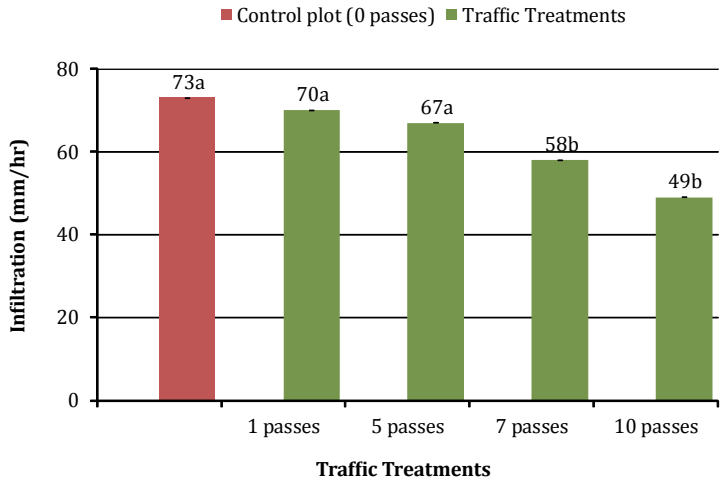
Table 5. Effect of traffic treatments on rut depth (mm).

Tabla 5. Efecto de los tratamientos de tráfico sobre la profundidad de huella (mm).

Traffic Treatments	Mean of rut depth (mm)
1 pass	37 a
5 passes	60 b
7 passes	87 c
10 passes	99 d

Different letters within each traffic treatments shows significant differences ($P < 0.01$ Duncan's multiple range test).

Diferentes letras dentro de cada tratamiento de tráfico muestran diferencias significativas ($P < 0,01$ prueba de rango múltiple de Duncan).



Bars with the same letter are not significantly different (P < 0.01) Duncan's multiple test.

Barras con las mismas letras indican que no hay diferencias significativas entre los tratamientos (P < 0,01) prueba de rango multiple de Duncan.

Figure 1. Average infiltration values (mm/h) in the 0 to 200 mm depth range for the five traffic treatments.

Figura 1. Valores medios de infiltración (mm/h) en el intervalo de 0 a 200 mm de profundidad para los 5 tratamientos de tráfico.

Table 6. Total porosity of soil (%) estimated for three depth intervals under different degrees of tractor traffic (0, 1, 5, 7 and 10 passes of a light-weight 2WD tractor).

Tabla 6. Valores porosidad total del suelo (%) calculados para tres rangos de profundidad bajo diferentes grados de tráfico del tractor (0, 1, 5, 7 y 10 pasadas de un tractor 2WD liviano).

Depth (mm)	Control plot	1 pass	5 passes	7 passes	10 passes
0 - 150	53.2 a	52.4 a	51.3 a	45.6 b	37.7 c
150 - 300	50.9 a	49.8 a	46.0 a	38.4 b	36.9 b
300 - 450	42.6 a	42.2 a	41.8 a	38.1 b	37.3 b

Values with different letters (horizontally) are significantly different at each depth (P < 0.01 Duncan's multiple range test).

Los valores con letras diferentes (horizontalmente) son significativamente diferentes para cada profundidad (P < 0,01 Prueba de rango múltiple de Duncan).

Such level of compaction can be similar to that commonly found, for example, after a single pass with much heavier (*e.g.*, 10-12 t axle load) equipment (*e.g.*, 1). Finnally, data from parameters analyzed within this study showed that traffic compaction by light-weight tractors (*e.g.*, ≈15 kN overall load) induced significant changes to the topsoil (0-150 mm) and subsoil properties

(150-450 mm). These results confirm those previously reported by Håkansson (1987), who indicated that the lasting effects of compaction are related to soil type, the number of passes, and the number of years after compaction was impossed. Hence, the data showed in this work support the hypothesis formulated prior to this study. Technological developments such as in (ultra)-low ground pressure systems (*e.g.*, IF and VF marked tyres) offer promise to mitigate the effects of traffic on soil compaction, and this is an area that merits a research priority within intensively-managed horticultural systems such as the one described in our study. Reduction in contact pressures by using tyres at the lowest (safest) operating pressure aided by the use of central tyre-inflation-pressure control systems may also be recommended if this was a more cost-effective option than IF/VF marked tyres.

Conclusions

Given the experimental conditions of this study, the following conclusions can be drawn:

One and five passes of a light-weight tractor (15 kN overall load) did not increase soil bulk density or cone index significantly at shallow depths (0-150 mm).

Only 7 and 10 repeated passes of a light-weight tractor induced significant increases in both soil cone index and soil bulk density to a depth of 450 mm.

The work reported in this article showed that soil compaction caused by a light-weight tractor can significantly decrease total soil porosity and water infiltration into soil.

The traffic treatments applied within this study affected all soil measured parameters in the topsoil and resulted in soil physical conditions that would be unsuitable for crop establishment and development.

There is a need to investigate the feasibility of using low (ground) pressure tyre technology in light-weight vehicles commonly used in almond production (and similar tree plantations) in Mediterranean soils. The utilisation of low (ground) pressure tyre technology may be a cost-effective alternative to mitigate the effect of traffic compaction in these systems compared with deep tillage.

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Current knowledge and future prospects of lima bean (*Phaseolus lunatus*)-rhizobia symbiosis

Conocimiento actual y perspectivas de futuro de la simbiosis frijol de lima (*Phaseolus lunatus*)-rizobia

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REVIEW

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Abstract

Lima bean (*Phaseolus lunatus)* is an important species of the genus *Phaseolus* for human consumption in tropical regions. The seeds are important source of protein for people from South America, Africa and Mexico. In addition, as a legume plant, lima bean presents the ability to perform the biological nitrogen fixation (BNF) through the symbiosis with nitrogen-fixing bacteria. The studies about diversity and efficiency of lima bean-rhizobia symbiosis have increased worldwide, mainly in Latin America. These studies have shown *Bradyrhizobium* and *Rhizobium* as the main symbionts, although *Sinorhizobium*, *Mesorhizobium* and *Allorhizobium* have been found associated with lima bean. Also, there is a large variation in the efficiency of N fixation by the current isolates of rhizobia and some rhizobia have presented high capability for fixing N. This review aims to explore the studies about diversity and efficiency of rhizobia in symbiosis with lima bean.

Keywords
BNF-efficiency • *Phaseolus* • rhizobia diversity

Resumen

El frijol de lima (*Phaseolus lunatus*) es una especie importante del género *Phaseolus* para consumo humano en regiones tropicales. Las semillas son una fuente importante de proteínas para las personas de América del Sur, África y México. Además, como leguminosas, el frijol de lima presenta la capacidad de realizar la fijación biológica de nitrógeno (BNF) a través de la simbiosis con bacterias fijadoras de nitrógeno. Los estudios sobre la diversidad y la eficiencia de la simbiosis de frijol de lima-rizobio han aumentado en todo el mundo, principalmente en América Latina. Estos estudios han demostrado que Bradyrhizobium y Rhizobium son los principales simbiontes, aunque Sinorhizobium, Mesorhizobium y Allorhizobium se han asociado al frijol de lima. Además, existe una gran variación en la eficacia de la fijación de N por los aislamientos actuales de rizobios y algunos rizobios han presentado una alta capacidad para reparar N. Esta revisión tiene como objetivo explorar los estudios sobre la diversidad y la eficacia de rizobios en simbiosis con frijol lima.

Palabras clave
BNF-eficiencia • *Phaseolus* • diversidad de rizobios

Introduction

Legume plants belonging to the genus *Phaseolus* present economic importance worldwide. This genus comprises five species: *P. vulgaris* L., *P. lunatus* L., *P. coccineus* L., *P. acutifolius* A. Gray and *P. polyanthus* Greeman (14). Lima bean (*P. lunatus* L.) is considered the second most cultivated species from the genus *Phaseolus*, being an important species of plant for humans in tropical regions (11, 17, 23).

This legume is originated from Perú and was domesticated in the Andean Mountains, specifically in southern Ecuador-northwestern Perú, and central-western Mexico (25). The study of Mota-Aldana *et al.* (2010) also identified three main varieties: "Big Lima" (big seeds) domesticated in the Andean Mountains; "Sieva" and "Potato" (small seeds) originated in central-western Mexico. Lima bean is predominantly autogamous, although it has outcrossing rates up to 48% (6). It has been characterized by an indeterminate growth habit, a prolonged flowering period, and production of a large number of pods (37).

Lima bean is considered an important source of protein for people from South America, Africa, and Mexico. Interestingly, this crop is economically important in some regions of United States. For example, California has about 24,000 acres of lima bean with a value of about $32 million in 2017 (35). The seeds present high content of protein (210-260 $g \cdot kg^{-1}$) and carbohydrate (550-640 $g \cdot kg^{-1}$), low fat (10-23 $g \cdot kg^{-1}$) levels and high fiber levels (32-68 $g \cdot kg^{-1}$), high levels of the minerals K, Zn, Ca, and Fe, and low levels of Na and P (7).

It is also a relevant plant for Brazil, mainly in the Northeast region, where it is used as an alternative food source. Currently, Brazil produces approximately 14,951 t of lima bean from an area of about 37,521 ha. Since the Northeast region presents high annual temperature and drought periods, lima bean is adapted to these conditions and presents rusticity and tolerance to long and dry periods (5).

On the other hand, lima bean also presents the ability to associate symbiotically with nitrogen-fixing bacteria, commonly known as rhizobia, and therefore can perform the process of biological nitrogen fixation (BNF) (33). Since this plant needs about 100 kg N to produce 1.5 t of seeds (18), the BNF could be an ecological and economical alternative for supplying N to the plant.

Biological nitrogen fixation in lima bean

BNF is an important source of nitrogen (N) to the plants, which is important for plant growth. It has suggested that after photosynthesis, the process performed by plants to produce the energy necessary for their survival, BNF is regarded as the second most important biological process on the earth (http://cnx.org/content/m47338/latest/). This process occurs through of a symbiotic system between legumes and rhizobia (36), and the fixed N_2 represents a renewable source of N within agriculture. The amount of N fixed by plants is ranged from 100 to 900 kg ha^{-1} $year^{-1}$. For example, in soybean, an important crop worldwide, the contribution of N derived from the atmosphere via BNF represents 0 to 337 kg N ha^{-1} (9).

The symbiosis rhizobia-legume occurs in root nodules that are established by legume in response to appropriate rhizobia (2, 33). Thus, the nodulation is usually a host-specific interaction where some specific bacteria infect a limited range of legumes and the N-fixing nodules are formed as a consequence of this interaction (30). Rhizobia able to nodulate

and fix N_2 in legumes comprise bacteria distributed among the genera *Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Burkholderia, Cupriavidus, Devosia, Herbaspirrilum, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium, Rhizobium, Shinella* and *Sinorhizobium* (8).

The success of symbiosis depends on the availability of compatible rhizobia to the specific legumes. Considering the genus *Phaseolus*, the studies have presented information about nodulation and BNF in common bean (*P. vulgaris*), which form symbiotic associations with several species from the genus *Rhizobium* (20, 21). There are variations among the efficiency of BNF in common bean. Recently, Moreira *et al.* (2017) evaluated the contribution of rhizobia isolates on N derived from the atmosphere, via BNF, to *P. vulgaris* and found a range of 509 to 1037 mg plant^{-1} which represented 46% to 75% of the total N found in the plants. For lima bean, the studies about symbiosis with rhizobia have shown a miscellaneous of genera able to nodulate this legume with different efficiencies. In this way, studies about the diversity of rhizobia which present ability for associating with lima bean are increasing in the last years. Thus, this review aims to explore the studies about diversity and efficiency of rhizobia in symbiosis with lima bean.

Diversity of lima bean-nodulating rhizobia

Rhizobia are common inhabitants of diverse soil types and the number of cells can substantially differ from a few cells to 10^5 bacteria per gram of soil (13). The wide geographical distribution of rhizobia may be related to the large genetic diversity among these bacteria (22). The knowledge about diversity of rhizobia provides information on host preferences, predominance of isolates, genetic structure and also source of efficient strains for using as inoculant (21).

The majority of legume-nodulating rhizobia belong to α-proteobacteria group that include *Rhizobium, Sinorhizobium, Azorhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium, Bradyrhizobium* and *Methylobacterium* (16). Usually, each genus is associated and nodulates with specific legumes. For example, *Rhizobium* is associated with common bean (*Phaseolus vulgaris* L.), while *Bradyrhizobium* nodulates soybean (*Glycine max* L. Merrill) and cowpea (*Vigna unguiculata* L.) (15).

Previous old host-based classification scheme included symbionts of lima bean in the same group of rhizobia associated with slow-growing cowpea (*Vigna unguiculata)* (29). This group was a diverse assemblage of strains that were later included in the genus *Bradyrhizobium* (1, 34). In these studies, the rhizobial isolates associated with lima bean were obtained from areas where this plant species is not native and the studies have only focused on morphological, physiological and symbiotic characteristics. Thus, the limitation of these methods did not address properly the existing diversity.

Currently, studies have been conducted with molecular tools that provide the possibility to identify taxonomic groups of rhizobia in soils and have shown several genera with ability to associate with lima bean (table 1, page 284). Indeed, molecular tools have identified a diverse group of rhizobia-nodulating lima bean comprising bacteria belonging to *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Allorhizobium* (4, 14, 26, 28, 32).

Table 1. Rhizobia genera isolated from lima bean nodules.
Tabla 1. Géneros de Rhizobia aislados de nódulos de frijol de lima.

Genera	Location	Method	Reference
Bradyrhizobium	USA	Morphology and physiology	Thies *et al.*, 1991
B. yuanmingense *Bradyrhizobium sp*	Perú	16S rDNA and *dnaK*, *nifH*, and *nodB* genes	Ormeño-Orillo *et. al.*, 2006
Bradyrhizobium	Mexico	16S rDNA and recA, nodZ and nifH genes.	Lopez-Lopez *et al.*, 2013
Bradyrhizobium	Perú	16S rDNA and recA, atpD, glnII, dnaK and gyrB genes.	Duran *et al.*, 2014
Bradyrhizobium, Mesorhizobium, Rhizobium	Brazil	endonucleases MboI, HaeIII, and NheI	Santos *et al.*, 2011
B. yuanyrense *B. liaoningense* *B. paxllaeri* *B. icense*	Perú	16S rRNA	Matsubara and Zúñiga-Dávila, 2015
R. mesosinicum *R. alamii*	Perú	16S rRNA	Matsubara and Zuñiga-Dávila, 2015
Rhizobium *Bradyrhizobium,* *Allorhizobium*	Brazil	16S rDNA	Araujo *et al.*, 2015

The majority of studies about the diversity of rhizobia in lima bean have assessed a relative wide collection from geographic locations including Perú, Mexico and Brazil (4, 14, 19, 23, 26, 32). Perú is considered the center of diversity of this plant species and the first study on the diversity of rhizobia, by using molecular tools, has characterized some symbionts of lima bean (26). Thus, Ormeño-Orrillo *et al.* (2017) evaluated the molecular diversity of rhizobial isolates associated with lima bean and found divergent bradyrhizobial lineages (*B. yuanmingense* and *Bradyrhizobium* sp.) according to Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the *rpoB, dnaK*, *nifH* and *nodB* genes and sequencing of the 16S rDNA. This finding could confirm that *Bradyrhizobium* is the main genus effectively associated lima bean. Indeed, the study conducted later by Duran *et al.* (13), by using a polyphasic approach, confirmed *Bradyrhizobium*-nodulating lima bean in Perú. However, the authors found two different lineages (*B. paxllaeri* sp. and *B. icense*) those described by Ormeno-Orrillo *et al.* (2006). The hypothesis that *Bradyrhizobium* is the most important genus found in Perú was addressed by Matsubara and Zuñiga-Dávila (2015) who evaluated rhizobia associated with lima bean in Perú and found species belonging to *Bradyrhizobium* genus (*B. yuanyrense, B. liaoningense, B. paxllaeri,* and *B. icense*). However, this study also reported the genus *Rhizobium* (*R. mesosinicum* and *R. alamii*). Thus, these studies showed that there is a high diversity of rhizobia associated with lima bean in Perú and it indicates a relative promiscuity of lima bean in contrast to previous statements that this plant species has a restricted symbiont (28).

As an important center of domestication (25), Mexico also has a high diversity of rhizobia able to nodulate lima bean. The first study about diversity of rhizobia-nodulating lima bean in Mexico was conducted by Lopez-Lopez *et al.* (2013) who sequenced *rpoB*, *recA*, *nodZ*, and *nifH* genes of the rhizobia and found isolates described as *Bradyrhizobium*. It was the first report of nodule bacteria from lima bean in Mesoamerican region (center of origin and domestication) and it confirms *Bradyrhizobium* as the main bacteria associated with lima bean. Therefore, *Bradyrhizobium* seems to be the most abundant genus nodulating lima bean in Perú and Mexico.

Brazil is an important region of dispersion of lima bean and the studies have shown a high diversity of rhizobia associated with this plant species. Thus, a study about genetic diversity of native rhizobia associated with lima bean in Brazil was conducted by Santos *et al.* (2011) and shows a broad spectrum of rhizobial groups. In this study, rhizobia isolates were obtained and placed into groups based on divergence in their morphological, physiological and genetic traits. The restriction patterns obtained with endonucleases MboI, HaeIII, and NheI showed sufficient variability and identified isolates belonging to the genera *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. Later, Araujo *et al*. 2015, sequenced the 16S rDNA of these isolates and found species belonged to the genus *Bradyrhizobium*, *Sinorhizobium* and *Rhizobium.* These results confirm that lima bean may be associated by diverse rhizobia species and also showed different groups than those reported in Perú and Mexico. Indeed, Araújo *et al.* (2015) found 14 isolates of rhizobia genetically different that those observed in Perú by Ormeño-Orillo *et al.* (2006). The isolates found by Araujo *et al.* (2016) were classified as *Rhizobium, Bradyrhizobium* and *Allorhizobium.*

Efficiency of rhizobia on BNF in lima bean

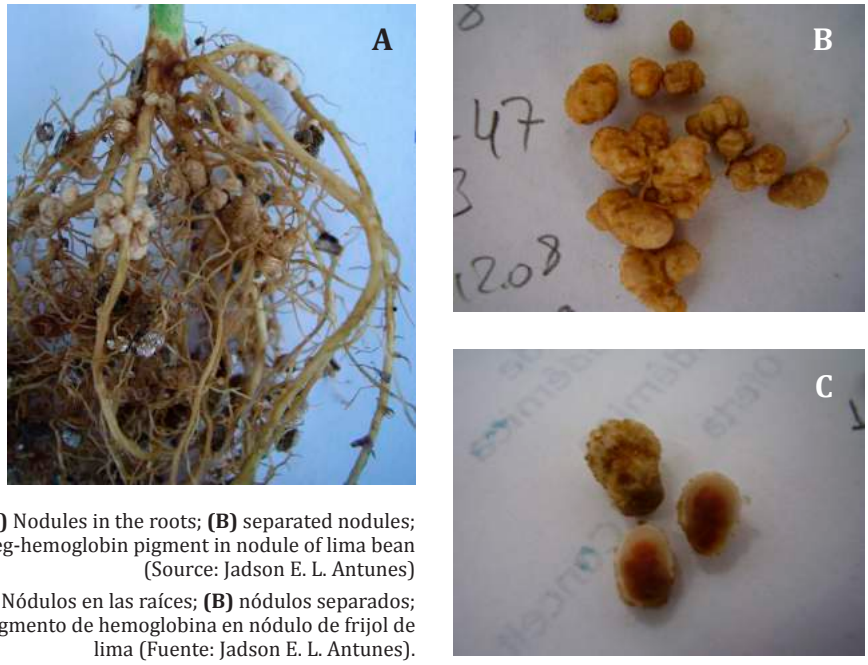
Studies about nodulation and efficiency of BNF have increased in the last ten years and the main objective is to evaluate suitable strains for recommendation of inoculation. These studies have focused on the evaluation of rhizobia in different genotypes of lima bean (3, 10, 11). Indeed, the first study about nodulation in lima bean evaluated different genotypes (12). The main question was to determine if the nodule formation coincides with plant development and they found a close relationship between plant and nodule growth. Later, Santos *et al.* (2009) evaluated the ontogeny of nodulation in lima bean and found that the highest nodule number and biomass occurred between 45 and 60 days after plant emergence, which correspond to the flowering period of the plant. Additionally, Santos *et al.* (2009) found red colored nodules as an indication of leghemoglobin (photo 1, page 286). However, the confirmation that the rhizobia had high efficiency in fixing N in lima bean was reported by Antunes *et al.* (2004) who evaluated the symbiotic effectiveness of 17 rhizobial isolates associated with lima bean and compared with two strains of reference CIAT 899 and NGR 234. They found eight isolates with higher N accumulation and N_2-fixation efficiency compared with the strains CIAT 899 and NGR 234. Rhizobia isolated from different legume species seem to present ability for nodulating and fixing N in lima bean. Thus, Costa *et al.* (2017) evaluated rhizobia isolates from nodules of *Vigna unguiculata*, *Campsiandra surinamensis*, *Inga sp*. and *Swartzia sp.* on lima bean and found isolates with high efficiency in N fixation. Therefore, the prospection of

rhizobia able to nodulate lima bean should be also done in different legume species.

As reported above, the main genus found in association with lima bean is *Bradyrhizobium*, although *Rhizobium* and other genera are able to associated with this plant species. Thus, in order to evaluate the efficiency of *Bradyrhizobium* and *Rhizobium* on nodulation and efficiency of BNF in lima bean, Costa Neto *et al.* (2017) found *Bradyrhizobium* more efficient than *Rhizobium* in establishing the symbiotic association with lima bean varieties. These authors also found isolates with high efficiency as compared with the other symbiotic pairs. Thus, the studies are in advance to find suitable and potential strains for inoculation in lima bean.

Future prospects

Lima bean is an important legume in tropical regions and the studies have shown a great diversity of rhizobia associated with this plant species. Also, there is a large variation in BNF efficiency observed in the current isolates of rhizobia. Therefore, further studies have to focus on: a) classification of rhizobia associated with lima bean in different regions; b) selection of specific and efficient strains for using as inoculant; c) improvement of N fixation ability by native rhizobia and adapted to different regions; d) selection of lima bean genotypes combined with high efficient strains; e) implementation of programs of lima bean breeding and management in Perú, Mexico and Brazil; f) analysis of the advantages of an adequate symbiosis for improving the content of protein and better quality of the nutritional offer for human nutrition.



(A) Nodules in the roots; **(B)** separated nodules; **(C)** Leg-hemoglobin pigment in nodule of lima bean (Source: Jadson E. L. Antunes)

(A) Nódulos en las raíces; **(B)** nódulos separados; **(C)** Pigmento de hemoglobina en nódulo de frijol de lima (Fuente: Jadson E. L. Antunes).

Photo 1. Nodules in lima bean roots at 45 days after plant emergence.

Foto 1. Nódulos en raíces de frijol de lima a los 45 días de la emergencia de la planta.

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Effects of food-related health concerns and risk perception on the consumption frequency of fresh vegetables

Efectos de la preocupación en salud asociada a los alimentos y la percepción de riesgo en la frecuencia de consumo de vegetales frescos

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Abstract

Consuming fresh vegetables certainly brings health benefits; however, these types of products may also contain biological, chemical and technological elements that can affect people´s health due to lack of food safety. We developed a conceptual model to explain the main relationships between food-related health concerns (FHCs) and risk perceptions (RPs) on consumption frequency of fresh vegetables (CFFV) from a food safety point of view. We applied a structured questionnaire to 1028 consumers in the Central and South Central zones of Chile, where the main agricultural production of the country is concentrated. Through a structural equation model, we determined the moderator effect of RP on the relationship between FHC and CFFV. As a result, CFFV is less if RP is present in the minds of the consumer, impacting the direct effect of FHC on CFFV. Finally, our results suggest that reducing risks associated with the production and commercialization of fresh vegetables can improve health concerns related to food and the consumption of fresh vegetables. Therefore, the state must improve surveillance systems of fresh vegetables commercialized in local markets.

Keywords
Food safety • consumers • structural equation model (SEM) • moderator effect • fresh vegetables

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Resumen

Consumir vegetales frescos sin duda trae beneficios a la salud, sin embargo, este tipo de productos también pueden contener elementos biológicos, químicos y tecnológicos que afecten la salud de las personas por falta de inocuidad de los alimentos. Con base en estudios previos, desarrollamos un modelo conceptual para explicar las principales relaciones entre la preocupación en salud ligada a los alimentos (FHC) y la percepción de riesgo (RP) sobre la frecuencia de consumo de hortalizas frescas (CFFV) desde el punto de vista de la inocuidad alimentaria. Se aplicó un cuestionario estructurado a 1028 consumidores en las zonas Centro y Centro Sur de Chile, donde se concentran los principales sistemas de producción agrícola del país. A través de un modelo de ecuaciones estructurales se determinó el efecto moderador de RP sobre la relación entre FHC y CFFV, lo que significa que CFFV es menor si RP está presente en las mentes de las personas. Impactando el efecto directo de FHC en CFFV. Por último, los resultados sugieren que la reducción de los riesgos asociados con la producción y comercialización de hortalizas frescas, mejorará la preocupación por la salud relacionada con los alimentos y el consumo de hortalizas frescas. Por lo tanto, el Estado debe mejorar los sistemas de vigilancia de las hortalizas frescas comercializadas en el mercado local.

Palabras claves

Inocuidad alimentaria • Modelo de ecuaciones estructurales (SEM) • efecto moderador • vegetales frescos

Introduction

Different studies report the importance of vegetable consumption to avoid non-communicable diseases (10, 22). Nevertheless, these types of products may also contain risky elements that can affect human health. These risks in fresh vegetables are associated to three different sources: a) Biological hazards such as *E. Coli* and listeriosis; b) Chemical hazards, *e.g.* excessive pesticide residues and c) Potential risks associated with technology such as gene modification, irradiation and nanomaterials (8, 46, 52). However, risk perception (RP) toward fresh vegetables by the consumer is low, at least until they suffer an adverse health incident related to them (48). However, people with high food-related health concerns attempt to avoid incidents associated to food consumption (17, 41). In this context, food risk perception and food related health concerns are concepts associated to food safety. According to authors such as Grunert (2005) and the World Health Organization (WHO) (2014), food safety is the probability of not contracting a disease as consequence of consuming a certain food, and it is considered a public health concern around the world. Different incidents associated to the lack of food safety have been reported in many countries during the last thirty years, and fresh vegetables do not escape these incidents. More recently, in the scientific literature there are examples such as fenugreek sprouts imported from Egypt which were contaminated with *E. coli*, affecting the French and German populations during 2011; maize contaminated with aflatoxins affected southern Europe in 2013; Listeriosis in

frozen vegetables affected populations in the EU and the USA in 2016 (13). All these examples associated to vegetables show food risks which can cause health problems. This given, the relationship between food risk, health concern and frequency of vegetable consumption from the perspective of food safety has not yet been analyzed in-depth in the literature. In addition, the lack of food safety is perceived in different ways by consumers from developed and developing countries. In developed countries, consumers are concerned about the risk associated with food (44). Meanwhile in developing countries, these health problems generate less concern in the population; and therefore exists a major propensity to consume fresh vegetables lacking safety controls (4, 5, 7). Our research attempts to explain the main relationship between food-related health concerns (FHC) and risk perceptions (RP) on the consumption frequency of fresh vegetables (CFFV) in Chile as a study case for Latin America. The following sections discuss the conceptual model and the research hypotheses, continuing with the methodology and our findings. Finally, we present the discussion, conclusions and implications for future research.

Conceptual model and development of the hypotheses

Vegetable consumption frequency

Vegetables are basic components of a balanced diet, and their consumption have health benefits to the population. These benefits are associated to the reduction of non-comunicable deseases such as cancer, cardiovascular complications and nutritional deficiencies. For these reasons, the WHO (2017) recommends consuming at least 400 grams of fruit and vegetables per day. However, in many countries (such as USA, New Zealand, Chile and others), the consumption is only half the WHO-recommended level (21, 34). There are different factors that influence the vegetable consumption frequency. Some of these factors are psychological, and others have to do affordability, economy and socio-demographics (5, 9, 36, 39). However, there still exists a gap in research regarding vegetable consumption frequency from a food safety perspective.

Food-related health concerns, risk perception and vegetable consumption

People's health concerns are associated with their health-related behavior (40). According to Sun (2008), the relationship between health concern about developing diseases and attitudes toward healthy eating is mediated by food choice motives. In that sense, consumers choose fresh vegetables for consumption in attempt to have a balanced diet and engage in healthy eating. These behaviors reduce the population's health concerns about contracting a diet-associated disease. This is because of the perception that the main benefits of consuming fresh vegetables are to reduce the incidence of obesity and cardiovascular diseases, and that they supply vitamins, minerals, fiber, antioxidants, among other nutrients needed to maintain good health (24, 34, 40, 49). Therefore, assuming food-related health concerns as a latent dimension are a good proxy for determining consumption frequency of fresh vegetables, we hypothesized that:

H_1. Food-related health concerns influence the consumption frequency of fresh vegetables.

To examine food risk from the consumer's point of view, it is necessary to address the concept of their risk perception about food. From the perspective of cognitive theory, the risk

perception is the consumer concern caused by the uncertain effects on their health generated by insalubrious foods (6). All types of contamination in vegetables from chemical, biological and/or technological sources represent a food risk to the population.

Recent studies show that risky events associated to contaminated vegetables represent a public health concern. For example, the necessity of reducing the consumer exposure to active pesticides in vegetables (44); contamination with heavy metals and pathogens (1, 35). It is important to highlight that if risky events associated with vegetables that affect society are publically reported, RP will increase. This implies that the consumer reacts by reducing or postponing the vegetable consumption until the food alert is lifted. Likewise, they may prefer to purchase labeled, branded or quality assured vegetables (5). Therefore, based on this information, we hypothesized that:

H_2. Food-related health concerns are related to risk perception

H_3. Risk perceptions directly influence the consumption frequency of fresh vegetables.

According to Sun (2008), consumer health concerns are related with diet. Consumer demand for healthy food products has increased in the last decade. Some examples are the proliferation of functional foods, the rise in demand for organic produce among others (3, 47). Therefore, consumer health concerns could influence a higher consumption of fresh vegetables. But, food risk could likewise generate a contrary effect, since the consumption frequency of fresh vegetables, depends on the level of consumers' RP towards different hazards such as the presence of pesticide, microorganisms and technological alterations (27, 30). Based on these antecedents, we hypothesized that:

H_4 Food-related health concerns indirectly influence fresh vegetable consumption frequency through RP.

H_5. Risk perception as a moderator latent variable affects the relationship between food-related health concerns and the consumption frequency of fresh vegetables.

Based on the hypothesis proposed, our conceptual model is presented in figure 1 (page 293).

Materials and methods

Sample and procedures

Two of the most important zones for vegetable production in Chile were chosen. These zones included the cities of Santiago, Talca and Temuco, corresponding to the central and south central regions of the country. Data were compiled through convenience sampling of 1,200 vegetable buyers, over the age of 18 (400 fresh vegetable buyers in each city), using face to face surveys administered from September to November 2012. The measuring instrument was applied in public places close to stores, banks and supermarkets. Previously, a pre-test of 10% of the sample was carried out to detect biases in comprehension.

The questionnaire was structured to investigate consumer opinion about the following items: risk perception toward fresh vegetables they consumed; health concerns associated with fresh vegetables; frequency of vegetable consumption and socio-demographic characteristics. Following Byrne (2010), outliers were detected and deleted in order to continue with the analysis. Finally, the sample used in the study was 1,028 cases. Table 1 (page 294) shows the socio-demographic data from the surveyed sample.

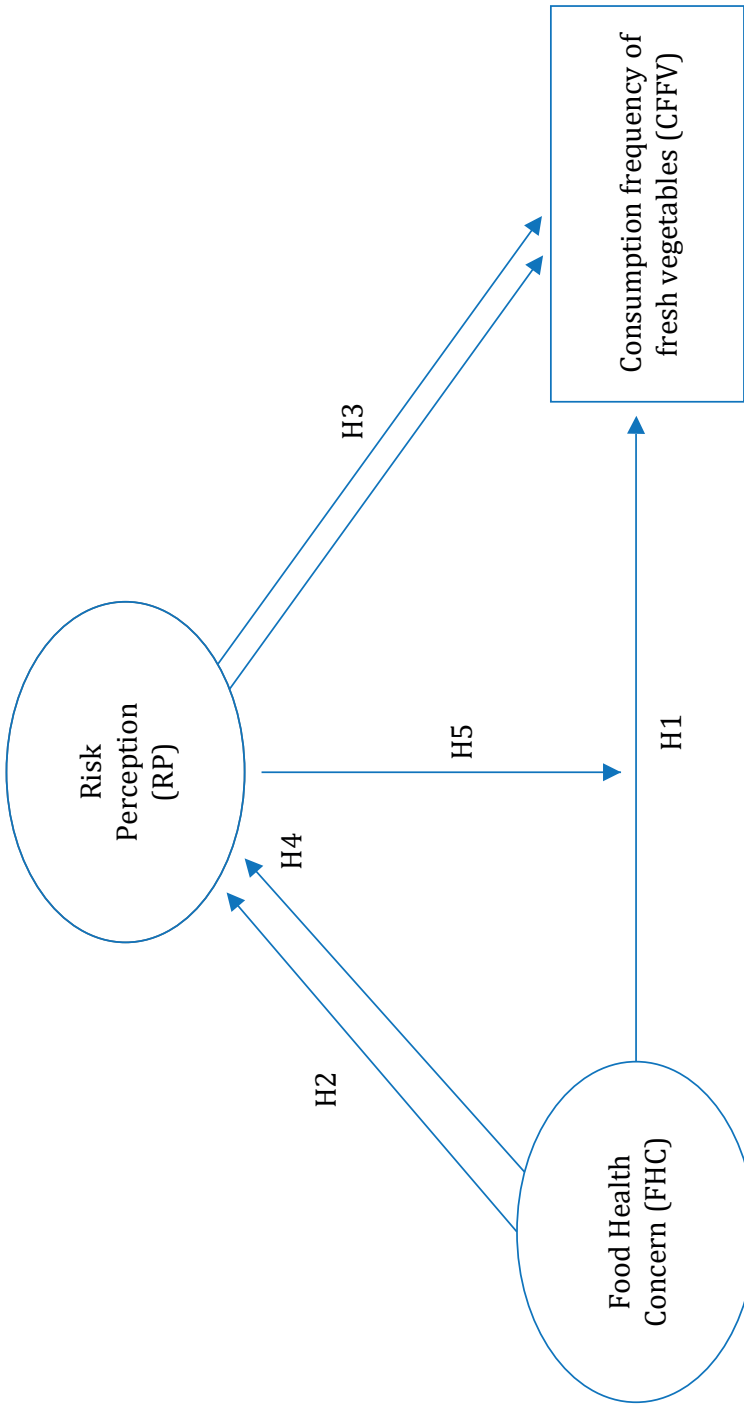


Figure 1. Conceptual model of factors that affect the frequency of fresh vegetable consumption in consumers, from a food safety point of view.

Figura 1. Modelo conceptual de los factores que afectan la frecuencia de consumo de vegetales frescos en los consumidores, desde un punto de vista de la inocuidad alimentaria.

Table 1. Socio-demographic statistics.
Tabla 1. Estadística socio-demográfica.

Items	**Values**
Sample size	1028
Gender (%)	
Male	30.5
Female	69.5
Age (mean ± st. dev.)	38.9 ± 3.53
Education (%)	
Elementary	5.9
High School	32.5
Incomplete technical college	2.5
Complete technical college or incomplete university	48.2
Complete university or more	10.9
Income (%)	
More than US$3.700 US$1.401 to US$3.700 US$1.121 to US$1.400 US$500-US$1.120 Less than US$500	13.0 43.3 26.7 12.5 4.5
Frequency of vegetable consumption (%)	
Occasionally	5.1
Once a week	8.9
Three times a week	37.5
Daily	48.6
Main fresh vegetable consumed (%)	
Lettuce	50.6
Tomato	22.6
Other	26.8
Places for buying fresh vegetables (%)	
Municipal markets	29.6
Greengrocers	32.3
Food distribution centers	7.6
Supermarkets	30.5
Self-declared food safety knowledge (%)	
Low food safety knowledge	32.2
Medium food safety knowledge	52.7
High food safety knowledge	15.1

Data collection instrument

The measuring instrument was structured with observed variables and measurement scales used in previous studies. A 5-point importance scale was used to measure each indicator (1 to express the lowest level and 5 to indicate the highest level), except for the frequency of vegetable consumption and socio-economic variables. The measurement of risk perception toward vegetables consumed was based on previous literature on food products, and the statements were adapted from measures contained in Brewer and Prestat (2002), Tucker *et al.* (2006) and Yeung and Morris (2006). The measurement of opinion about health concerns associated with food was adapted from Gil *et al.* (2000); Sánchez and Gil (2000). In the analysis, an observable variable was included as a dependent variable named vegetable consumption frequency (15, 24), which received the effects from all the constructs. Table 2 (page 296) presents the observable variables (indicators) which defined each construct (latent variables).

Statistical analysis

Data were analyzed by the structural equation model (SEM), which represented the relationships between the constructs of food-related health concerns (FHC) and risk perception (RP) and the observed variable consumer frequency of fresh vegetables (CFFV). The steps involved in the analysis were: a) Measurement modeling through confirmatory factor analysis (CFA); b) Construct validity; c) Structural modeling to test the causal relationship; d) Mediation test through direct and indirect effects obtained by the Sobel and Bootstrapping test and e) Moderation test through techniques for constructs proposed by Zainudin (2012). To evaluate the model's goodness of fit, various indicators were used: Chi-square to df ratio (χ^2/df < 5.0); comparative fit index (CFI close to 0.9 or 1.0), goodness fit index (GFI close to 0.9 or 1.0) and normed fit index (NFI close to 0.9 or 1.0) and the robustness of the mean squared error approximation (RMSEA) with values lower than 0.08 (20, 26). The analysis was performed with AMOS 20 and IBM SPSS 20.

Results

Descriptive analysis

This study was carried out with the participation of fresh vegetable buyers, over 18 years old. Most of the interviewees were women who had either completed or not completed higher education. In terms of household income, the highest proportion of interviewees received monthly income between US$1.401 and US$3.700. The majority of the consumers surveyed declared that they ate fresh vegetables daily. The most consumed fresh vegetable was lettuce, followed by tomatoes. Of the sample, 61.9% bought their produce at traditional markets, such as municipal markets, greengrocers and food distribution centers. Most interviewees reported a medium level of food safety knowledge (more details in table 1, page 294).

Measurement and structural model

The first step carried out was to validate the scales through confirmatory factor analysis (CFA). Results of the CFA showed good fit of the datasets $\chi 2$ = 82.727, df = 30, $\chi 2$/df = 2.76, p=0.000, RMSEA = 0.041, CFI = 0.984, GFI = 0.984, NFI = 0.976 (table 2, page 296).

Table 2. Measurement model, reliability and validity of the scales used in the analysis.
Tabla 2. Modelo de medida, confiabilidad y validez de las escalas utilizadas en el análisis.

Construct	Observed variable	Mean	SD	β(t-value)	AV	CR	Cronbach-α	Measurment Model
								$\chi2$ = 82.727
Risk perception (RP)	*What is the risk perception level on the fresh vegetables consumed?*				0.50	0.85	0.85	
	Pesticide presence	2.97	1.52	0.81 (15.97)				df = 30
	Polluted irrigation water	3.03	1.43	0.88 (15.82)				
	Microorganism contamination	3.20	1.39	0.78 (15.68)				$\chi2$ /df = 2.76
	Faulty Food-Handling in restaurants	3.20	1.45	0.61 (13.94)				
	Irradiation UV	2.40	1.38	0.57 (16.82)				p = 0.000
	Genetically modified organisms	2.74	1.45	0.53a				
								RMSEA = 0.041
Food-related Health concerns (FHC)	I have a healthy diet	3.72	1.14	0.57a	0.39	0.71	0.71	CFI = 0.984
	I eat fruits and vegetables frequently	4.18	0.94	0.54 (12.13)				
	I am worried about my diet and its effects on my health	4.17	0.97	0.76 (14.18)				GFI = 0.984
	I am interested in food related information	4.05	1.01	0.59 (12.77)				
								NFI = 0.976
	Frequency of vegetable consumption	3.30	0.83	n.i.				

In addition, the scales used in the analysis satisfied the composite reliability test (above 0.7); average variance extracted values (close to 0.5) and the internal consistency (Cronbach's α above 0.7), showing good indicators of reliability and validity. Therefore, the measurement model presented adequate internal validity.

Once the scales were validated, the structural model was tested through Maximum Likelihood (figure 2, page 298). The structural model had a good fit of the dataset, and indices were within acceptable limits, but exceeded the minimum values recommended in the literature $\chi2 = 94.326$, df = 35, $\chi2/df = 2.69$, p=0.000, RMSEA = 0.041, CFI = 0.984, GFI = 0.984, NFI = 0.975. All direct proposed relationships were significant. In terms of hypotheses with direct relationships, it was found that the construct FHC was positively associated with CFFV (H_1) (β = 0.57, t =8.74, p = 0.000). Furthermore, FHC was positively associated with RP (H_2) (β = 0.22, t =4.87, p = 0.000), and RP was positively associated with CFFV (H_3) (β = 0.09, t =2.83, p = 0.05). Therefore, our results confirm that CFFV was determined mainly by FHC, and to a lesser extent by cosumers' RP of toward fresh vegetables.

Mediation analysis

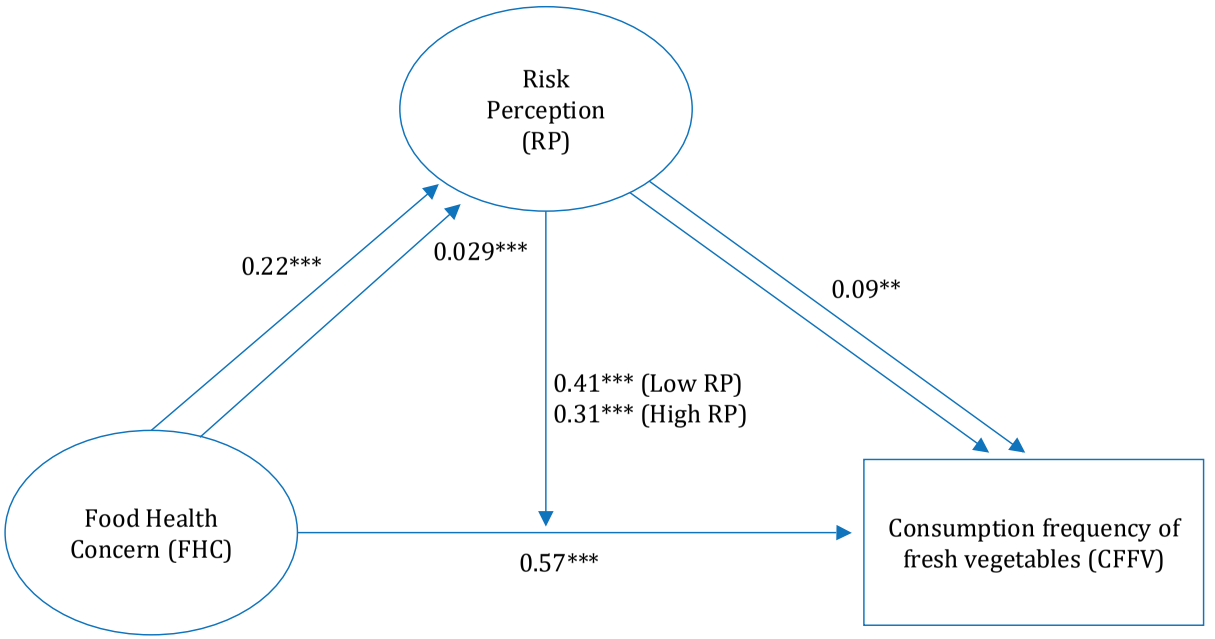
Regarding mediation analysis, we highlight that the construct RP mediated the effect between FHC and CFFV (H_4)(β = 0.029, p = 0.000) (figures 1, page 293 and 2, page 298). Table 3 (page 299) shows the indirect effect model for the mediator. Significant p values of the Sobel test indicated that independent variable FHC predicted CFFV to a lesser but significant extent, through RP (mediator), confirming H_4. The bootstrap method also revealed that the mediating effect of RP, between FHC and CFFV, did not lie within zero for the predicted confidence interval. Therefore, the mediated effect was significantly different from zero at p = 0.000.

Moderation analysis

Moderation analysis was carried out using RP as a latent variable moderator of the relationship between FHC and CFFV. Following the steps for AMOS, proposed by Zainudin (2012), it can be confirmed that RP acts as a moderator of the proposed relationship. This is because the difference of the $\chi2$ value and degree of freedoms between the constrained and unconstrained models are greater than 3.84 (table 4, page 299). Therefore, the proposed hypothesis (H_5) is supported. This means that the moderator effect of RP on FHC and CFFV for the standardized estimate path decreased from the general model (H_1 = 0.57) to 0.41 for the Low RP group and 0.31 for the High RP Group.

Discussion

The majority of the interviewees expressed that they consume fresh vegetables daily (mainly lettuce and tomatoes), which is beneficial for consumers from the perspective of health concerns (14, 31). Consequently, the results obtained in our research contribute to the knowledge about CFFV and what is determined by FHC and RP from perspective of food safety. According to our results, the CFA and structural equation model suggest that there is an interaction between the constructs FHC, RP, and the dependent variable CFFV. The results show that the main construct that determines the frequency of vegetable consumption is mainly FHC and, to a lesser extent, the consumer's RP. Our first hypothesis (H1) confirms that FHC directly and significantly influences CFFV.



The significance of the mediation and moderation variable is: *p = 0.05; **p = 0.01; ***p = 0.001. $\chi 2$ = 94.326, df = 35, $\chi 2$/df = 2.69, p=0.000, RMSEA = 0.041, CFI = 0.984, GFI = 0.984, NFI = 0.975

Significancia estadística de la variable de mediación y moderación: *p = 0,05; **p = 0,01; ***p = 0,001. $\chi 2$ = 94,326, df = 35, $\chi 2$/df = 2,69, p=0,000, RMSEA = 0,041, CFI = 0,984, GFI = 0,984, NFI = 0,975

Figure 2. Structural model representing the relationships between risk perception, food-related health concerns and consumption frequency of fresh vegetables. (Ovals represent latent variables or factors, while rectangles represent observed variables. One-headed arrows represent predictive values between factors and observed variables).

Figura 2. Modelo estructural que representa la relación entre percepción de riesgo, preocupación en salud asociada a los alimentos y frecuencia de consumo de vegetales frescos. (Óvalos representan variables latentes o factores, mientras que el rectángulo representa la variable observada. Flechas de una cabeza representan valores predictivos entre factores y variable observada).

Table 3. Indirect effect among food-related health concerns (FHC) and consumption frequency of fresh vegetables (CFFV) through risk perception (RP).

Tabla 3. Efecto indirecto entre la preocupación en salud ligada a los alimentos (FHC) y la frecuencia de consumo de hortalizas frescas (CFFV), a través de la percepción de riesgo (RP).

Mediation confidence interval								
Independent variable	Mediator	Dependent variable	Value	Se	Lower	Upper	Z values	p-value
FHC	RP	CFFV	0.029	0.0073	0.0148	0.0432	4.0024	0.000

Table 4. The moderation test for Low and High Risk Perception of fresh vegetable buyers.

Tabla 4. Prueba de moderación para una Alta y Baja Percepción de Riesgo de los compradores de vegetales frescos.

Groups	χ^2 constrained model	DF constrained model	χ^2 unconstrained model	DF unconstrained model	$\Delta \chi^2$	Result on Moderation (H5)	Standard estimate path
Low RP	27.026	5	7.134	4	19.892	***	0.41
High RP	19.329	5	2.513	4	16.816	***	0.31

This finding is in line with Lee *et al*. (2013) who found that health concerns have a significant impact on behavioral intentions. In addition, FHC is a relevant dimension for consumers at the moment of purchasing vegetables, due to their health benefits (24, 34, 40, 49).

Furthermore, people concerned about their health and healthy eating habits can for example, opt for the consumption of functional foods (16) and/or organic products as a way to reduce the chemical and technological risks associated with food (2, 3). Another finding of this study is the confirmation of the second hypothesis (H2), which concludes that FHC is significantly related to RP. This finding is supported by the previous study about organic foods of Naspetti and Zanoli (2006), who noted that the risk perception is influenced by a generalized health concerns. In addition, it is important to highlight that RP influences CFFV in a minimum, but significant (H3). This finding is based on the logic that a consumer is not thinking about RP at the time of purchase. However, this RP is not triggered until the consumer is faced with an event that affects their health. In this context, Kaptan *et al* (2018) noted that technologies applied to food production tend to potentially be associated with higher levels of RP, linked to perceptions that the food itself is unnatural. Notwithstanding, for some risks that involve biological irreversibility, moral or ethical concerns may be more important determinants of consumer responses than risk or benefit.

The tests of hypotheses of mediation (H4) and moderator effects (H5) of RP on the relationship the between FHC and CFFV are significant. However, the moderator effect of RP on the relationship between FHC and CFFV is higher than RP mediator effects. This means that the RP construct reduces the direct effect of FHC on CFFV when people consider vegetables a higher latent hazard. Therefore, our findings add evidence that consumers show concern about the RP construct for fresh vegetables despite of all their associated benefits. But RP is a dimension or construct present in the minds of consumers, which makes them question the healthiness of fresh vegetables, which can make people reduce or stop buying vegetables. However, RP as a dimension depends mainly on the personal and indirect food safety experience each person (42). For example, Abass *et al.* (2017) found that RP shown by consumers about urban-grown vegetables was low, even after a decade of vegetable contamination risks in Ghana.

Contrarily, as developing countries achieve higher levels of development, RP should grow following the trends observed in developed countries (23). This means that food safety issues are positioning themselves in developing countries, since some consumers seem to be aware of potential health implications when buying and consuming uncertified fresh vegetables. Hence, from a consumer´s point of view, it is necessary to tackle hygiene problems of traditional markets (28, 45). One mitigation option is to certify fresh vegetables as a food risk-reduction strategy; and a food safety labels can be used as a tool for this purpose (4, 54). However, according to Milne (2012), it is essential for the population itself to demand safer products and defend their own right to know how fresh vegetables are produced, stored and commercialized in domestic markets. Consumers do not wonder about whether pesticides comply or not with their withholding period or about whether products have water

quality certification or good storage conditions, among other risk factors related to fresh vegetables (5). Therefore, it is imperative for the Chilean government to improve surveillance systems on fresh vegetable producers in order to ensure the quality of the produce destined for domestic markets.

As for the limitations of the study, it is worth noting that the sample is not representative of Chile´s population distribution. However, the sample is made up of consumers who are in charge of buying vegetables for the household, as acknowledged by the higher proportion of female interviewees, a situation similar to developed countries (38, 47).

Conclusions

We found that the frequency of vegetable consumption is mainly determined by consumers´ food-related health concerns; and, to a lesser extent, by consumers´ risk perception. However, risk perception is a latent variable which reduces the direct effect of food-related health concerns on the consumption frequency of fresh vegetables through its moderator effect. Therefore, all stakeholders involved (fresh vegetable supply chain agents, government institutions and the private sector) must be aware of the risk associated with fresh vegetables, in order to avoid exposing consumer to potential health risks.

Besides keeping these risks controlled, governments, especially in developing countries must continue with promotional campaigns about vegetable and fruit consumption to prevent chronic diseases associated with poor eating habits and to improve the population's physical condition. In terms of food safety, as a public health policy, the state should strengthen the surveillance of vegetable production systems to reduce the long-term risk associated with traditional markets. Governments should also invest resources in GAPs training for vegetable producers, as well as in quality assurance systems for commercial agents dealing with domestic markets.

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Postharvest control of *Rhizopus stolonifer* on blackberry (*Rubus fruticosus*) by blackberry native crop bacteria

Control poscosecha de *Rhizopus stolonifer* en zarzamora (*Rubus fruticosus*) por bacterias nativas de zarzamora

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Abstract

The potential of four native bacterial strains of blackberries cv. Brazos (*Rubus fruticosus*): *Bacillus subtilis (BSS)*, *Bacillus subtilis (BSL), Bacillus licheniformis (BLI)* and *Leifsonia aquatica (LAQ)*, was evaluated for the postharvest control of soft rot caused by *Rhizopus stolonifer* in blackberry fruits. The fruits were treated with cell suspensions (CS) and cell-free supernatants (CFE) from each bacterial strain and were infected with two strains of *R. stolonifer (RSA and RSC)*. The severity and inhibition percentage of the disease were determined. Additionally, the inhibition by siderophores and the inhibition percentage of *R. stolonifer* spore germination were analyzed as possible control mechanisms. The CS of BSS inhibited RSA by 45.8%, followed by CFE of LAQ which controlled the same strain by 39.7%. The CS of BLI inhibited RSC by 37.7%, whereas the CFE of BSS and LAQ controlled it by 47.7 and 41.8%, respectively. All bacterial strains inhibited RSA and RSC by siderophores production (38.7 to 48.6 %) and the inhibition of spore germination of RSC was higher than 93% after 48 h. This work is one of the first to report *R. stolonifer* control by native bacteria CS and CFE, particularly LAQ in postharvested blackberry fruits. These results show the combination of mechanisms used by bacteria to control both *R. stolonifer* strains.

Keywords
Postharvest berries • Biocontrol • Cell-free Extract • *Leifsonia* • *Bacillus* • *Rhizopus stolonifer* • Soft rot

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Resumen

Fue evaluado el potencial de cuatro cepas bacterianas nativas de zarzamora var. Brazos (*Rubus fruticosus*): *Bacillus subtilis* (BSS), *Bacillus subtilis* (BSL), *Bacillus licheniformis* (BLI) y *Leifsonia aquatica* (LAQ), para el control en poscosecha de la podredumbre blanda causada por *Rhizopus stolonifer* en frutos de zarzamora. Los frutos fueron tratados con suspensiones celulares (CS) y extractos libres de células (CFE) de cada cepa bacteriana y fueron infectados con dos cepas de *R. stolonifer* (RSA y RSC). Se determinó el porcentaje de severidad e inhibición de la enfermedad. Además, fueron analizados como posibles mecanismos de control, la inhibición por sideróforos y el porcentaje de inhibición de la germinación de esporas de *R. stolonifer*. La CS de BSS inhibió un 45,8% a RSA, seguido por CFE de LAQ que controló la misma cepa en un 39,7%. El CS de BLI inhibió a RSC un 37,7%, mientras que el CFE de BSS y LAQ lo controlaron un 47,7 y 41,8%, respectivamente. Todas las cepas bacterianas inhibieron RSA y RSC por producción de sideróforos (38,7 a 48,6%) y la inhibición de la germinación de RSC por esporas fue mayor que 93% después de 48 h. Este trabajo es uno de los primeros en informar el control de *R. stolonifer* por CS y CFE de bacterias nativas, particularmente LAQ en poscosecha de frutos de zarzamora. Estos resultados muestran la combinación de mecanismos utilizados por las bacterias para controlar ambas cepas de *R. stolonifer*.

Palabras clave

poscosecha de frutillas • Biocontrol • Extracto libre de células • *Leifsonia, Bacillus* • *Rhizopus stolonifer* • Podredumbre blanda

Introduction

Global consumers' growing interest in including nutraceutical compounds in their diet has caused an increase in the market of fresh products rich in these compounds, such as beetroot and berries (1, 5); among them, blackberries, whose phytochemical and antioxidant content, in addition to their sweet flavor and smell, have contributed to make them a well-renowned and widely consumed product (12, 29). One of the most frequent production problems is the high susceptibility to mechanical damage during postharvest manipulation, which in turn contributes to the development of fungal diseases (8), such as soft rot, caused by *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill, thereby causing great economic losses in this crop. *R. stolonifer* control has been primarily performed through the application of synthetic fungicides (4, 16, 34). However, various collateral effects, such as the development of fungal resistance, environmental and human health damage, have been attributed to those products (2, 11). Postharvest disease control exacerbates the problem as producers face the dilemma of offering damage-free products with good external quality and innocuous at the same time. Biological control with the use of antagonistic microorganisms has showed positive results in the control of different fruit pathogens, and it is considered a viable alternative to chemical control since it is also environmentally friendly and low-cost (3, 6, 13, 22, 31, 36). An example of the above are the works of Arrebola, *et al.* (2010) and Govender *et al.* (2005), where *Bacillus* species strains were used to control postharvest diseases in citrus fruits and mango, respectively.

Furthermore, it has been posed that the use of cell-free supernatants, produced by phytopathogen antagonistic bacteria, is less risky than the use of bacterial cells; in this context, some works have reported that cell-free supernatants of *Pseudomonas* and *Bacillus* reduced the occurrence of phytopathogenic fungi in apple and tangerine fruits (17, 19, 21). For the control of *Rhizopus stolonifer,* it has been proposed to apply yeast (35) and bacteria (7, 34) in tangerines, peaches, apricots and grapes in postharvest; however, this is, as far as you know, the first work exploring its control in blackberry fruits. Due to the above, the objective of this work was the evaluation of the *Rubus fruticosus* native bacteria's potential, as well as its cell-free supernatants on the control of soft rot in blackberry fruits (*Rubus fruticosus*). Moreover, tests were conducted to explore possible mechanisms to control *Rhizopus stolonifer* used by the bacterial strains analyzed.

Materials and methods

Biological material

Fungal strains

Two strains of *Rhizopus stolonifer*, RSA and RSC, were used, which were selected because they presented different degrees of pathogenicity. Both were isolated from Brazos variety blackberries collected in Los Reyes, Michoacán, Mexico. The fruits were placed in a humid chamber and incubated in a growing room at 25°C during 5 d until the characteristic mycelium appeared; then, 5-mm-diameter sections were taken from the fruits covered with mycelium, and they were placed in Petri dishes with Potato Dextrose Agar (PDA) medium and were incubated at 25°C. Once the mycelium sporulated, serial dilutions were performed to obtain individual spores, which were placed in tubes with Potato Dextrose Broth (PDB) to obtain pure cultures (16). The isolates were identified by observation in an inverted microscope at 40X (1X71S8F-3, Olympus America Inc., Center Valley, PA, USA) and a stereoscope at 10X (SMZ-1500, Nikon Incorporated, Melville, NY, USA) using the keys reported by Schipper (1984). The strains identified were inoculated again in Petri dishes with PDA and incubated at 25°C during 5 d, at the end of which they were used to follow the Koch's postulates to confirm their pathogenicity. In both strains of *R. stolonifer*, blackberry fruits with export quality, uniform size, shape, weight and color, and free of physical damage, were used. To disinfect them, they were submerged in a sodium hypochlorite solution at 1.5% (vv^{-1}) during 5 min and were rinsed with sterile distilled water. The fruits were placed in humid chambers and inoculated with 50 µL of a spore suspension of the corresponding strain of *R. stolonifer* ($1x10^5$ spores mL^{-1} grown in PDB) and incubated in a digital incubator (M-815, Thermo Electron Corp., Marietta, OH, USA) at 25°C during 72 h. Fifteen fruits were evaluated per treatment with three replicates each.

Bacterial strains

Four bacterial strains isolated from a commercial crop of Brazos variety blackberries, located in Los Reyes, Michoacán, Mexico, were used. The antagonist activity against *R. stolonifer* was verified in a previous study (10): *Bacillus subtilis* isolated from soil (BSS), *Bacillus subtilis* (BSL), *Bacillus licheniformis* (BLI) and *Leifsonia aquatica* (LAQ) isolated from leaves, which were identified at a molecular level through the extraction of chromosome DNA in accordance with Harwood and Cutting (1990), amplification of 16S rDNA region, with the universal oligos: 27F TACGGYTACCTTGTTACGACTT and 2RAGAGTTTGATCMTGGCTCAG; the

amplified products were identified by the company Macrogen Corp. USA.

The comparison of the amplified products from 16S rDNA with the database (BLAST, nr/nt) revealed that *B. subtilis S* (BSS) (1403 pb), *B. subtilis* L (BSL) (1401pb), *B. licheniformis* (BLI) (1402pb) and *L. aquatica* (LAQ) (1393pb) corresponded to the species previously identified with the biochemical tests. The strains were preserved in Petri dishes with nutrient agar (Bioxon and Becton Dickinson, Mexico City, Mexico) at 4°C and were subcultured in the same growth medium 24 h before their use in the bioassays.

Antagonism by reduced siderophore production

The siderophore production by the bacterial strains was confirmed by growth in PDA medium with chrome azurol. To relate the production of siderophores to the inhibition of growth of both *R. stolonifer* (RSA and RSC), an antagonism test in iron-rich medium was performed under the supposition that, upon this element's high bioavailability, the microorganisms would not be forced to compete for it. PDA added with 100 mg of $FeCl_3$ (Baker Analyzed Reagent, J. T. Baker, Phillipsburg, NJ, USA) per liter of culture media (Rachid and Ahmed, 2005) was used. At four equidistant points of a 98 mm diameter Petri dish, the bacterial strains were inoculated by means of a roast, spreading them in an area of 1 cm in diameter at each point. Agar discs with mycelium were placed with 5-mm-diameter of each strain of the pathogen (RSA or RSC) in the center of the Petri dishes and were incubated in a digital incubator (M-815, Thermo Electron Corp., Marietta, OH, USA) at 28°C during 72 h; the growth diameter of the discs with mycelium was measured with a digital Vernier every 24 h. As control, iron-free PDA plates were used inoculating the pathogen as described above. The test ended when the mycelium fully covered the dishes of the control treatment. The formula used to calculate the antifungal index was Antifungal Index (%) = $(1 - Da\ Db^{-1})\times100$; where: Da is the diameter of the growth zone of the disc (cm) in the specimen dish, and Db is the diameter of the growth zone of the disc (cm) in the control dish (32). Three plates were used as an experimental unit and each treatment had 3 replicates.

Inhibition test of *R. stolonifer* spore germination

This test was performed in accordance with Bryk *et al.* (1998) with modifications. Bacterial suspensions of all the strains were prepared by inoculations thereof in 50 ml of PDB, they were incubated at 25°C during 24 h under constant stirring and adjusted to a $1X10^6$ cfu mL^{-1} density. Suspensions of RSA and RSC fungal strains were prepared by inoculations in PDB at 25 °C for 72 h, to obtain a final concentration of $1x10^5$ spores mL^{-1}. The suspensions of each bacterial strain (BSS, BSL, BLI and LAQ) were mixed with the RSA or RSC spore suspension in test tubes in a 1:2 proportion (v:v bacteria: fungi, respectively). The tubes were incubated at 25°C for 12, 24 and 48 h. As control, the spore suspension of the corresponding strain mixed with PDB in the same proportion as the remaining treatments was used. After each incubation time, an aliquot of each treatment was taken to be observed under the microscope. Three replicates per treatment were used. Ten spores in 10 fields were examined in each sampling time with a compound microscope at 40X (Axiolab-450907, Carl Zeiss Inc., Thornwood, NY, USA).

The amount of germinated and non-germinated spores was recorded and expressed as germination percentages of *R. stolonifer* present in the different strains.

Antagonism test (*in vivo*)

Suspensions of each bacterial strain were prepared in 150 ml of nutrient broth (Baker Analyzed Reagent, J. T. Baker, Phillipsburg, NJ, USA) incubating at 25°C for 24 h under constant stirring. Cell-free supernatants (CFE) were prepared to know the strains' capacity to produce water-soluble extracellular compounds with antifungal activity. These were obtained through CS centrifugation (RMC-2, Wheaton Science Products, Millville, NJ, USA) at 10 000 rpm per 10 min, the supernatant was filtered through a 0.22 µm nylon membrane (Millipore Corporation, Bedford, MA, USA).

Brazos variety blackberry fruits with export quality, uniform size, shape, weight and color, and free of physical damage, were used, which were disinfected as previously described and submerged in the corresponding bacterial suspension (CS) or cell-free extract (CFE) during 2 h, sprinkled with 10 ml of a spore suspension ($1x10^5$ spores mL^{-1}) of RSA or RSC strains, placed in humid chambers and incubated at 25 °C during 72 h. No damage was inflicted to the fruits prior to inoculation of the *R. stolonifer* strains. Fifteen fruits per treatment were evaluated with three replicates each. The treatments analyzed were: Fruits inoculated with the RSA or RSC strain (control with pathogen); fruits inoculated with PDB only (control without pathogen); fruits inoculated with cell suspensions (CS) of the 4 respective bacteria compared with the RSA or RSC strain; fruits inoculated with cell-free supernatants (CFE) of the 4 respective bacteria compared with RSA or RSC.

The severity of the infection was determined every 24 h during 5 d using the scale previously described in the pathogenicity test. The severity index was calculated with the formula reported by Pérez, *et al.* (1995) and the pathogen inhibition percentage with the same formula, but expressing the severity levels as percentages.

Statistical analysis

All of the experiments were performed with a completely random design. The fungal inhibition percentage data were transformed with the Arcsine of the square root. The data were processed by means of an analysis of variance and a Tukey means comparison using SAS® System for Windows 9.0 version (SAS Institute, Cary, NC, USA). The differences between the means were considered significant with a $p \leq 0.05$.

Results

***R. stolonifer* strains pathogenicity**

The identity of the *R. stolonifer* (RSA and RSC) strains obtained from the blackberry plants was confirmed by the Schipper (1984) keys. The fruits inoculated with both strains developed mycelium after 24 h, covered the fruit and generated exudates, the infection and softening appeared after 48 h, and after 72 h the fruits were degraded and with signs of fermentation. The infection percentage was 90% for RSA and 100% for RSC.

Antagonism by reduced siderophore production

The results showed that LAQ had a controlling effect on the RSA strain significantly higher than the remaining treatments for it presented an antifungal index

0.8 times higher than BSS and 0.7 times higher than BSL; followed by the BLI strain, which exceeded 0.9 and 0.8 times the inhibitory power of BSS and BSH, respectively. The RSC strain was controlled by BLI, which showed the higher antifungal index exceeding 0.8 times BSS and 0.85 times BSH, followed by LAQ whose antifungal index exceeded 0.9 and 0.8 times that presented by BSS and BSH, respectively (table 1). It is worth mentioning that BLI and LAQ, grown in an enriched medium, presented inhibition percentages on the growth of both *R. stolonifer* strains evaluated higher than those obtained in medium without $FeCl_3$.

Inhibition test of *R. stolonifer* spore germination

The results obtained showed that BSS and BLI allowed the germination of 6.10 and 18.7% of the RSC spores after 48 h, whereas, in the control, there was observed the germination of 90% of the spores after the same time (table 2, page 312).

In BSL and LAQ treatments, the amount of RSC spores reduced by 96% and 91% respectively after 12 h; this was due to their lysis, since fragments thereof were observed in all of the fields analyzed. In that time, no spore germination was found in said treatments; however, the remaining spores germinated after 48 h (3.5 and 5.2%, respectively) (table 2, page 312). Furthermore, the treatments applied had no effect on the germination of RSA spores, since they reached similar germination percentages to those of control (87-90%) after 48 h (Data not shown).

Antagonism test (*in vivo*)

The four native bacterial strains of blackberry plants showed positive effects to prevent soft rot caused by the RSA and RSC fungal strains.

Table 1. Antagonism of native bacterial strains of blackberry (*Rubus fruticosus* cv. Brazos) against R. *stolonifer* in medium enriched in $FeCl_3$.

Tabla 1. Antagonismo de cepas bacterianas nativas de zarzamora (*Rubus fruticosus* var. Brazos) contra *R. stolonifer* en medio enriquecido en $FeCl_3$.

Antagonist Agent	Antifungal Index (%)			
	With $FeCl_3$		Without $FeCl_3$	
	RSA	RSC	RSA	RSC
BLI	46.1±0.5^{c}	48.3±0.7^{b}	41.4±2.3^{d}	42.7±2.4^{d}
BSS	42.7±0.6^{d}	43.1±0.4^{d}	40.0±1.3^{d}	41.6±2.1^{d}
LAQ	48.6±0.3^{b}	47.0±0.3^{c}	36.4±2.0^{e}	41.9±2.9^{d}
BSL	38.7±0.5^{e}	41.2±1.2^{e}	37.6±1.1^{e}	36.2±1.1^{e}
Control (-)	0.00±0.0^{f}	0.00±0.0^{f}	0.00±0.0^{f}	0.00±0.0^{f}
Control (+)	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}

Means ± standard deviation are presented. The different superscript letters in the same column indicate significant differences according to the Tukey test ($p \leq 0.05$, n=3). RSA: *Rhizopus stolonifer* A strain; RSC: *Rhizopus stolonifer* C strain. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica*; *BSL: Bacillus subtilis* L; Control (-): PDB medium; Control (+) *Rhizopus stolonifer* A or C strains, respectively.

Se presentan las medias ± desviación estándar. Letras superíndices diferentes en la misma columna indican diferencias significativas de acuerdo a la prueba de Tukey ($p \leq 0,05$, n=3). RSA: *Rhizopus stolonifer* cepa A; RSC: *Rhizopus stolonifera cepa* C. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica*; *BSL: Bacillus subtilis* L; Control (-): medio PDB; Control (+) *Rhizopus stolonifer* cepas A ó C respectivamente.

Table 2. Inhibition of germination of spores of *R. stolonifer* (RSC) by the native bacterial strains of Blackberry (*Rubus fruticosus* cv. Brazos).

Tabla 2. Inhibición de la germinación de esporas de *R. stolonifer* (RSC) por cepas bacterianas nativas de Zarzamora (*Rubus fruticosus* var. Brazos)

Treatments	Spore germination percentage of RSC (%)		
	12 h	24 h	48 h
BLI+RSC	3.8±0.7^{b}	4.7±0.8^{c}	6.1±0.1^{c}
BSS+RSC	9.2±0.5^{b}	15.8±0.3^{b}	18.7±0.4^{b}
LAQ+RSC	0.0±0.0^{c}	1.8±0.5^{d}	5.2±0.7^{d}
BSL+RSC	0.0±0.0^{c}	1.1±0.7^{d}	3.5±0.2^{d}
RSC	81.3±6.7^{a}	89.0±2.6^{a}	89.9±1.1^{a}

Means ± standard deviation are presented. The different superscript letters in the same column indicate significant differences according to the Tukey test ($p \leq 0.05$, n=3). RSC: *Rhizopus stolonifer* strain C; BLI: *Bacillus licheniformis*, BSS*: Bacillus subtilis S*, LAQ: *Leifsonia aquatica, BSL: Bacillus subtilis* L.

Se presentan las medias ± desviación estándar. Letras superíndices diferentes en la misma columna indican diferencias significativas de acuerdo a la prueba de Tukey ($p \leq 0,05$, n=3). RSC: *Rhizopus stolonifer* cepa C; BLI: *Bacillus licheniformis*, BSS*: Bacillus subtilis S*, LAQ: *Leifsonia aquatica, BSL: Bacillus subtilis* L.

The fruits treated with CS of BSS showed a significant inhibition (45.8%) on the growth of the RSA strain, which was 1.6, 1.6 and 2.5 times higher than that presented by BLI, LAQ and BSL treatments, respectively. This severity level was 1.8 times lower than that showed by the RSA control (4.25). Furthermore, the treatment with CFE of LAQ inhibited 39.7% the production of mycelium, which was 2.1, 1.9 and 1.7 higher than the inhibition percentage presented by CFE of BLI, BSS and BSL respectively; showing a severity level of 2.5, which was 1.7 times lower than that presented by the RSA control ($p \leq 0.05$). The fruits of the treatment with CFE of BSS showed an inhibition on the growth of RSC strain of 47.7%, with a severity level of 2.5, which was 1.8 and 1.6 times higher than that of CFE treatments of BLI and BSL, respectively, and 1.7 times lower than that presented by the RSC control. Similarly, the treatment with CFE of LAQ permitted the inhibition of fungal growth by 48.8%, which was 6 and 1.4 times higher than that of CFE treatments of BLI and BSL, with a severity level of 2.8, which was 1.5 lower than that of the RSC control (table 3, page 313).

Discussion

In the last years, postharvest pathogen biocontrol strategies have been developed for a wide range of fruits, including compact fruits with thick and waxy cuticle, such as: mangoes (*Manguifera indica*); citrus fruits such as oranges (*Citrus sinensis*) and tangerines (*Citrus reticulata*); tomatoes (*Solanum lycopersicum*), papaya (*Carica papaya*) and peaches (*Prunus persica*), among others (3, 6, 28). In the aforementioned fruits, it is necessary to make a small cut on the epidermal cuticle so that the pathogens enter successfully and cause an infection (18). However, berries, such as blackberries, are highly vulnerable to the attack of postharvest pathogens, even without any injuries, due to their soft and thin cuticle.

Table 3. Control of *R. stolonifer* with bacterial suspensions (CS) and cell-free extracts (CFE) of native bacterial strains of blackberry (*Rubus fruticosus* cv. Brazos).

Tabla 3. Control de *R. stolonifer* con suspensiones bacterianas (CS) y extractos libres de células (CFE) de cepas bacterianas nativas de zarzamora (*Rubus fruticosus* var. Brazos).

Treatments	Antagonist Agent	Severity Index *		Inhibition (%)**	
		RSA	RSC	RSA	RSC
Cell Suspension (CS)	*BLI*	3.0±0.1^{c}	3.05±0.05^{cd}	28.2±0.1^{c}	37.7±0.05^{cd}
	BSS	2.3±0.1^{b}	3.4±0.05^{de}	45.8±0.1^{b}	30.6±0.05^{de}
	LAQ	3.1±0.1^{cd}	3.2±0.05^{cde}	27.06±0.1^{cd}	33.6±0.05^{cde}
	BSL	3.3±0.1^{cd}	3.2±0.05^{cde}	22.3±0.1^{cd}	33.6±0.05^{cde}
Cell-Free Extract (CFE)	*BLI*	3.4±0.05^{d}	3.6±0.05^{e}	18.8±0.05^{d}	25.5±0.05^{e}
	BSS	3.4±0.3^{dc}	2.5±0.2^{b}	20.0±0.3^{cd}	47.7±0.2^{b}
	LAQ	2.5±0.1^{b}	2.8±0.1^{bc}	39.7±0.1^{b}	41.8±0.1^{bc}
	BSL	3.3±0.1^{cd}	3.4±0.3^{de}	22.3±0.1^{cd}	29.5±0.3^{de}
Control	Control (-)	1.0±0.0^{a}	1.0±0.0^{a}	100.0±0.0^{a}	100.0±0.0^{a}
	Control (+)	4.2±0.05^{e}	4.9±0.1^{f}	0.0±0.0 ^{e}	0.0±0.0^{f}

Means ± standard deviation are presented. The different superscript letters in the same column indicate significant differences according to the Tukey test ($p \leq 0.05$, n=3). *Expressed as severity level based on a scale. **Calculated based on severity levels. RSA: *Rhizopus stolonifer* A strain; RSC: *Rhizopus stolonifer* C strain. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica; BSL: Bacillus subtilis* L; Control (-): PDB medium; Control (+) *Rhizopus stolonifer* A or C strains, respectively.

Se presentan las medias ± desviación estándar. Letras superíndices diferentes en la misma columna indican diferencias significativas de acuerdo a la prueba de Tukey ($p \leq 0,05$, n=3). * Nivel de severidad basado en una escala. **Calculado con base en los niveles de severidad. RSA: *Rhizopus stolonifer* cepa A; RSC: *Rhizopus stolonifera cepa* C. BLI: *Bacillus licheniformis*; BSS*: Bacillus subtilis S*; LAQ: *Leifsonia aquatica*; *BSL: Bacillus subtilis* L; Control (-): medio PDB; Control (+) *Rhizopus stolonifer* cepas A ó C respectivamente.

In this study, the *R. stolonifer* strains isolated from blackberries were capable of causing soft rot in the fruits even if they did not have any mechanical damage, for they are polydrupes, and this type of aggregates makes them susceptible to damages and hinders their treatment before selection for trade. Therefore, these berries are often packed without ensuring their preservation from pathogen attacks (20).

In this work, the effect of native bacteria of blackberry plants was evaluated, as well as their cell-free supernatants (CFE) in the control of soft rot of Brazos variety blackberry fruits (*Rubus fruticosus*). Interestingly, the growth of RSA was significantly reduced upon applying the CS of BSS, showing an inhibition percentage of 45.8%. These results coincide with those reported by Wang, *et al.* (2013) who used the *B. subtilis* SM21 strain to reduce by 37.2% the rot caused by *R. stolonifer* in peaches. Bonaterra *et al.* (2003) also reported the control of *R. stolonifer* in fruits damaged and subsequently treated by the *Pantoea agglomerans* EPS125 strain in different cultivars, such as nectarines (80-100%), apricots (60-97%) and peaches (76-86%).

Moreover, the use of cell-free supernatants (CFE) of the 4 bacterial strains tested in this study against *R. stolonifer*, allowed to confirm that they may be effective to control the pathogen, thus reducing the potential risks of applying bacterial directly cells to the fruits. This coincides with the proposal of Janisiewicz, W. and Roitman, J. (1988), who have proposed that the use of cell-free supernatants poses a lower risk

to human health than the application of living bacterial cells. Said authors applied *B. subtilis* 155 *in vitro* cell-free supernatants to inhibit the growth of gray mold caused by *Botrytis cinerea* with positive results. In contrast to the aforementioned study, in this work the evaluation was *in vivo*; however, the effect of CFE of BSS on postharvest disease control was shown with a 47.7% RSC development inhibition. In addition, the controlling capacity of CFE of LAQ was observed with a 39.7% RSA growth inhibition and a 41.8% of RSC and, as far as you know, this is the first report of the antagonistic effect of this bacterium and its CFE against soft rot caused by *R. stolonifer* in blackberries. Similar results were reported by using CFE of *Bacillus licheniformis* (EN74-1) to control gray mold in apple fruits, caused by *Botrytis mali,* accomplishing 58.8% of the pathogen growth (17). The CFE of *Pseudomonas syringae* showed antifungal activity against *Penicillium digitatum*, although this was lower than that obtained when the bacterial cells were applied (22).

Biocontrol may be understood as a dynamic process where interactions among fruit-pathogen-antagonist play a decisive role to preserve the system's integrity. Therefore, the biocontrolling activity of the four bacterial strains used in this study may have been the result of a combination of different mechanisms. In this regard, it has been proven that the strains pertaining to the *Bacillus* species, including *B. subtilis* and *B. licheniformis*, are capable of producing surfactants, such as: surfactin, iturin A and amicoumacin, with antagonistic activity capable of restricting pathogenic action (14, 25). This does not exclude the controlling capacity of *B. subtilis* through the production of antibiotics, siderophores and certain volatile compounds (more than 21 different volatile compounds with biocontrol activity have been reported) (3). Regarding the test with $FeCl_3$-enriched medium, an increase in the inhibition percentages was observed in two of the four bacterial strains used in comparison with the iron-free medium tests. LAQ was capable of inhibiting RSA growth with an antimycotic index of 48.6% *versus* a 36.4% of the iron-free medium and the RSC with an antifungal index of 47% of the $FeCl_3$ medium *versus* a 41.9% of the iron-free medium, which suggests that one of the mechanisms used by both strains to accomplish the control of the pathogen was the production of siderophores.

On the other hand, the results obtained in the inhibition of spore germination of the RSC strain showed that the four strains studied produce substances capable of inhibiting their germination; BLI and BSS strains were able to obtain high spore germination inhibition percentages (93.9% and 81.2%, respectively) after 48 h. Furthermore, BSL and LAQ treatments were able to lyse the RSC spores after 12 h (96 and 91%, respectively). These results coincide with the observations by Bryk *et al.* (1998), who reported that this mechanism was used by *Erwinia herbicola* to control *B. cinerea* and *P. expansum*.

Other authors attribute similar effects to the production of lytic enzymes, such as chitinases and glucanases by bacteria such as *B. subtilis*, which act on the cell wall of the pathogen compromising its integrity (21). Thus, the lysis of the spores is a mechanism that may have contributed to the inhibition of the pathogen germination observed. This early lysis capacity of the pathogen's spores may be fundamental to provide blackberry fruits with protection against postharvest diseases, given that the antagonist must act during the first stages of the infection of the fruit.

These results coincide with those reported by Panebianco *et al.* (2015), who observed that the *P. digitatum* spore germination inhibition by a *P. syringae* strain in liquid medium after 24 h ranged between 70-100%. Similar results were also observed by Ghosh *et al.* (2015), who reported *R. stolonifer* VBAM1 spore inhibition germination by the CFE of *Burkholderia cenocepacia* VBC7, *Pseudomonas poae* VBK1 (95%) and three lactic acid bacteria strains (97%). Even though the role of the *Leifsonia* species in the blackberry agroecosystem has not been yet elucidated, it is well known that this bacterial species are capable of producing biofilms involved in protective and food competition effects (27). It has been reported that oligopeptides, surfactants, and siderophores produced by some bacterial strains are capable of acting as inducing molecules that increase enzymes' activity, such as phenylalanine ammonia-lyase, polyphenol oxidase and superoxide dismutase, which are related to fruit defense mechanisms; as well as catalase and peroxidase which act as postharvest detoxifying agents in the fruit, as it was observed with the application of a cell suspension of *Pseudomonas putida* in papaya (31, 37). In this work, the four strains used were capable of producing siderophores with an important RSA and RSC growth inhibition activity by BLI and LAQ, which may have induced the production of some of the abovementioned enzymes, thereby causing the control effect observed.

Although the *Bacillus subtilis* is a well-known biological control agent, whose efficacy in postharvest control has been reported in many papers and from which the secondary metabolites that it actively produces have been isolated and characterized (23, 33), it is worth mentioning that there is no evidence of any work focused on postharvest control of fungal diseases in blackberry fruits. Additionally, *Leifsonia aquatica* has not been reported as an antagonist, producer of antifungals. Further research is required to characterize the action mechanisms involved in the fungicide effect of the bacteria related to blackberries in order to develop biotechnologies to control soft rot.

This study reports the effect of biocontrol of native bacterial strains associated with (*Rubus fruticosus*) Brazos variety, and their cell-free extracts, to control soft rot in the fruit, highlighting the potential of innovative biological agents such as *Leifsonia aquatica* and its metabolites, which represent an effective alternative for postharvest soft rot control.

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Resistance to *Meloidogyne enterolobii* in sweet potatoes

Resistencia a *Meloidogyne enterolobii* en batatas

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Abstract

The present work was designed to select for sweet potatoes clones (*Ipomoea batatas*) resistant to *Meloidogyne enterolobii* (Syn. *M. mayaguensis*) as well as evaluate the efficiency of the selection methods used by estimating their genetic (VCg) and environmental (VCe) variation coefficients as well as broad sense heritability. A total of 142 sweet potato genotypes were tested, including four commercial varieties (Brazlândia Rosada, Brazlândia Roxa, Brazlândia Branca, and Palmas) as well as the Santa Clara tomato cultivar (utilized as a susceptibility standard). The experimental design was completely randomized blocks, in two repetitions of six plants each. Resistance levels were classified according to the numbers of eggs per gram of roots, the reproduction factor (RF), and the reproduction index (RI) relative to the Santa Clara tomato cultivar. The *b*= *VCg/VCe* ratio and broad sense heritability were high in terms of the numbers of eggs per gram of roots, as well as in terms of the reproduction factor and reproduction index, demonstrating the efficiency of the methodology used in the selection of resistant genotypes. Thirty-one sweet potato genotypes resistant to *M. enterolobii* were identified as having significant potential for continuing the breeding program.

Keywords
Ipomoea batatas • plant breeding • reproduction index • root-knot nematode

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Resumen

Este trabajo fue realizado con el objetivo de seleccionar clones de batata (*Ipomoea batatas*) resistentes a *Meloidogyne enterolobii* (Syn. M. *mayaguensis*) y evaluar la eficiencia del método de selección empleado, por la estimación de los coeficientes de variación genética (CVg) y el medio ambiente (CVe), y de las estimaciones de las herencias en el sentido amplio. Se utilizaron 142 genotipos de batata, entre ellos cuatro cultivares comerciales: Brazlândia Rosada, Brazlândia Roxa, Brazlândia Branca y Palmas, y el tomate cv. Santa Clara (utilizado como patrón de susceptibilidad). El diseño experimental utilizado fue de bloques aleatorizados completos con dos repeticiones de seis plantas cada uno. La clasificación de los niveles de resistencia fue realizada de acuerdo con el número de huevos por gramo de raíz, factor de reproducción (FR) e índice de reproducción (IR) relativo a lo observado en el tomate Santa Clara. La relación b = CVg / CVe y la heredabilidad en sentido amplio fueron altas tanto para el número de huevos por gramo de raíz como para el factor de reproducción e índice de reproducción, demostrando la eficiencia del método empleado para la selección de genotipos resistentes. Se identificaron, como prometedores para dar continuidad al programa de mejoramiento genético, 31 genotipos de batata resistentes a *M. enterolobii*.

Palabras clave

Ipomoea batatas • mejoramiento genético vegetal • índice de reproducción • nematodo del nudo

Introduction

Sweet potato [*Ipomoea batatas* (L.) Lam.] is one of the most valuable crops cultivated in tropical and subtropical regions for diverse forms of use (22). Sweet potato has multiple uses as food sources for humans (cooked sweet potatoes), or they can be processed industrially to produce starch and flower. They can also be used in animal feeds or as an alternative source of ethanol as a biofuel (1, 3, 7, 15).

Even though sweet potatoes have the potential for high production levels in Brazil, there productivity is generally low, principally due to the use of obsolete and degenerated genetic material that can make them susceptible to pests and diseases (1, 11). Root-knot nematodes (*Meloidogyne* spp.) are one of the major pathogens responsible for the low productivity of this staple in Brazil (13.5 $t.ha^{-1}$) (6, 10).

The principal nematode species that infect sweet potato are *M. javanica* and *M. incognita*, races 1, 2, 3 and 4. Although sweet potatoes are considered "false non-hosts", as they frequently do not display symptoms most characteristic of those pathogens (galls, produced by females ovipositioning on the roots), the secondary roots of these plants can host dense populations of nematodes and the females will penetrate the roots (principally secondary roots) to deposit their egg masses (2, 6).

The use of nematicides as a control method is expensive and inefficient, and the inadequate application of these chemicals can contaminate workers, the sweet potatoes to be consumed, and the general environment (2).

As such, the use of resistant genotypes is indicated for controlling these soil pathogens as this technique does not increase production costs and does not impact the environment (5, 24).

A number of sweet potato varieties have demonstrated resistance to species of *Meloidogyne* knot-root nematodes, such as *M. incognita* (races 1, 2, 3 and 4) and *M. javanica* (12, 23), although new species of these pests have been described that are able to overcome plant defenses (12, 14, 17).

Among the new nematode species with potential for infecting and damaging sweet potatoes cultivars that demonstrate resistance to *M. incognita* and/or *M. javanica,* is *Meloidogyne enterolobii* (Syn. *M. mayaguensis*). The occurrence of *M. enterolobii* (26) was reported for the first time in Brazil in Petrolina (PE). In addition to its ability to disseminate quite rapidly, *M. enterolobii* is polyphagic and has been reported parasitizing ornamental plants, tobacco, soybeans, coffee trees, papaya plants, acerola, araçá, and a number of other horticultural plants (8, 25).

There is currently only scarce information available in Brazil concerning the resistance of commercial cultivars of sweet potatoes to *M. enterolobii* (4). Melo *et al.* (2011) evaluated the resistance of various genotypes of tomatoes, lettuce, common beans, peppers, green peppers, and sweet potatoes to *M. enterolobii* and reported that not a single tomato genotype analyzed (with or without the Mi allele that confers resistance to *M. incognita* or *M. javanica*) was resistant to that nematode. Only one cultivar of beans, two varieties of *Capsicum chinense*, and three of *Capsicum annuum* demonstrated moderate resistance levels.

Only five cultivars of lettuce and two clones of sweet potatoes (UFLA07-49 and UFLA07-53) demonstrated high resistance. Therefore, considering the aggressiveness of *M. enterolobii* and its capacity to infect a wide number of hosts, it will be extremely important to identify cultivars or additional varieties of sweet potatoes with resistance to this predator.

The present work therefore sought to identify sweet potato clones resistant to *M. enterolobii* and evaluate the efficiency of the selective methods used by estimating the genetic and environmental variation coefficients of those plants as well as heritability (in the broad sense).

Material and methods

The present research was undertaken at the Horticulture Experimental Station of HortiAgro Sementes S.A., Fazenda Palmital, in the municipality of Ijaci, Minas Gerais State, Brazil (-21°14'16" x -45°08'00"; 918 m a. s. l.). Sweet potato clones with preliminary productivities evaluated at near or above 50 t of roots per hectare (116 total varieties) were identified and denominated 2007HSF-*xxx*-*yy*, where *xxx* = number of the family of half-sibs and *yy* = the clone number selected within the family (table 2, page 324; table 3 page 325-326 and table 4, page 327-328). A total of 142 sweet potato genotypes were examined (table 2, page 324; table 3 page 325-326 and table 4, page 327-328), including: four commercial cultivars (Brazlândia Rosada, Brazlândia Roxa, Brazlândia Branca, and Palmas), the 116 clones of sweet potatoes 2007HSF-xxx-yy, 11 varieties (denominated UFLA-07-01, UFLA-07-02, UFLA-07-05, UFLA-07-10, UFLA-07-11, UFLA-07-12, UFLA-07-14, UFLA-07-31, UFLA-07-43, UFLA-07-49, and UFLA-07-53, and 11 clones (denominated Amanda, Anaclara, Barbara, Beatriz, Carolina, Duda, Itajubá, Izabela, Julia,

Livia, and Marcela) from among cultivars being examined by the breeding program at the Tocantins Federal University. The Santa Clara tomato cultivar was used as the standard nematode host.

Styrofoam trays (with 72 wells) containing a commercial substrate (approximately 120 ml of substrate per cell) were used to plant branches approximately 20 cm long with 3 to 4 internodal buds. Nematode inoculation was performed 30 days after sowing, using *M. enterolobii* eggs extracted from previously inoculated tomato plants (cv. TOM-684, containing the *Mi* gene and susceptible to *M. enterolobii*), following Hussey and Barker (1973). After washing, the tomato plant roots were cut into small pieces, processed in a blender for 30 seconds in a solution of 0.5% sodium hypochlorite, and subsequently passed through a 0.074 mm sieve (200 mesh) and retained on a smaller sieve (0.028 mm / 500 mesh) in abundant water. The 0.074 mm sieve retained the remnants of the tomato roots while the 0.028 mm sieve collected the eggs of *M. enterolobii*, which were subsequently transferred to a holding beaker (using pure water).

The water level in the beaker was then completed to 1000 ml, and three 1 ml volumes were removed from the homogenized slurry of eggs with the aid of a pipette and subsequently counted in a Peters chamber using a stereomicroscope. An aliquot of this suspension containing 2000 eggs was then used to inoculate the sweet potato plants (using a veterinary syringe to inject eggs in the commercial substrate).

The viability of the inoculum was evaluated in an eclosion chamber, indicating that 63.95% of the eggs were viable, corresponding to an initial population of 1,279 viable eggs per inoculum.

The plants were treated in two repetitions of six plants each. The plants were arranged in trays with 11 rows holding six plants each, with rows of the Santa Clara tomato cultivar alternating with different sweet potato genotypes. As such, one repetition represented a trial with six plants. Sixty days after inoculation with the nematode eggs, the plants were carefully removed from the polystyrene trays and their roots washed to extract the eggs. The numbers of eggs were estimated by counting 1 mL of the suspension in a Peters chamber, using a stereomicroscope. The total numbers of collected eggs were determined by extrapolating from these sample counts.

The parameters used as resistance parameters were: numbers of eggs per gram of roots; reproduction factor (RF = final population / initial population of viable eggs); and the reproduction index. To calculate the number of eggs per gram of roots, the total number of eggs extracted from each radicular system was divided by the fresh mass of the roots. The reproduction factor (RF) was used to define resistance (RF<1) and susceptibility (RF≥1), following Oostenbrink (1966).

The reproduction index of *M. enterolobii* was determined using tomato plants as standard controls (100%). The values of the final populations (Pf) encountered in the sweet potato genotypes were divided by the populations encountered in the tomato controls. Based on these values, it was could defined the levels of resistance of each sweet potato genotype to *M. enterolobii* according to the reproduction index (RI) established by Taylor (1967), in which: S = a susceptible plant (normal reproduction), ratio > 51% in relation to the tomato standard controls; SR = slightly resistant, from 26% to 50%; MoR = moderately resistant, from 11% to 25%; VR = very resistant, from 1% to 10%; HR/I = highly resistant/immune, <1%.

To attend the prerequisites of analyses of variance, the values obtained for all of the parameters were submitted to log transformation (x+1), in which "x"

represents the number of eggs per gram of roots. The transformed data was then submitted to analysis of variance using the SAS software package (18). The averages of the treatments were compared using the Scott and Knott test. Based on the expected mean squares of the analysis of variance, it was estimated the genetic (σ_g^2) and environmental (σ_e^2) variances and broad sense heritability (h_a^2) for each character, as described by Tsegaye *et al.* (2007), using the following equation:

$$h_a^2 = (\sigma_g^2/(\sigma_g^2 + \sigma_e^2))x100$$

The genetic (VCg) and environmental (VCe) variation coefficients, as well as the b index (VCg/VCe) for the characters evaluated were estimated (21), based on the following expressions:

$$VC_g(\%) = (((\sigma_g^2)^{0.5})/\mu)x100,$$

$$VC_e = (((\sigma_e^2)^{0.5})/\mu)x100$$

Results and discussion

There were significant differences between the numbers of eggs per gram of roots, the reproduction factors (RF), and the reproduction indices (RI) of *M. enterolobii* among the sweet potato genotypes, indicating significant genetic variability among those plants (table 1, page 323).

In this respect, it is important to emphasize that the contribution of epigenetic mechanisms involved cannot be ruled out. These may be defined as those mechanisms that cause changes in the genome, although inheritable during cell division, but which do not involve a change in the DNA sequence.

According to Tang and Ho (2007), epigenetic patterns are sensitive to environmental changes that may cause phenotypic changes that will be transmitted to offspring, which do not alter the DNA sequence but are inheritable by mitosis and over generations.

The estimates of the coefficients of environmental variation (VCe) were 7.69%, 22.03% and 11.05%, for the number of eggs per gram of roots, the reproduction factor (RF), and the reproduction index (RI) respectively. These estimates of the coefficient of variation were inferior to those commonly observed in experiments examining nematode resistance in sweet potatoes (13). Very elevated estimates for the coefficients of genetic variation (VCg) were observed in the present work as compared to the estimates of VCe, these being 18.90%, 48.21% and 32.20% for the numbers of eggs per gram of roots, the reproduction factor, and the reproduction index respectively. These results imply that there is a high degree of variability among the samples, and therefore very favorable conditions for the selection of resistant clones - as seen by the values of the ratio *b*= VCg/VCe, which were greater than 2.0 (table 1, page 323) for the three characteristics analyzed.

The estimates of broad sense heritability (h_a^2) were high (above 80%) for the three variables analyzed (table 1, page 323), reinforcing the probability that most of the phenotypic variability was of a genetic nature, and indicating that selection based on these characteristics could be undertaken with efficiency, as was noted above. Similar values of heritability were also described by Andrade Júnior *et al.* (2016), who evaluated sixty-three clones of sweet potato for resistance to *Meloidogyne javanica*. Considering the both classification criteria (reproduction factors and reproduction index), 71.43% of sweet potato clones were classified as resistant.

Based on the criterion of the numbers of eggs per gram of roots, 80.99% of the

genotypes tested were considered susceptible, as their parameters did not differ significantly by the Scott-Knott test at a 5% level of probability from those of the Santa Clara tomato cultivar used as a susceptible control (table 2, page 324).

The sweet potato cultivars 'Brazlândia Rosada', 'Brazlândia Roxa', 'Brazlândia Branca', and 'Palmas' tested here were found to be susceptible to *M. enterolobii*. Marchese *et al.* (2010) noted that the cultivars Palmas and Brazlândia Roxa were resistant to race 1 of *M. incognita*, while 'Brazlândia Rosada' and 'Brazlândia Branca' were susceptible to it. Resistance to *M. incognita* was therefore not found to be associated with resistance to *M. enterolobii.* Twenty-seven sweet potato genotypes (19.01%) were classified as resistant to *M. enterolobii* as they fell into groups that were distinct from the Santa Clara tomato cultivar, by the Scott-Knott test at a 5% level of probability (table 2, page 324).

Fully 78.87% of the sweet potato genotypes tested were considered susceptible by the criteria used by Oostenbrink (1966), with their reproduction factors being equal to or more than 1.0 (table 3, page 325-326); these results were likewise confirmed by the numbers of eggs per gram of roots for the cultivars Brazlândia Branca, Brazlândia Roxa, Brazlândia Rosada, and Palmas.

It was encountered susceptible to highly resistant or immune genotypes according to their reproduction indices and the reproduction criteria established by Taylor (1967), (table 4, page 327-328).

Among the genotypes tested, 78.16% (S= susceptible and LR= slightly resistant) were classified as susceptible, including the varieties Brazlândia Branca, Brazlândia Roxa, Brazlândia Rosada, and Palmas; 4.93% were classified as moderately resistant; 15.49% as very resistant; and 1.40% as highly resistant or immune (table 4, page 327-328).

Table 1. Results of the analyses of variance of the numbers of eggs per gram of root, reproduction factor (RF), and reproduction index (RI) of *Meloidogyne enterolobii* on the roots of sweet potato clones.

Tabla 1. Resultados de los análisis de varianza de los números de huevos por gramo de raíz, factor de reproducción (FR) e índice de reproducción (IR) de *Meloidogyne enterolobii* en las raíces de los clones de batata.

Sources of variation	DF	N° of eggs/g of root	Reproduction factor	Reproduction index
Blocks	1	0.8664626**	0.23411711**	0.57411078**
Genotypes	142	0.7354280**	0.20888558**	0.66287558**
Error	138	0.0563091	0.01975456	0.03684918
Average (μ)		3.083961	0.637924	1.73772
VCe (%)		7.69	22.03	11.05
VCg (%)		18.9	48.21	32.2
b= VCg/VCe		2.46	2.19	2.91
h_a^2 (%)		85.78	82.72	89.47

VCe, coefficient of environmental variation; VCg, coefficient of genetic variation; b index, VCg/VCe; and h_a^2, broad sense heritability.^{ns}Not significant. * and ** Significant at 5% and 1% levels of probability respectively, by the F test. (data expressed as log (x+1), in which x represents the value of the number of eggs/g in the root, reproduction factor, and reproduction index). DF = Degrees of Freedom.

VCe, coeficiente de variación ambiental; VCg, coeficiente de variación genética; índice b, VCg / VCe; y h_a^2, heredabilidad de sentido amplio. ^{ns} No es significativo. * y ** Significativo a niveles de probabilidad de 5% y 1%, respectivamente, mediante la prueba F (datos expresados como log (x + 1), donde x representa el valor de la cantidad de huevos/g en la raíz, el factor de reproducción y el índice de reproducción). DF = Grados de libertad.

Table 2. Average numbers of *Meloidogyne enterolobii* eggs per gram of roots [1] of 142 sweet potato genotypes and the Santa Clara tomato cultivar, utilized as the standard control for susceptibility.

Tabla 2. Promedio de huevos de *Meloidogyne enterolobii* por gramo de raíces [1] de 142 genotipos de batata y el cultivar de tomate Santa Clara, utilizado como control estándar para la susceptibilidad.

GENOTYPE	N° of eggs/g root	GENOTYPE	N° of eggs/g root	GENOTYPE	N° of eggs/g root
2007HSF001-01	1669d[2]	2007HSF010-12	2447d	2007HSF027-05	3095d
2007HSF001-09	912d	2007HSF010-17	2146d	2007HSF027-07	1235d
2007HSF001-16	1913d	2007HSF010-23	41a	2007HSF027-08	299c
2007HSF001-17	5158d	2007HSF010-25	38a	2007HSF027-09	3600d
2007HSF001-21	72a	2007HSF010-30	854d	2007HSF027-10	80b
2007HSF001-22	1607d	2007HSF010-31	6637d	2007HSF027-12	23a
2007HSF001-23	2311d	2007HSF010-33	2376d	2007HSF027-16	3387d
2007HSF001-24	2640d	2007HSF010-35	2297d	2007HSF028-05	1399d
2007HSF001-25	2167d	2007HSF010-37	2050d	2007HSF028-06	2014d
2007HSF001-26	1546d	2007HSF010-41	3198d	2007HSF028-08	3875d
2007HSF001-28	3344d	2007HSF010-47	216c	2007HSF028-11	2020d
2007HSF001-37	3313d	2007HSF011-01	2020d	2007HSF028-16	2724d
2007HSF001-40	2007d	2007HSF011-02	3579d	2007HSF029-01	3192d
2007HSF001-41	1605d	2007HSF011-05	1774d	2007HSF029-02	294c
2007HSF001-45	2905d	2007HSF011-06	2864d	2007HSF029-03	3159d
2007HSF001-47	1451d	2007HSF011-10	5734d	2007HSF029-09	1856d
2007HSF001-58	586d	2007HSF012-02	458c	2007HSF030-02	1349d
2007HSF002-02	196c	2007HSF013-03	2202d	2007HSF030-10	1805d
2007HSF002-04	2662d	2007HSF013-04	1637d	2007HSF031-04	1911d
2007HSF002-05	5074d	2007HSF014-04	3863d	2007HSF031-02	961d
2007HSF002-08	708d	2007HSF014-05	2007d	UFLA07-01	2293d
2007HSF002-11	3062d	2007HSF016-05	113b	UFLA07-02	2248d
2007HSF002-14	24a	2007HSF018-03	2751d	UFLA07-05	3486d
2007HSF002-19	2782d	2007HSF019-01	3213d	UFLA07-10	2750d
2007HSF004-03	318c	2007HSF020-05	2245d	UFLA07-11	3488d
2007HSF004-04	360c	2007HSF020-07	3583d	UFLA07-12	917d
2007HSF004-06	2457d	2007HSF020-08	62a	UFLA07-14	2104d
2007HSF004-08	39a	2007HSF020-12	1844d	UFLA07-31	1312d
2007HSF005-01	3092d	2007HSF021-01	1181d	UFLA07-43	2402d
2007HSF005-03	1706d	2007HSF022-02	2712d	UFLA07-49	146c
2007HSF005-06	2530d	2007HSF022-03	3808d	UFLA07-53	63a
2007HSF006-08	600d	2007HSF022-04	308c	AMANDA	1396d
2007HSF006-13	104b	2007HSF022-05	2665d	ANACLARA	290c
2007HSF006-16	877d	2007HSF022-06	8959d	BARBARA	4785d
2007HSF006-17	2113d	2007HSF022-09	1243d	BEATRIZ	3141d
2007HSF007-04	2559d	2007HSF022-10	1903d	CAROLINA	1058d
2007HSF007-10	781d	2007HSF022-12	23a	DUDA	99b
2007HSF007-15	1032d	2007HSF022-16	77b	ITAJUBA	1747d
2007HSF007-16	849d	2007HSF022-19	2897d	IZABELA	5034d
2007HSF007-17	1298d	2007HSF023-08	177c	JULIA	988d
2007HSF007-18	2500d	2007HSF024-01	313c	LIVIA	45112d
2007HSF007-21	2736d	2007HSF024-02	1994d	MARCELA	2800d
2007HSF007-26	1605d	2007HSF024-04	3683d	BrazlândiaBranca	4083d
2007HSF007-27	1654d	2007HSF024-06	3317d	BrazlândiaRosada	3034d
2007HSF009-06	4243d	2007HSF025-04	3322d	BrazlândiaRoxa	4174d
2007HSF010-01	2608d	2007HSF026-01	78b	Palmas	3727d
2007HSF010-06	2617d	2007HSF026-02	9368d	Tomato cv. Santa	3388d
2007HSF010-08	2790d	2007HSF026-05	894d	Clara	

[1] Statistical analyses refer to the numbers of eggs/gram of root after log (x+1) transformation of the data, presenting the original data. [2] Averages followed by the same letter belong to be same group by the Scott-Knott test at a 5% level of probability.

[1] Los análisis estadísticos se refieren al número de huevos / gramo de raíz después de la transformación (x + 1) de los datos, presentando los datos originales. [2] Los promedios seguidos por la misma letra pertenecen al mismo grupo según la prueba de Scott-Knott a un nivel de probabilidad del 5%.

Table 3. Averages of the reproduction factor (RF) of *Meloidogyne enterolobii* in the 142 sweet potato genotypes evaluated and the Santa Clara tomato cultivar, and the classification of these genotypes in terms of their resistance (R) and susceptibility (S) to nematodes.

Tabla 3. Promedios del factor de reproducción (FR) de *Meloidogyne enterolobii* en los 142 genotipos de batata evaluados y el cultivar de tomate Santa Clara, y la clasificación de estos genotipos en términos de su resistencia (R) y susceptibilidad (S) a los nematodos.

GENOTYPE	RF	Class[2]	GENOTYPE	RF	Class	GENOTYPE	RF	Class
2007HSF001-01	3.40c[1]	S	2007HSF010-12	2.18b	S	2007HSF027-05	7.47d	S
2007HSF001-09	3.13c	S	2007HSF010-17	4.70c	S	2007HSF027-07	1.55b	S
2007HSF001-16	5.62c	S	2007HSF010-23	0.10a	R	2007HSF027-08	0.77b	R
2007HSF001-17	9.75d	S	2007HSF010-25	0.06a	R	2007HSF027-09	8.91d	S
2007HSF001-21	0.04a	R	2007HSF010-30	2.00b	S	2007HSF027-10	0.16a	R
2007HSF001-22	4.93c	S	2007HSF010-31	8.04d	S	2007HSF027-12	0.04a	R
2007HSF001-23	6.77d	S	2007HSF010-33	3.48c	S	2007HSF027-16	6.60d	S
2007HSF001-24	4.97c	S	2007HSF010-35	5.03c	S	2007HSF028-05	2.60c	S
2007HSF001-25	5.63c	S	2007HSF010-37	4.00c	S	2007HSF028-06	2.15b	S
2007HSF001-26	5.26c	S	2007HSF010-41	6.91d	S	2007HSF028-08	4.77c	S
2007HSF001-28	6.21c	S	2007HSF010-47	0.20a	R	2007HSF028-11	5.12c	S
2007HSF001-37	8.86d	S	2007HSF011-01	4.59c	S	2007HSF028-16	6.07d	S
2007HSF001-40	4.16c	S	2007HSF011-02	8.97d	S	2007HSF029-01	5.91c	S
2007HSF001-41	3.19c	S	2007HSF011-05	5.95c	S	2007HSF029-02	0.66a	R
2007HSF001-45	5.59c	S	2007HSF011-06	7.54d	S	2007HSF029-03	7.57d	S
2007HSF001-47	4.10c	S	2007HSF011-10	10.67d	S	2007HSF029-09	6.46c	S
2007HSF001-58	1.09b	S	2007HSF012-02	0.86b	R	2007HSF030-02	3.94c	S
2007HSF002-02	0.23a	R	2007HSF013-03	5.15c	S	2007HSF030-10	4.83c	S
2007HSF002-04	7.10d	S	2007HSF013-04	6.38c	S	2007HSF031-04	4.48c	S
2007HSF002-05	9.46d	S	2007HSF014-04	9.86d	S	2007HSF031-02	2.57c	S
2007HSF002-08	0.96b	R	2007HSF014-05	8.39d	S	UFLA07-01	1.95b	S
2007HSF002-11	4.82c	S	2007HSF016-05	0.13a	R	UFLA07-02	2.74c	S
2007HSF002-14	0.05a	R	2007HSF018-03	5.73c	S	UFLA07-05	4.27c	S
2007HSF002-19	7.14d	S	2007HSF019-01	9.52d	S	UFLA07-10	5.68c	S
2007HSF004-03	0.48a	R	2007HSF020-05	6.22c	S	UFLA07-11	5.03c	S
2007HSF004-04	0.18a	R	2007HSF020-07	6.77d	S	UFLA07-12	2.33b	S

Table 3 (cont.). Averages of the reproduction factor (RF) of *Meloidogyne enterolobii* in the 142 sweet potato genotypes evaluated and the Santa Clara tomato cultivar, and the classification of these genotypes in terms of their resistance (R) and susceptibility (S) to nematodes.

Tabla 3 (cont.). Promedios del factor de reproducción (FR) de *Meloidogyne enterolobii* en los 142 genotipos de batata evaluados y el cultivar de tomate Santa Clara, y la clasificación de estos genotipos en términos de su resistencia (R) y susceptibilidad (S) a los nematodos.

GENOTYPE	RF	Class[2]	GENOTYPE	RF	Class	GENOTYPE	RF	Class
2007HSF004-06	4.08c	S	2007HSF020-08	0.11a	R	UFLA07-14	2.27b	S
2007HSF004-08	0.10a	R	2007HSF020-12	3.15c	S	UFLA07-31	2.58c	S
2007HSF005-01	10.99d	S	2007HSF021-01	2.65c	S	UFLA07-43	3.47c	S
2007HSF005-03	3.49c	S	2007HSF022-02	9.35d	S	UFLA07-49	0.24a	R
2007HSF005-06	4.61c	S	2007HSF022-03	7.10d	S	UFLA07-53	0.12a	R
2007HSF006-08	0.08a	R	2007HSF022-04	0.82b	R	AMANDA	3.12c	S
2007HSF006-13	0.19a	R	2007HSF022-05	5.76c	S	ANACLARA	0.48a	R
2007HSF006-16	1.79b	S	2007HSF022-06	11.30d	S	BARBARA	7.48d	S
2007HSF006-17	3.02c	S	2007HSF022-09	4.04c	S	BEATRIZ	4.23c	S
2007HSF007-04	8.81d	S	2007HSF022-10	4.09c	S	CAROLINA	0.78b	R
2007HSF007-10	1.30b	S	2007HSF022-12	0.08a	R	DUDA	0.14a	R
2007HSF007-15	1.50b	S	2007HSF022-16	0.12a	R	ITAJUBA	5.76c	S
2007HSF007-16	1.56b	S	2007HSF022-19	6.92c	S	IZABELA	12.07d	S
2007HSF007-17	1.88b	S	2007HSF023-08	0.32a	R	JULIA	1.30b	S
2007HSF007-18	5.29c	S	2007HSF024-01	0.52a	R	LIVIA	31.24d	S
2007HSF007-21	5.49c	S	2007HSF024-02	5.63c	S	MARCELA	4.07c	S
2007HSF007-26	5.24c	S	2007HSF024-04	8.19d	S	BrazlândiaBranca	10.66d	S
2007HSF007-27	5.16c	S	2007HSF024-06	5.30c	S	BrazlândiaRosada	4.63c	S
2007HSF009-06	13.09d	S	2007HSF025-04	8.50d	S	BrazlândiaRoxa	7.00d	S
2007HSF010-01	8.39c	S	2007HSF026-01	0.12a	R	Palmas	9.19d	S
2007HSF010-06	8.95d	S	2007HSF026-02	14.43d	S	Tomato cv. Santa Clara	4.84c	S
2007HSF010-08	5.15c	S	2007HSF026-05	3.10c	S			

[1] Separations of the averages by the Scott-Knott test at a 5% level of probability were performed based on the log (x+1) transformation of the data. Averages expressed based on non-transformed data.

[1] Las separaciones de los promedios por la prueba de Scott-Knott a un nivel de probabilidad del 5% se realizaron sobre la base de la transformación logarítmica (x + 1) de los datos. Promedios expresados en base a datos no transformados.

[2] Classification according to Oostenbrink (1966). / [2] Clasificación según Oostenbrink (1966).

Table 4. Averages of the reproduction indices (RI) of *Meloidogyne enterolobii* and their classification in terms of resistance to 142 sweet potato genotypes and the Santa Clara tomato cultivar (utilized as the standard control for susceptibility).

Tabla 4. Promedios de los índices de reproducción (IR) de *Meloidogyne enterolobii* y su clasificación en términos de resistencia a 142 genotipos de batata y el cultivar de tomate Santa Clara (utilizado como control estándar para la susceptibilidad).

GENOTYPE	RI (%)	Class[2]	GENOTYPE	RI (%)	Class	GENOTYPE	RI (%)	Class
2007HSF001-01	70.0d[1]	S	2007HSF010-12	70.0d[1]	S	2007HSF027-05	70.0d[1]	S
2007HSF001-09	64.3c	S	2007HSF010-17	64.3c	S	2007HSF027-07	64.3c	S
2007HSF001-16	117.0d	S	2007HSF010-23	117.0d	S	2007HSF027-08	117.0d	S
2007HSF001-17	199.4e	S	2007HSF010-25	199.4e	S	2007HSF027-09	199.4e	S
2007HSF001-21	0.8a	AR/I	2007HSF010-30	0.8a	AR/I	2007HSF027-10	0.8a	AR/I
2007HSF001-22	102.5d	S	2007HSF010-31	102.5d	S	2007HSF027-12	102.5d	S
2007HSF001-23	140.0d	S	2007HSF010-33	140.0d	S	2007HSF027-16	140.0d	S
2007HSF001-24	101.7d	S	2007HSF010-35	101.7d	S	2007HSF028-05	101.7d	S
2007HSF001-25	115.9d	S	2007HSF010-37	115.9d	S	2007HSF028-06	115.9d	S
2007HSF001-26	109.0d	S	2007HSF010-41	109.0d	S	2007HSF028-08	109.0d	S
2007HSF001-28	127.5e	S	2007HSF010-47	127.5e	S	2007HSF028-11	127.5e	S
2007HSF001-37	183.3e	S	2007HSF011-01	183.3e	S	2007HSF028-16	183.3e	S
2007HSF001-40	85.2d	S	2007HSF011-02	85.2d	S	2007HSF029-01	85.2d	S
2007HSF001-41	66.2d	S	2007HSF011-05	66.2d	S	2007HSF029-02	66.2d	S
2007HSF001-45	116.2e	S	2007HSF011-06	116.2e	S	2007HSF029-03	116.2e	S
2007HSF001-47	85.9d	S	2007HSF011-10	85.9d	S	2007HSF029-09	85.9d	S
2007HSF001-58	22.6c	MoR	2007HSF012-02	22.6c	MoR	2007HSF030-02	22.6c	MoR
2007HSF002-02	4.9b	MR	2007HSF013-03	4.9b	MR	2007HSF030-10	4.9b	MR
2007HSF002-04	146.8e	S	2007HSF013-04	146.8e	S	2007HSF031-04	146.8e	S
2007HSF002-05	195.2e	S	2007HSF014-04	195.2e	S	2007HSF031-02	195.2e	S
2007HSF002-08	19.9c	MoR	2007HSF014-05	19.9c	MoR	UFLA07-01	19.9c	MoR
2007HSF002-11	99.0c	S	2007HSF016-05	99.0c	S	UFLA07-02	99.0c	S
2007HSF002-14	1.1a	MR	2007HSF018-03	1.1a	MR	UFLA07-05	1.1a	MR
2007HSF002-19	149.1e	S	2007HSF019-01	149.1e	S	UFLA07-10	149.1e	S
2007HSF004-03	9.8b	MR	2007HSF020-05	9.8b	MR	UFLA07-11	9.8b	MR
2007HSF004-04	227.5e	S	2007HSF020-07	54.6c	S	UFLA07-12	71.7e	S
2007HSF004-06	72.3d	S	2007HSF020-08	193.4e	S	UFLA07-14	5.0b	MR

Table 4 (cont.). Averages of the reproduction indices (RI) of *Meloidogyne enterolobii* and their classification in terms of resistance to 142 sweet potato genotypes and the Santa Clara tomato cultivar (utilized as the standard control for susceptibility).

Tabla 4 (cont.). Promedios de los índices de reproducción (IR) de *Meloidogyne enterolobii* y su clasificación en términos de resistencia a 142 genotipos de batata y el cultivar de tomate Santa Clara (utilizado como control estándar para la susceptibilidad).

GENOTYPE	RI (%)	Class[2]	GENOTYPE	RI (%)	Class	GENOTYPE	RI (%)	Class
2007HSF004-08	94.7d	S	2007HSF020-12	146.7e	S	UFLA07-31	2.5a	MR
2007HSF005-01	3.3a	MR	2007HSF021-01	16.9b	MoR	UFLA07-43	63.5c	S
2007HSF005-03	3.9a	MR	2007HSF022-02	119.4e	S	UFLA07-49	10.0b	MR
2007HSF005-06	37.1c	LR	2007HSF022-03	234.9f	S	UFLA07-53	155.6e	S
2007HSF006-08	62.4d	S	2007HSF022-04	83.7c	S	AMANDA	88.2e	S
2007HSF006-13	181.4e	S	2007HSF022-05	84.6d	S	ANACLARA	16.3c	MoR
2007HSF006-16	27.0c	LR	2007HSF022-06	1.6a	MR	BARBARA	2.9a	MR
2007HSF006-17	42.9c	LR	2007HSF022-09	2.4a	MR	BEATRIZ	119.7d	S
2007HSF007-04	32.2c	LR	2007HSF022-10	144.6e	S	CAROLINA	250.0e	S
2007HSF007-10	38.9c	LR	2007HSF022-12	6.7b	MR	DUDA	71.7e	S
2007HSF007-15	109.8e	S	2007HSF022-16	10.8b	MR	ITAJUBA	5.0b	MR
2007HSF007-16	113.2e	S	2007HSF022-19	116.5d	S	IZABELA	2.5a	MR
2007HSF007-17	109.1d	S	2007HSF023-08	168.0e	S	JULIA	26.8c	LR
2007HSF007-18	106.7d	S	2007HSF024-01	111.1e	S	LIVIA	645.6g	S
2007HSF007-21	273.2e	S	2007HSF024-02	175.4e	S	MARCELA	83.9e	S
2007HSF007-26	176.1d	S	2007HSF024-04	2.4a	MR	BrazlândiaBranca	220.8e	S
2007HSF007-27	185.7e	S	2007HSF024-06	300.6f	S	BrazlândiaRosada	95.9e	S
2007HSF009-06	107.3e	S	2007HSF025-04	64.4c	S	BrazlândiaRoxa	144.8e	S
2007HSF010-01	227.5e	S	2007HSF026-01	54.6c	S	Palmas	189.2e	S
2007HSF010-06	72.3d	S	2007HSF026-02	193.4e	S	Tomato cv. Santa Clara	100.0e	S
2007HSF010-08	94.7d	S	2007HSF026-05	146.7e	S			

[1] Separations of the averages by the Scott-Knott test at a 5% level of probability, based on log (x+1) transformed data. Averages expressed based on non-transformed data. [2] S - Culture susceptible (normal reproduction), above 51% in relation to the tomato control; LR -slightly resistant, from 26% to 50%; MoR - moderately resistant, from 11% to 25%; MR -very resistant, from 1% to 10%; AR/I – highly resistant/immune, below 1% (adapted from Taylor, 1967).

[1] Separaciones de los promedios por la prueba de Scott-Knott con un nivel de probabilidad del 5%, basado en datos transformados logarítmicos (x + 1). Promedios expresados en base a datos no transformados. [2] S - Cultivo susceptible (reproducción normal), superior al 51% en relación con el control del tomate; LR: levemente resistente, del 26% al 50%; MoR: moderadamente resistente, del 11% al 25%; MR: muy resistente, del 1% al 10%; AR / I - altamente resistente / inmune, por debajo del 1% (adaptado de Taylor, 1967).

The clones UFLA-07-49 and UFLA-07-53 were among the genotypes most resistant to the nematode *M. enterolobii* according to the three criteria utilized. The clones denominated UFLA-07-*xx* represent genotypes that have already been tested in advance phases of the breeding program at UFLA and demonstrated high productivity and commercially interesting attributes-which, summed with their resistance to the nematode *M. enterolobii*, make them very promising for commercial markets. In recent studies, these same two genotypes demonstrated resistance to the nematode *M. incognita* race 1 (13).

Using the criteria established by Taylor (1967), 31 genotypes could be selected for further study as they represent clones that are highly resistant/immune, very resistant, and moderately resistant to *M. enterolobii* (table 4, page 327-328). Marchese *et al.* (2010), in their search for sweet potato genotypes resistant to *M. incognita* race 1, considered only plants that were highly resistant/immune or very resistant as worthy of selection. However, that study did not use multiple comparison procedures that might recommend the inclusion of moderately resistant genotypes.

In comparing the reproduction factor with reproduction index criteria, it was observed that genotype 2007HSF001-58 would not be considered resistant according to the first criterion, even though demonstrated an average RF of 1.09 (table 3, page 325-326), a value very close to the cutoff value for selected genotypes; it was, however, considered moderately resistant according to the criteria established by Taylor (1967).

Twenty-seven genotypes (19.01%) were classified as resistant according to all three selection criteria considered together (tables 2, page 324; table 3, page 325-326 and table 4, page 327-328).

The genotypes 2007HSF001-58, 2007HSF002-08, 2007HSF006-08, and the Carolina variety did not show consistency in terms of their resistance according to all of the criteria used, and would not be selected based on the numbers of eggs per gram of roots (table 2, page 324).

However, comparisons of the averages of these genotypes with that of the Lívia genotype indicated considerable differences between them, as the populations of nematodes in the latter grew by more than 30X, as was noted above.

In general, the three criteria were coherent among themselves and efficient in the identification and selection of genotypes resistant to *M. enterolobii.* It could be observed, however, that the reproduction index generated a wider distribution of distinct classes (AR/I, MR, MoR, LR and S), allowing more flexibility in establishing a cut-off level for genotype selection. As such, we selected 31 genotypes with some level of resistance according to the reproduction index criterion to continue the sweet potato breeding program.

Of the 142 sweet potato genotypes evaluated in the present study, 121 had been used in experiments undertaken by Marchese *et al.* (2010). Comparing these two studies indicated that the genotypes that demonstrated a certain degree of resistance to both race 1 of *M. incognita* and *M. enterolobii* were clones 2007HSF001-21, 2007HSF002-02, 2007HSF002-08, 2007HSF002-14, 2007HSF004-08, 2007HSF006-13, 2007HSF010-25, 2007HSF012-02, 2007HSF020-08, 2007HSF023-08,

2007HSF024-01, 2007HSF027-08, UFLA07-49, and UFLA07-53. These genotypes appear to be promising in terms of the sweet potato breeding program at UFLA, as the definition of genotypes that demonstrate resistance to large numbers of knot-root nematode species should generate more confidence among farmers and therefore more acceptance.

The observations that there are genotypes resistant to both *M. incognita* and *M. enterolobii*, and that some genotypes demonstrate resistance to race 1 of *M. incognita* but susceptibility to *M. enterolobii*, and vice-versa, indicate that the genes that confer resistance to *M. incognita* are not the same as those conferring resistance to *M. enterolobii*.

Conclusions

The relationships between estimates of the coefficients of genetic and environmental variation, and broad sense heritability were high in terms of the numbers of eggs per gram of roots as well as for the reproduction factors and reproduction indices, demonstrating the efficiency of the methodology used here for selecting resistant genotypes.

Thirty-one sweet potato genotypes resistant to *M. enterolobii* (21.84% of the clones evaluated) were selected for continuing the breeding program.

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Sodium N-methyldithiocarbamate impact on soil bacterial diversity in greenhouse tomato (*Solanum lycopersicum* L.) crop

Impacto de metam sodio en la diversidad bacteriana de un suelo cultivado con tomate (*Solanum lycopersicum* L.) en invernadero

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Abstract

The constant use of sodium N-methyldithiocarbamate (metam sodium: MS) in protected agriculture in México has attracted the attention of researchers and producers on their effects on the environment. The objective of this study was to evaluate the impact of MS on the bacterial community structure in an agricultural soil with tomato crop (*Solanum lycopersicum* L.) considering the different phenological stages of the crop. The experiment was carried out in a greenhouse, with a completely randomized block design with two treatments: 1) without MS and 2) with application of 400 L·ha^{-1} of MS. For the determination of the bacterial structure, the biodiversity indexes of richness (S), diversity (H') and equity (J'), identification of operational taxonomic units (OTU) were used through the T-RFLP technique. Application of MS in soil showed no significant effect on bacterial richness. However, the application of MS does alter the structure of the bacterial community (H' and J') in each of the tomato phenological stages. Finally, future studies which include the evaluation of the effects of MS on the physiology of intensive crops and functions in the different soil types are need.

Keywords
biodiversity indexes • soil • T- RFLP • bacterial community

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Resumen

El uso constante del N-metil ditiocarbamato de sodio (metam sodio: MS) en la agricultura protegida en México ha atraído la atención de investigadores y productores sobre sus efectos en el medio ambiente. El objetivo de este estudio fue evaluar el impacto del MS en la estructura de la comunidad bacteriana en un suelo agrícola con cultivo de tomate (*Solanum lycopersicum* L.) considerando las diferentes etapas fenológicas del cultivo. El experimento se llevó a cabo en un invernadero, con un diseño de bloques completamente al azar y dos tratamientos: 1) sin MS y 2) con aplicación de 400 $L \cdot ha^{-1}$ de MS. Para la determinación de la estructura bacteriana, se utilizaron los índices de biodiversidad de riqueza (S), diversidad (H') y equidad (J'), identificación de unidades taxonómicas operacionales (UTO) mediante la técnica T-RFLP. La aplicación de MS en el suelo no mostró un efecto significativo sobre la riqueza bacteriana. Sin embargo, la aplicación de MS altera la estructura de la comunidad bacteriana (H' y J') en cada una de las etapas fenológicas del tomate. Finalmente, se necesitan estudios futuros que incluyan la evaluación de los efectos del MS sobre la fisiología de los cultivos intensivos y las funciones en los diferentes tipos de suelos.

Palabras clave
índices de biodiversidad • suelo • T-RFLP • comunidad bacteriana

Introduction

Sodium N-methyldithiocarbamate (metam sodium: MS) is a disinfectant for farming soil which belongs to the thiocarbamates group. MS is applied to the tomato crops in greenhouse conditions. The use of MS in Mexico has increased due to the expansion of protected agricultural surface. The current surface of this system is over 23000 hectares (28). Greenhouse tomato growth is done through intensive monocultivation, which is what places most of the application of MS during the beginning of every agricultural year, in order to prevent any fungal diseases caused by *Fusarium* spp., *Phytophthora* spp., *Pyhlum* spp., *Rhizoctonia* spp., *Verticillium* spp., and *Sclerotinia* spp., among other species (10, 17). These applications affect the native populations of microbes and the non-target populations of the soil (9). The diversity of the bacteria populations in the soil, according to the impact of the MS, can be estimated through the biodiversity indexes, richness (S), diversity (H') and equity (J') (28), obtained from the operational taxonomical units (OTU), utilizing assigned determined phylogenetic through a molecular technique of T-RFLP (Terminal restriction fragment length polymorphism) (30), this technical molecular has advantage with independent culture methods. Where less than 1% of soil microorganisms can be grown (31). When considering the limitations of microcosmic experiments in the laboratory, it is suggested (21) that the composition and behavior of the microbes that inhabit the soil after fumigation should be evaluated more accurately by field trials. The objective was to evaluate the impact of MS application on the soil microbial diversity at different tomato phenological stages in a greenhouse cropping system.

Materials and methods

This study was carried out at the Faculty of Agronomy and Veterinary of the Autonomous University of San Luis Potosi, Mexico (22°13'48" N and 100°51'35" W, 1834 m a. s. l.). Soil characteristics were: silt 20%, sand 61%, clay 19%, organic matter 0.15%, interchangeable potassium 1.2 $C^{+}mol \cdot kg^{-1}$, extractable phosphorous 6 $mg \cdot kg^{-1}$, inorganic nitrogen 10 $mg \cdot kg^{-1}$, pH 8.17, electrical conductivity 0.86 $dS \cdot m^{-1}$ (23). Soil moisture at the time of the MS application was at holding capacity.

The soil was prepared a week previous to applying the MS with two steps of dredge in addition at a dose equivalent to 4 $t \cdot ha^{-1}$ of poultry manure of the brand Vertia® (20). The treatments involved in the study were: 1) without MS application and 2) with a single application of 400 $L \cdot ha^{-1}$ of MS. The MS was applied manually according the farmer practice.

The experimental design was performed by blocks at random with three replicates and the experimental units were plots of 2.2 m^2. Twenty-two days after applying of MS, the seedlings of Hannibal tomato variety (Harris Moran, USA) were transplanted. Mineral fertilizers were applied along the crop cycle (table 1) (5).

Samples were taken at four soil sampling dates: 1) 15 days before MS application (0 day); 2) 22 days after MS application (at transplanting); 3) 40 days after MS application (at flowering); and 4) 70 days after MS application (fructification).

The soil sampling procedure consisted on taking three samples from each plot at a depth of 0-25 cm (30 g per sample) and by mixing them, a compound sample was created for each plot.

Table 1. Tomato crop fertilization chronogram.

Tabla 1. Cronograma de fertilización del cultivo de tomate.

Fertilizer	8 a 25 DAT	26 a 45 DAT Kg $ha^{-1}day^{-1}$	46 a 70 DAT
N	1.0	2.0	6.0
P_2O_5	1.0	1.5	1.5
K_2O	2.0	4.0	10.0
Ca	1.8	3.0	3.5
Mg	0.6	1.0	2.0

DNA extraction - T- RFLP Analyses

The total DNA was extracted from samples composed of 10 g of soil with the DNA Power Soil kit (MoBio, Carlsbad, California, USA) following the manufacturer's instructions. The genomic DNA extracted was purified by the Clean DNA and Concentrator kit (Zymo Research, Irvine, California, USA), following the manufacturer's instructions.

The total DNA extracted from the soil samples was used as a mold for the amplification through PCR from a fragment of 1.5 kb of the ribosomal DNA region 16S, with a pair of universal bacterial (F27, 5'-AGA GTT TGA TCM TGG CTC AG- 3' y R1492, 5' TAC GGY TAC CTT GTT ACG ACT-3') (17), were digested with the endonuclease restriction *NdeI* (thermos Fisher Scientific).

The size of the final restriction fragments (T-RF) was determined in an automatized capillary sequencer ABI 3130 (Applied Biosystems, Foster City, CA) generating the electropherograms, with an internal size pattern of BTO 550 (Qiagen, USA), within a range of 50 to 550 pairs of bases (pb) (3).

The genetic profile was expressed in terms of the peak intensity and size of the T-RF, and was analyzed with the Genemapper software V3.7 (Applied Biosystems. Inc., USA) with a peak detection hight of 50 fluorescent units, Assignment Tool (PAT) (15). Found in the Microbial Community Analysis webpage (MICA). Considering as a basis the Silva data (R106) 16/18S rRNA (22). To compensate for differences in the PCR product quantity and T-RFLP profile intensity among samples, the peak relative heig of heach sample (OTU) divided by the sum of all peak heights from the corresponding sample (29).

Diversity indices calculations

The microbial genetic diversity was estimated by mean of three indices, which reflex the bacterial structure:

Richness (S) represented by the number of bacteria species. Was calculated by the presence or absence of T-RF band electropherrograms present in the samples, represented in operational taxonomical units (OTU) (30). An OTU is a group of phylogenetically related organisms without specifying a taxonomical range (24).

Shannon index (H'), which represents the level of bacteria population diversity in the soil, was calculated as:

$$H' = -\Sigma(p_i)\ (\ln p_i)$$

where:

p_i = relative abundance of each OTU in relation to the total population, which was in turn calculated based on the peak area of each T-RF divided by the sum of the total areas of T-RF in the corresponding samples (9).

The H' values range from 0 to 5, which are generally between 1.5 y 3.5, it is highly uncommon that they exceed 4.5. If H' presents a value of 0, then it was have one OTU present, which is interpreted as low biodiversity (24).

Evenness index (J'), which represents the distribution of the abundance of the distinct OTUs in the soil, was calculated as J' = H'/ ln S. The values of J' vary from 0 al 1 (maximum value) (4).

Biodiversity indices were evaluated ($p \leq 0.05$), the richness data were logarithmically transformed (13), then ANOVA ($p \leq 0.05$) was performed to compare treatments, phenological stages and principal components analysis (PCA), using SAS program (version 9.0, USA).

Results and discussion

The genetic profile can be seen in terms of the intensity and size of the T-RF, which is the sequential longitude of the bases of paired units (50-500 pb) (8), just as indicated by the electropherograms for each of the treatments (figure 1, page 337). Each T-RF comes from a particular sequence of 16S rDNA, to which each T-RF is assumed as operational taxonomical unit (OTU) (28).

The results of the richness (S) in the flowering stage (40 days) a reduction in S is observed with respect to the previous stage, to increase again in the fructification stage, in both treatments. The richness was not affected by the MS application within the each phenological stage ($p \leq 0.05$) (table 2, page 337).

The bacterian richness of the soil (number of different species), cultivated with tomato in intense conditions was able to overcome the initial disturbance caused by the application of the MS. This phenomenon demonstrates an elevated soil resilience, *i.e.* the capacity of a community to try and recover its original state before a disturbance (2).

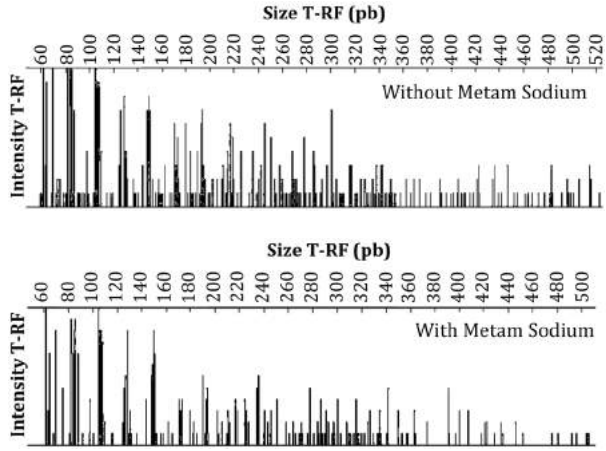


Figure 1. Soil bacterial genetic profile obtained by molecular technique T-RF (size and intensity) in without MS and with MS treatments, considering all sampling dates (0, 22, 40 and 70 days after transplanting). MS: sodium N-methyldithiocarbamate.

Figura 1. Perfil genético bacteriano obtenido por la técnica molecular T-RF (tamaño e intensidad) de suelo sin tratamiento con MS y suelo tratado con MS, al considerar todas las etapas del experimento (0, 22, 40 y 70 días después del transplante). MS: N-metil ditiocarbamato de sodio.

Table 2. Richness (S), Shannon (H') and Shannon evenness (J') indices (mean ± SD) for with MS and without MS treatments in each tomato phenological stage. MS: sodium N-methyldithiocarbamate.

Tabla 2. Índices de riqueza (S), Shannon (H') y Shannon evenness (J') (media ± DE) para los tratamientos sin MS y con MS en cada etapa fenológica del tomate. MS: N-metil ditiocarbamato de sodio.

Sampling	Richness		Shannon (H')		Shannon evennes (J')	
	without MS mean ±SD	**with MS** mean ±SD	**without MS** mean ±SD	**with MS** mean ±SD	**without MS** mean ±SD	**with MS** mean ±SD
0 Days	3.4 ± 0.42	-	2.92 ± 0.067	-	0.919 ± 0.047	-
22 Days (vegetative growth)	3.7 ± 0.30a	4.1 ± 0.16	3.7 ± 0.329b	3.74 ± 0.08a	0.887 ± 0.052b	0.911 ± 0.041a
40 Days (flowering)	2.7 ± 0.20a	2.3 ± 0.23	2.97 ± 0.071a	2.52 ± 0.212b	0.904 ± 0.007b	0.982 ± 0.011a
70 Days (fructification)	3.7 ± 0.41a	3.2 ± 0.44	3.46 ± 0.079a	3.82 ± 0.357b	0.890 ± 0.013a	0.851 ± 0.020b

Diverse causes could have contributed to maintaining the bacterian richness similar to farming soil after applying MS along the passing of time: a) Fertilization; the elevated nutrient levels applied to the tomato crop in an intensive system (1 to 6 kg $N \cdot ha^{-1} \cdot day^{-1}$, table 1, page 335). such nutrients could have been utilized by the bacterian community (12, 32); b) Root functions (14), which release chemical compounds in a secretion such as sugar, amino acids, flavonoids, proteins and so on throughout the different tomato crop phenological stages (6); c) The accelerated decomposition of the MS, even after one single fumigation increases decomposing bacteria in the soil (10); d) The application of a low dosis (400 $L \cdot ha^{-1}$), which according to the product data sheet, can be applied up to 1200 $L \cdot ha^{-1}$ (Buckman Lab., USA).

The result of a disturbance, such as fumigation, generates high reproduction rates of surviving bacteria (25). Contrarily to the richness (S), the Shannon (H') and the evenness (J') indices, showed a significant change between treatments in each vegetative stage ($p \leq 0.05$), (table 2), thus also the vegetative stages show significant differences with the application of the MS ($p \leq 0.05$), (figure 2, page 339). These results show changes in the structure of the community caused by the applying chemical fertilizers and pesticides (MS) to the soil, which provoke modifications in the nutrient contents, organic carbon in the soil, pH, and humidity amongst others (25). This could explain the results obtained where significant changes can be seen in the diversity and evenness of the OTU.

There were other two studies where there was also a change in diversity and evenness after fumigation (19, 32). Future studies must demonstrate if the changes of diversity found in this research (a fluctuation of H' between 10 and 18%; in J' they were of 3 and 8%), representing an effectuation over the crop soil sustainability in an intensive system (greenhouses).

Relative abundance in Operational Taxonomical Units (OTUs)

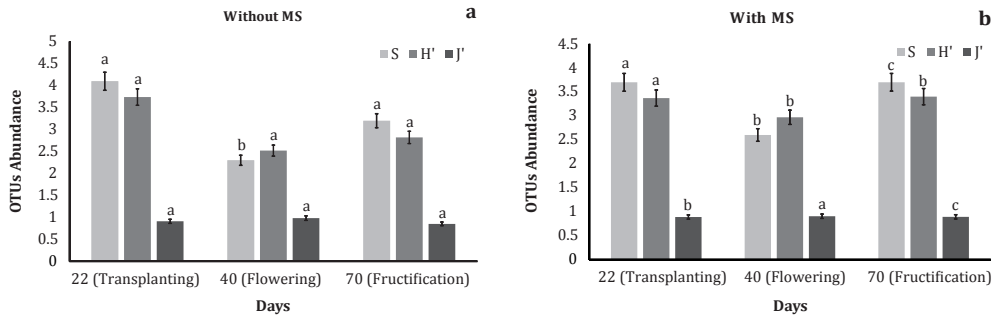
The Phylogenetic assigning of the OTU, was in this case, coincidentally carried out by the same MICA data base (27). There were eight phylogenetic groups of bacteria adjusted to the T- RFLP profiles generated by this research.

The behavior of each group with relative abundance bases of the bacterian OTU showing changes in the different phenological stages, is illustrated in figure 3 (page 339): a-*Proteobacteria*. d-*Proteobacteria*, g-*Proteobacteria*, *Firmicutes*, *Cyanobacteria* and *Terrabacteria* were conspicuous in the treatment without MS.

And for treatment with MS: *Proteobacteria*, a-*Proteobacteria*, b-*Proteobacteria*, d-*Proteobacteria*, g-*Proteobacteria*, *Firmicutes*, *Cyanobacteria* and *Terrabacteria*. The *phylas* show changes in the relative abundance percentages of the operational taxonomical units (OTU) throughout the tomato crop phenological stages evidentiating changes in the bacterian communities for both treatments (without MS *vs* with MS), and were coincidental in the H' indicator (diversity).

The structural changes in the community were marked by the *phyla* a-*Proteobacteria*, g-*Proteobacteria*, *Firmicutes* and *Agrobacterium*, which were found within the most abundant in farming soils, during the whole evolutionary stages and are particularly known for their potentialities to promote plant growth (7).

From this *in situ* composition of the identified bacterian community, the most abundant *phyla proteobacteria,* which is a diversely metabolic group of four *subphylas* (α-, β-. γ- y δ-), was commonly reported in the soil (1).



Different letters indicate statistical differences ($p \leq 0.05$) between treatments.

Letras diferentes indicant diferencias estadísticas ($p \leq 0,05$) entre tratamientos.

Figure 2. Comparison between the phenological stages of the tomato crop with the biodiversity indices of richness (S), diversity (H') and equity (J') for without MS (a) and with MS (b) treatments. MS: sodium N-methyldithiocarbamate.

Figura 2. Comparación entre las etapas fenológicas del cultivo de tomate con los índices de biodiversidad de riqueza (S), diversidad (H') y equidad (J') para tratamientos sin MS (a) y con MS (b). MS: N-metil ditiocarbamato de sodio.

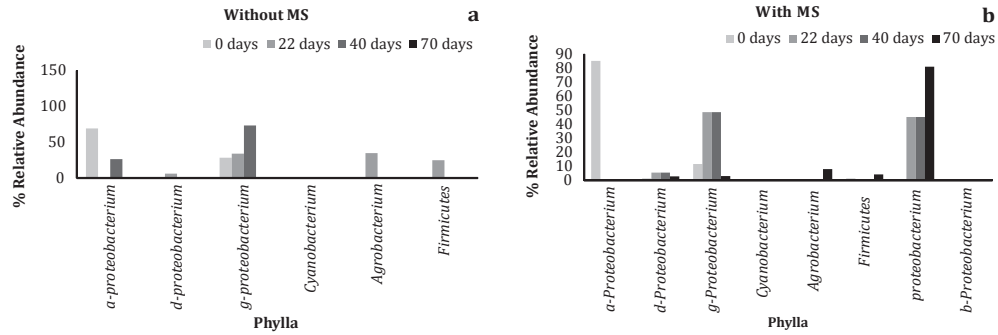


Figure 3. *Phylla* (phylogenetic assignment) *vs* % Relative abundance OTUs for treatments without MS (a) and with MS (b). MS: sodium N-methyldithiocarbamate. Within each sampling date.

Figura 3. Phylla (asignación filogenética) *versus* % Abundancia relativa UTOs. para tratamientos sin MS (a) y con MS (b). MS: N-metil ditiocarbamato de sodio. Dentro de cada fecha de muestreo.

Proteobacterias represent more than 40% of all the publicly validated prokariot genre and exhibit an extreme metabolic diversity (16).

Given the phylogenetic results and relative abundance, there is a possibility for an initial bacterian succession which usually occurs in disturbed ecological systems in as much as by natural as for anthropogenic causes (25). However, this must be corroborated in later studies with longer evaluation periods.

Principal components analysis (PCA)

The treatment without MS, the principal component analysis (PCA1), explained 26% of the relative abundance corresponding to 0 day. While, the PCA2 which corresponds to the transplanting stage explains 47.7% of the accumulated; the PCA3 the 60.54% of the variables corresponding to the flowering stage, and the PCA4 within the fructification stage explained 72.6%. An interaction rate of 56% between the OTUs and the phenological stages were detected.

In the with MS treatments, the PCA1 explained 24.45%, identified at 0 days; the PCA2 the 43.8% at the transplanting stage; PCA3 the 58% at the flowering; and the PCA4 corresponding to the fructification stage demonstrated a total accumulated in relative abundances of 70%.

Grouped OTU's for both treatment (without MS and with MS) at 0 days were identified by observing the highest relative abundance, considering that perhaps no disturbance had been caused by the MS application to the soil. All of the phenological stages have an accumulated of 72% in MS treatment and of 70% in without MS, indicating difference in the microbial community structures, which is in agreement with the results of H' and the OTUs relative abundances (table 2, page 337 and figure 4, respectively).

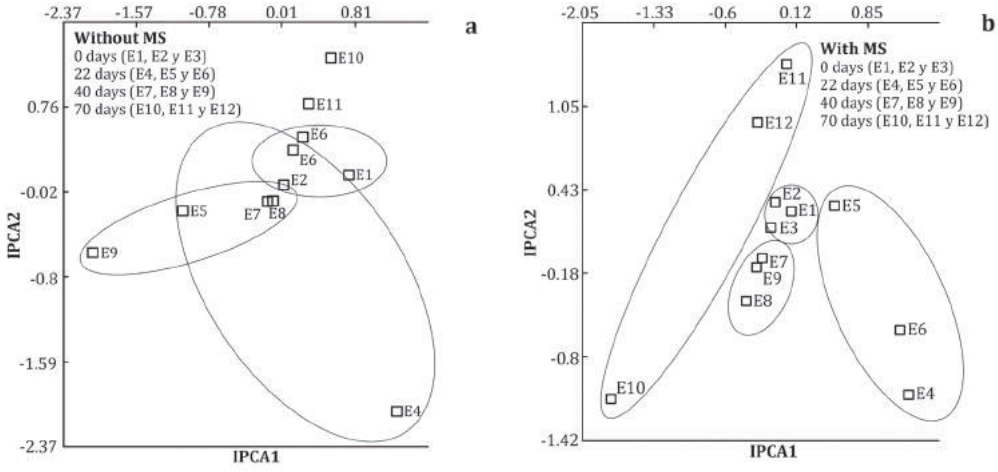


Figure 4. Principal component analysis in without MS (a) and with MS (b) treatments, discriminated by each sampling date. MS: sodium N-methyldithiocarbamate. E: replicates in each sampling date.

Figura 4. Análisis de componentes principales en tratamientos sin MS (a) y con MS (b), discriminados por cada fecha de muestreo. MS: N-metil ditiocarbamato de sodio. E: réplicas en cada fecha de muestreo.

Conclusion

The fact of that MS application modifies the biodiversity index (H' and J') of the soil bacterial community in all tomato phenological stages suggests that the use of this product in terms of dosage and frequency of application needs more attention. Moreover, future studies that consider the crop physiology and the effects on the basic soil functions are need.

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Dispersal of the pea leaf miner *Liriomyza huidobrensis* (Blanchard, 1926) (Diptera: Agromyzidae): a field experiment

Dispersión del minador de hojas *Liriomyza huidobrensis* (Blanchard, 1926) (Diptera: Agromyzidae): un experimento a campo

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Abstract

Movement of herbivore insects within agroecosystems can ultimately determine the level of damage to crops. It was evaluated the dispersal of *Liriomyza huidobrensis* (Blanchard, 1926) (Diptera: Agromyzidae) under field conditions. In addition, it was evaluated if body size was related to dispersal distance. Eight hundred individuals of *L. huidobrensis* were released in the central point of a series of concentric circles (6, 12, 24, 48, and 96 m in radius), where 117 yellow sticky traps were set to insect recapture. Circular Statistics were used to evaluate flight direction whereas hurdle models were applied to analyze dispersal probability in relation to distance. Five percent of the released individuals were recaptured, being 36 m the median distance of recapture. The distribution of recaptured insects was not random around the circles, but the preferential dispersal direction was not explained by wind direction. The incidence of recaptured *L. huidobrensis* of both sexes significantly decreased at increasing distances from the release point, but decayed faster for females. No effect was found of body size on the distance of recapture. The results suggest that *L. huidobrensis* dispersed mainly over short distances with males being capable of performing longer flights than females.

Keywords
body size • herbivore insect pest • movement • wind

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Resumen

El movimiento de insectos herbívoros dentro de agroecosistemas puede finalmente determinar el nivel de daño a los cultivos. Se evaluó experimentalmente la dispersión de *Liriomyza huidobrensis* (Blanchard, 1926) (Diptera: Agromyzidae) a campo y se analizó si el tamaño corporal se relacionó con la distancia de dispersión. Se liberaron ochocientos individuos en el punto central de una serie de círculos concéntricos (6, 12, 24, 48 y 96 m de radio), donde se colocaron 117 trampas pegajosas amarillas para recapturar los insectos. Se utilizó estadística circular para evaluar la dirección del vuelo, y modelos "hurdle" para analizar la probabilidad de dispersión en relación a la distancia. Se recapturó el 5% de los individuos liberados siendo 36 m con la distancia mediana de recaptura. La distribución de insectos alrededor de los círculos no fue aleatoria, pero la dirección de dispersión preferencial no se explicó por la dirección del viento. La incidencia de *L. huidobrensis* de ambos sexos disminuyó significativamente al aumentar la distancia, pero decayó más rápidamente para hembras. El tamaño corporal no influyó la distancia de recaptura. Estos resultados sugieren que *L. huidobrensis* se dispersó principalmente a distancias cortas, siendo los machos capaces de desarrollar vuelos más largos que las hembras.

Palabras clave
tamaño corporal • insecto herbívoro plaga • movimiento • viento

Introduction

Dispersal is a key process in ecology affecting the dynamics and persistence of insect populations (6). The term 'dispersal' can be used to describe both the movements towards a breeding location which implies gene flow across space, and 'trivial' movements represented by short foraging flights (3). Both active (locomotory) and passive (wind) components can account for the dispersal of flying insects, with the relative contribution of each one varying among species (17, 23).

Active dispersal can be affected by both environmental factors and by the movement ability of individuals which in turn depends on certain factors such as age, stage, sex and body size (3). Differential resource allocation between sexes contributes to sex-biased dispersal. In many insect species, female dispersal is limited due to investments of resources in egg production (3). Regarding body size, it is expected that larger individuals have a higher active dispersal rate than smaller ones (10, 13). It has been shown that relatively longer wings would increase acceleration capacity and improve the efficiency of prolonged flights (4). Passive dispersal by wind is likely to be important for minute insects (8) but in general little is known about how the dispersal of insects is influenced by wind in the landscape (12, 20).

In agro-ecosystems, the dispersal of herbivores can ultimately determine its spatial distribution and the level of damage to crops. Thus, understanding the dispersal process in insect pests might be helpful to improve management strategies (15).

The pea leaf miner, *Liriomyza huidobrensis* (Blanchard, 1926) (Diptera: Agromyzidae), is a polyphagous species that causes serious damage to several horticultural crops (2, 19). Originally from South America, this species has expanded its range and invaded many regions of the world, frequently reaching pest status (26). Damage to plants is mainly caused by larvae which excavate tunnels consuming mesophyll cells leaving intact the upper and lower leaf surfaces. This species tends to be resistant to commonly used pesticides, thus different management strategies are applied to control it (25).

For trap cropping, for example, it is relevant to know the dispersal behavior of the target herbivore in order to maximize pest control by adequately locating trap plants in relation to main crop (7).

Studying the movement of insects in their natural habitat is essential for understanding their biology and behavior but sometimes represents a great challenge, especially for small flying insects like adult dipteran leaf miners. One alternative to study dispersal patterns in insects is the Eulerian approach which relies on physical or biological 'traps' at some particular points where dispersers are sampled (16).

Although there are a few studies on flight patterns of *Liriomyza* species (5, 11, 18, 28), none of them tested dispersal through a release and recapture field experiment.

The aim of the present study was twofold: i) to evaluate the mean direction and distance of movement of *L. huidobrensis* individuals, and ii) to examine if body size was related to dispersal distance.

Material and methods

A release and recapture field experiment was performed in order to evaluate movement of *L. huidobrensis* individuals. The study was conducted in a 10-ha lawn field for commercial purposes. The field was placed in the outskirts of Córdoba city (31°16'58.5" S, 64°02'31.04" W), located in Córdoba province (Argentina), where no *L. huidobrensis* host plants were found after conducting a careful inspection of the field. A total of 800 unfed 1-2 days old individuals of *L. huidobrensis* reared on *Vicia faba* L. (broad bean) plants in the laboratory, were released in the experiment in a single release event.

Field collected *L. huidobrensis* adults were offered broad beans in the laboratory. Pots (28 cm 15 cm, 9 cm deep) containing 8-10 broad beans (6-10 leaves each) were placed in a cage (glass, wood and voile, 30 cm side length) with 20 pairs for 4 h, at room temperature, for mating and oviposition. This procedure was carried out simultaneously in several cages to obtain a high number of adults.

To recapture the insects, 117 yellow plastic and opaque sticky traps were used (20 cm x 20 cm) which are known to be highly attractive for leaf miners (14). Traps were placed in the field in a design of concentric circles at five distances from the central point (6, 12, 24, 48, and 96 m in radius), where flies were released (figure 1a, page 346).

The location of the releasing point was arbitrarily selected but any influence of this position on this results is unlikely given the spatial homogeneity of the lawn field. Traps were spaced out regularly every 9.5 to 10 m on each ring and placed at 50 cm above ground (from the base of the trap) as this is the height were *L. huidobrensis* usually disperses (14).

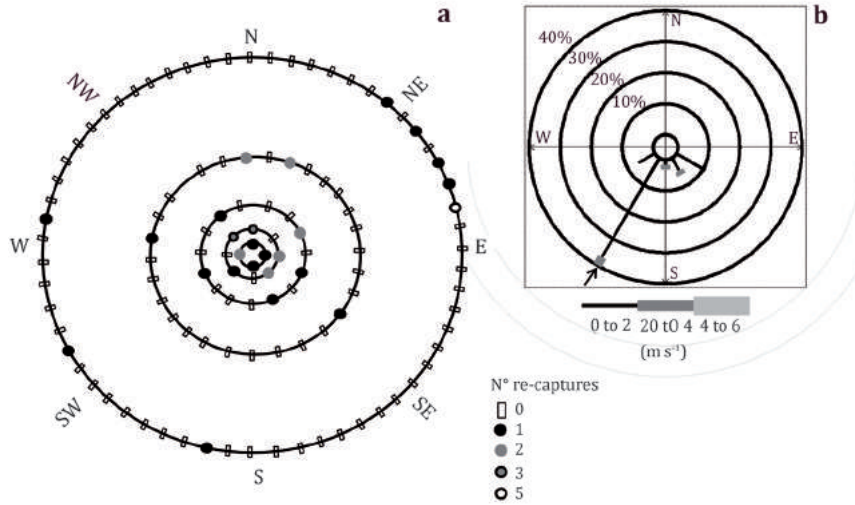

Figure 1. (a) Diagram of the field trial indicating the position of the 117 traps set at five distances (6, 12, 24, 48, and 96 m) from the central point where *Liriomyza huidobrensis* individuals were released. Different symbols indicate different number of re-captures (b) Circular histogram illustrating wind direction and speed during the experiment. Each ring represents the time proportion (%) of winds from different directions. The arrow indicates the prevailing wind direction.

Figura 1. (a) Diagrama del ensayo a campo que indica la posición de las 117 trampas establecidas a cinco distancias (6, 12, 24, 48 y 96 m) a partir del punto central donde se liberaron los individuos de *Liriomyza huidobrensis*. Diferentes símbolos indican diferente número de re-capturas (b) Histograma circular que ilustra las direcciones y velocidad del viento durante el experimento. Cada anillo representa la proporción de tiempo (%) en la que soplaron vientos desde diferentes direcciones. La flecha indica la dirección predominante del viento.

Leaf miners were released on July 16th 2014 at 11.30 am (after 15 min all flies leaved the box where they were transported) and traps were removed 24 h later and carried to the laboratory where they were inspected for *L. huidobrensis* adults.

The individuals collected were stored in tubes with 70% ethanol to posterior confirmation of its identity. For each individual, it was determined its sex and body size through the measurement of forewing length (mm).

The group of insects released had a female biased sex ratio (1.77:1 females to males). A meteorological station Nexus (TFA) placed nearby the experiment automatically recorded wind speed and direction every 20 minutes.

Data analysis

Flight direction of *L. huidobrensis* was analyzed by means of Circular Statistics. A Rayleigh test for Randomness was performed to determine if the distribution of captures was distinguishable from random. To test whether the leaf miner re-capture direction was the same as the wind direction, a one-sample test,

analogous to a one-sample t-test for data on a linear scale, was conducted using wind direction as a pre-assigned angle to be tested against the leaf miner flight direction. Particularly, it was tested if insects flew downwind by subtracting 180° to the mean wind direction.

The angle (degrees) of each trap was measured in relation to the release point (through a drawing program) and then transformed into radians, which is the unit that Circular Statistics use. The analyses were performed by using the package "circular" (1) in R 3.2.2 (21).

To assess leaf miner dispersal in relation to distance from release we analyzed data (N = 117) with hurdle models which are a type of zero-inflated models (30) allowing to model the excess of zeros in the trap recaptured population (figure 1, page 346). These models, which were performed separately for males and females, have two-parts whereby the first part is a binary outcome or incidence model, and the second part is a truncated count model (30).

In this models the explanatory variable for both parts was distance from release (m), the response variable was the number of recaptured flies per trap, and the assumed distributions were binomial for the incidence model and Poisson for the count model. Using this approach, it is possible to estimate the probability of recapture of each trap through the incidence model and then given *L. huidobrensis* individuals are present, estimate the relative mean number of individuals through the count model. Parameter estimates for distance were tested for significance using a Z- test. The hurdle models were performed using the package pscl (29) in R 3.2.2 (21).

Data on recapture distance were related to individual body size through General Lineal Models separately by sex since females of *L. huidobrensis* are generally larger than males (24).

Results

Five percent of the released individuals were recaptured in the traps. Although the liberated population was female biased there was a higher number of males recaptured (n=29) in relation to females (n=14). Considering the occupied traps (n=28), the mean distance of recapture of *L. huidobrensis* was of 46.71 m and the median 36 m.

The average wind direction during the experiment was 205° (*i.e.* direction from which wind blows, N = 0°, E = 90°, S = 180°, W = 270°), which indicates that the winds were preponderantly from the Southwest direction, with a wind speed of 0.4 m/s at the release event and with a mean of 0.65 m/s (range 0-2.4 m/s) during the whole duration of the experiment (figure 1b, page 346).

The distribution of recaptured insects (figure 1 a, page 346) was not random around the different circles according to the Rayleigh test (z=0.28, *P*=0.03), but their preferential direction was not explained by wind direction (z=0.27, *P*=0.005). In fact, the mean direction of insect recaptures was 188° which was upwind with respect to prevailing winds.

Hurdle models indicated that there was no effect of distance on the number of recaptured individuals (count model) of either sex (table 1, page 348).

However, the probability of recaptured *L. huidobrensis* (incidence model) significantly decreased with distance for both males and females (table 1; figure 2, page 348).

Table 1. Hurdle models for females and males of *Liriomyza huidobrensis* relating the distance from the release point to the probability that the species is present in a trap (incidence model) and the relative mean number of individuals in a trap (count model).

Tabla 1. Modelos "Hurdle" para hembras y machos de *Liriomyza huidobrensis* relacionando la distancia desde el punto de liberación y la probabilidad de que la especie esté presente en una trampa (modelo de incidencia) y el número medio relativo de individuos en una trampa (modelo de conteo).

Sex	Parts of the hurdle model	Estimate	Z	*P*
Females	Incidence model	-0.042	-3.228	0.001
	Count model	-0.019	-0.518	0.605
Males	Incidence model	-0.015	-2.112	0.034
	Count model	0.004	0.401	0.688

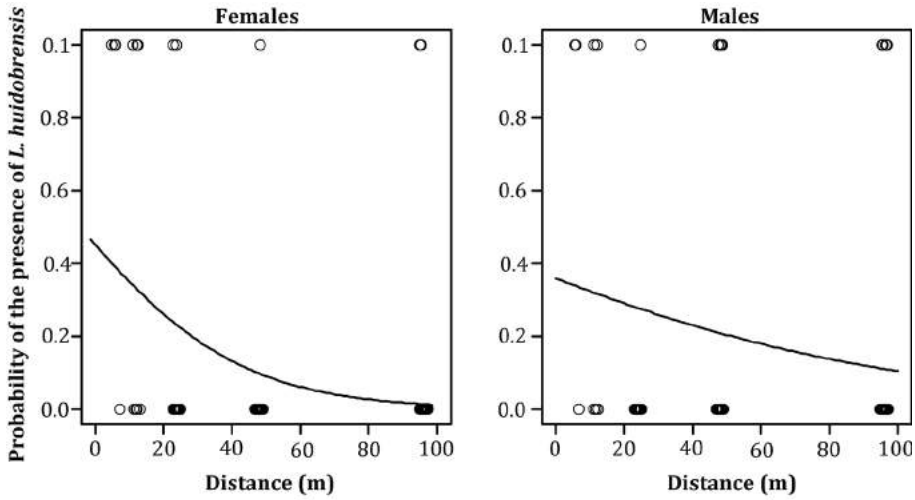

Figure 2. Relationship between the distance from the release point and the probability of the presence of *Liriomyza huidobrensis* females and males in each trap. There was a higher probability that males reached higher dispersal distances than females as it is shown by superimposed dots in the right panel.

Figura 2. Relación entre la distancia desde el punto de liberación y la probabilidad de presencia de hembras y machos de *Liriomyza huidobrensis* en cada trampa. Existe una mayor probabilidad de que los machos alcancen distancias de dispersión mayores que hembras tal como lo muestran los puntos super impuestos del panel derecho.

Although confidence intervals for distance parameters of females (95% CI: -0.072, -0.019) and males (95% CI: -0.03, -0.001) were superposed by 21%, the probability of recapture of each trap decayed faster with distance for females. Regarding body size, there was not a significant relationship between wing length and dispersal distance of flies for either sex (**Females**: $F_{1,12}$=0.001, P=0.97; **Males**: $F_{1,27}$=1.53, P=0.22).

Discussion

The results of this study show that the pea leaf miner dispersed mainly over short distances with males being capable of performing longer flights than females. Dispersal of flying insects from a specific point usually lead to a decline in density distribution at increasing distances (9, 22, 23).

Although could not be found a decreasing pattern for the abundance of flies recaptured in relation to distance, it was found that the higher probability to find *L. huidobrensis* was near to the release point, eventually falling with distance, and decaying faster for females. This result indicates that this sex was the most sensitive to longer distances, were a few individuals were re-captured. Increased captures of pea leaf miner males at long distances may be explained by high flight activity as they actively search for mates and food whereas females spend more time on leaves for oviposition (19).

Movement patterns could be affected by behavioral, morphological, physiological, genetic, and developmental attributes of focal individuals, as well as by environmental variables (16).

The higher number of males recaptured in relation to females, despite the released group was female biased, could be explained by differences in the level of attraction of the traps.

Martin *et al.* (2005), showed that translucent yellow sticky traps were more attractive for females than opaque traps, with no differences for males. Contrary to this expectations, it was could not detect an effect of the wing length of individuals with dispersal distance reached by the leaf miners of either sex, probably due to the low number of recaptures.

In addition, the low variability in wing length among released individuals of both sexes (CV<10%) could explain that result. Future experiments using a larger number of insects and low and high quality host plants would provide individuals with higher size differences (24).

Agromyzid flies are considered to be "moderate fliers" (27) because they tend to fly very short distances between host plants (28), but at the same time, they can move longer distances by wind dispersal (27). The results presented here showed that under the conditions of this experiment, wind had not a significant effect on *L. huidobrensis* dispersal suggesting that insects may be in part controlling their long distance movements (20). This is in agreement with the findings by Jones and Parrella (1986), for *L. trifolli,* a closely related species to *L. huidobrensis*. These authors showed that *L. trifolli* dispersed 26 m on average, but was capable to reach the furthest distance tested (102 m) in a greenhouse experiment free of wind.

In this study, wind speed at the moment of insect release was really low not exceeding 0.4 m/s and considering that mean direction of insect displacement was not downwind, it is unlikely that wind speed and direction did influence dispersal of the pea leaf miner in the trial.

However, as it was one release event it is uncertain what would happen with the dispersal pattern at higher wind speeds.

Aware that the models used to analyze *L. huidobrensis* dispersal are phenomenological, and thus fail to discriminate detailed components of the dispersal process (6), but the low rate of recaptures did not allow performing mechanistic models with reliable results.

Nevertheless, these analyses allow describing and predicting general patterns of dispersal for the pea leaf miner which represents the first step to begin understanding the dispersal process of this pest in the field. Knowledge of the dispersal potential of *L. huidobrensis* is relevant by different reasons. At local scale, it could be useful in crop pest control, for the application of trap cropping strategy being the knowledge on adult dispersal important to decide trap plants location (7).

In addition, the use of yellow traps as monitoring tool for the pea leaf miner could be improved by adjusting space between traps (11). At landscape scale, knowing mean dispersal distance of *L. huidobrensis* could be important to predict its potential of spread to other fields (19).

On the other hand, considering that *L. huidobrensis* is invasive in numerous parts of the world (26), the knowledge of dispersal parameters is relevant since they can have an overwhelming effect on invasion speed (15). At the same time, the dispersal information could serve to model the future areas of colonization (in altitude and latitude) of the pest under scenarios of global climate change (15). All in all, the information obtained in this field experiment could help to the future development of proper control strategies within an integrated pest management program and to develop forecasting systems to alert farmers about pest invasions and outbreaks (17).

Conclusions

In general the pea leaf miner showed a low dispersal capacity in a homogenous landscape. Nevertheless, the potential consequences of this behavior should be analyzed considering the generalist feeding habitat of the species. Future studies on the topic should incorporate different spatial configurations of the landscape in order to have bigger picture of the process. Obtaining field dispersal estimates, as those here showed is essential to understand the ecological and evolutionary bases of the dynamics and persistence of insect populations and would help to guide a proper design of management policies of this worldwide pest species.

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Chemical composition and *in situ* ruminal disappearance of sorghum silages grown in the mexican humid tropic

Composición química y degradabilidad ruminal *in situ* de ensilados de sorgo cultivados en el trópico húmedo mexicano

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Abstract

The purpose was to evaluate the chemical composition and *in situ* ruminal dry matter disappearance of four types of grain sorghum silages, one brown midrib sorghum (bmr) and, a corn silage, grown under rainfed conditions in a humid tropical region of Mexico. The crops were established at three sites. At harvest, three minisilos per treatment were filled with forage previously chopped. Minisilos opened at 55 days and samples of silage taken to dry and ground to 1 mm to determine the chemical composition and *in situ* ruminal dry matter disappearance. The crude protein was higher ($p<0.05$) in sorghum silages than corn silage. In sorghum silages, bmr sorghum had the lowest ($p<0.05$) cell wall content, and was equal ($p>0.05$) in ADF and ADL to corn silage. The degradation parameters (*a, b, c*) was higher ($p<0.05$) in bmr sorghum silage than grain sorghum silages. The effective degradability was equal ($p>0.05$) in bmr sorghum and corn silages. In the humid tropics, bmr sorghum silages are a good alternative to corn silage, especially in the dry season.

Keywords
Sorghum bicolor • silages • digestibility • forage quality • grasses • dry season

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Resumen

El objetivo fue evaluar la composición química y la degradabilidad ruminal *in situ* del ensilado de cuatro híbridos de sorgo para grano, uno de nervadura café (bmr) y un híbrido de maíz, bajo condiciones de secano en una región del trópico húmedo de México. Los cultivares se establecieron en tres sitios. A la cosecha, en cada sitio se llenaron tres minisilos de laboratorio por tratamiento. Los minisilos se abrieron a los 55 días y se tomó una muestra que fue secada y molida a 1 mm para determinar la composición química y la degradabilidad ruminal *in situ* de la materia seca. La proteína cruda fue mayor ($p<0,05$) en los ensilados de sorgo que en el ensilado de maíz. Entre los sorgos, el sorgo bmr presentó el menor ($p<0,05$) contenido de pared celular y fue similar ($p>0,05$) en FDA y LDA al ensilado de maíz. Los parámetros de degradación (*a, b, c*) fueron mayores ($p<0,05$) en el ensilado de sorgo bmr en comparación al de sorgos para grano. La degradabilidad efectiva fue igual ($p>0,05$) en el ensilado de sorgo bmr y el maíz. En el trópico húmedo, el ensilado de sorgo bmr, es una alternativa viable con referencia al ensilado de maíz, especialmente en la época seca.

Palabras clave

Sorghum bicolor • ensilado • digestibilidad • calidad del forraje • pastos • época seca

Introduction

The low quality and availability of pastures, especially in the dry season (January-May), is a recurrent problem in the humid tropical regions of Mexico (1). That reduces the intake of nutrients in the dual-purpose cattle (DPC) system and negatively influences animal performance. The 70% of producers with DPC consider the seasonal production forage as the main limitation of animal performance (1). Other factor that limits the availability of nutrients in tro pical grasses is the high lignin content, which can represent up to 10.5% of DM (28). Lignin is the primary indigestible component in plants cell walls that inhibits bacterial digestion in the rumen of fibrous carbohydrates (4).

In the tropics of Mexico, the conservation of forages as silage is promoted as an alternative to increase and maintain milk production in the year. Corn (*Zea mays* L.) is the crops to choice for producing high quality silages (22, 25); but this crop exhibits drawbacks during periods of water stress (6, 18). Under this scenario, sorghum [*Sorghum bicolor* (L.) Moench] is used as alternative crop to replaced corn silage, because can be sown late, is better in water use efficient, have high biomass yields (15, 21), increases the soil cover and has a lower nutrients and pesticides requirement compared with corn crop. Sorghum silage has acceptable levels of soluble carbohydrates (60-80 g kg^{-1} of DM), relatively low buffer capacity, dry matter content of more than 20% and physical structure to compact during silo filling and, represents lower production costs compared to corn (15, 27).

Sorghum silages is less used than corn silages, because sorghum exhibit higher lignin content and lower fiber digestibility, which increases rumen filling,

reduces DMI and limits milk yield. In the other hand, brown midrib (bmr) sorghums have lower lignin compared with conventional sorghums (without bmr gene), which makes them more digestible. Studies of *in situ, in vitro* and *in vivo* digestion, show that forage of plants with the bmr mutation, possess greater dry matter and NDF digestibility than its counterparts (3, 13, 21). Also, studies showed that bmr sorgos silage can equal the nutritional value of conventional corn silage, supporting similar milk yield (4, 21).

In central and northern of Mexico, sorghum silage is considered as an alternative to corn silage for cattle milk production. For high yields of dry matter (18 t ha^{-1}), digestibility (56%) and net energy lactation (1.45 Mcal kg^{-1}), sorghum silage is widely used by milk producers in these areas of country (20). However, under the environmental conditions of the tropics, the agronomic behavior of this forage species is different.

Overtime, selection of sorghum varieties originated plants with different morphological characteristics (26), among these, varieties with higher proportion of grain and lower proportion of stems. These characteristics can result in silage with high-energy value content, which can be used efficiently as a supplement in rations of DPC in the tropics to increase milk production throughout of year. However, there is no information about the behavior of sorghum in terms of silage yields and quality, produced in rainfed conditions, particularly in the dry season of the year (January-May).

Objective

Assess forage yield, chemical composition and *in situ* dry matter disappearance in different sorghums and corn silages cultivated under typical conditions of the humid tropics in Mexico.

Materials and methods

In December 2012, the grain type sorghums DK-67 (DK67, Dekalb®), Niquel (Niquel, Asgrow®), RB-Norteño (RBN, Inifap) and RB-Huasteco (RBH, Inifap), the two first adapted to the area and the two seconds non-adapted (from Tamaulipas state), a forage sorghum type gene-brown midrib Silo Miel 350 (SM350, Genex®) and a corn hybrid A7573 (A7573, Asgrow®), traditional in the zone, were established under rainfed conditions in three sites of Loma Bonita, Oaxaca, Mexico. The sites are located at coordinates 18°05' L N, 95°53' L W; 18°08' L N, 95°53' L W; 18°08' L N, 95°53' L W, between 0 and 200 m a. s. l. The soils in the three sites have a texture of crumbly sand, sandy clay loam and sandy loam; with 8, 51, 51 ppm of inorganic nitrogen; 52, 5.8 and 11 ppm of phosphorus; 3.68, 4.62 and 4.45 pH, respectively. The dominant climate in the area is warm humid with rains (81.7%) in summer (14). The average annual temperature is 25°C; maximum of 39°C and a minimum of 16°C and, 1,800 mm rain precipitation.

After weeding grass and two harrowing steps, sowing was done with a traditional zero- tillage machine at 20 kg ha^{-1} seed density. From each material, eight lines of twenty meters were planted with 0.75 m of space between lines, leaving a useful plot of 120 m^2. Fertilization was done manually, by apply 3 kg of urea (46-00-00) and 2 kg of di-ammonium phosphate (DAP, 18-46-00) per useful plot, equivalent to 417 kg ha^{-1} of fertilizer (60% urea and 40% DAP; 145 kg of N and 77.0 kg of P_2O_5 ha^{-1}), in

two applications: at 15 and 50 days after the sowing date. The crops tillage were the traditional: two foliar applications, insecticide and manual weeding.

The crops were harvested manually when the plant reached the milky-dough grain maturity stage (86 ± 5 d) and corn, when the grain reaches the half milk line (90 ± 3 d post-sowing). To the harvest, in each plot, the number of plants in 2.5 m per line were counted, cutting and weighed. Plant samples were taken diagonally in the lines until completing one line. With this information, the final density of plants, yield green forage and dry matter forage were calculated.

These plant samples were chopped with a gasoline stationary forage chopper. A sample of green material was taken and was frozen for further analysis. With the remaining material, three laboratory minisilos with a capacity of approximately three kilograms of green forage were filled. The minisilos were made of PVC with measures of 15.7" high x 4" diameter; with lower drainage for liquid and upper outlet for gases. After 55 days the minisilos were opened, the pH was measured and, approximately one kilogram of samples were taken and these were frozen for further analysis. Three replicates were made to measure the pH, by mixing in a blender jar for domestic use, 25 g of silage plus 250 ml distilled water, the mixture was liquefied for 30 seconds at maximum speed.

The solution was filtered through two layers of cheese cloth, after five minutes the measurement was made using HANNA potentiometer, model pH 209 (Instruments Inc. USA).

Chemical analysis of the samples

Fifty-four silage samples (nine per treatment) and eight-teen green forage (three per treatment) were unfreeze at room temperature and dry in triplicate in air oven at 60°C for 48 h; then, they was ground to 1 mm (Thomas A. Wiley Laboratory Mill, Model 4, Thomas Scientific, Swedesboro, NJ). The absolute dry matter (105°C for 8 h in oven), ash (550°C for 3 h in a muffle furnace) and crude protein (N x 6.25) were determined in the milled samples (2). The content of neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) was determined sequentially in the ANKOM^{200} Fiber Analyzer, using Ankom® F57 filter bags with a pore size of 25 µm.

***In situ* dry matter disappearance test**

This test was only done in Niquel, DK67, SM350 and A7573 silages. The preparation of subsamples was as follows: from each of the nine silage samples, 25 g were taken to form a representative subsample of each silage, which were used for the ruminal degradability test. These silages were selected for having presented better chemical composition, based on NDF, ADF and ADL content.

Two heifers of Beefmaster breed, with age of 26 months and average live weight of 450 kg, with permanent rumen cannula were used. One week before the incubation of the samples, each heifer received 8.8 kg daily (on dry basis) of a diet based on alfalfa (*Medicago sativa*) hay (32.4%), pangola (*Digitaria decumbens*) grass hay (35.0%), molasses (6.0%), salt (0.9%), minerals (0.4%) and commercial supplement (25.2%), which contained 12.3, 42.0 and 10.4% PC, NDF and ash, respectively.

The nylon bag technique (23) was used. In bags of 5 x 10 cm and pore size of 50 microns (Ankom Corp., Fairport, NY), were deposited 3 g of dry sample ground at 1 mm; two bags were used for each treatment and each

incubation time, for a total of 48 bags per heifer. The incubation times were: 6, 12, 24, 48 and 72 h. Bags were introduced into the rumen reverse manner, that is, the first time 72 h, ending with time 6 h.

In each incubation time, a bag without sample (white) was included to use it as a correction factor. The determination of soluble material at time zero, was done by immersing these bags group in distilled water at 39°C for 15 minutes.

After 72 h incubation bags were taken out of rumen at same time and placed in ice water to stop microbial activity. Afterwards, the bags were transferred to laboratory, where one by one these were washed manually with tap water until the water is clear. Bags were then dry in forced air oven at 65°C for 48 h to obtain the dry matter disappearance.

The rumen degradation parameters of dry matter (DMD) were estimated using the equation:

$$\% \text{ degradability} = \frac{Initial\ weight\ (g) - residual\ weight\ (g)\ x\ 100}{Initial\ weight\ (g)}$$

The DMD parameters were adjusted using the model described by Ørskov and Mc Donald (1979):

P (t) = a + b (1- exp (-c * t))

where:

P = amount of sample or nutrients that disappear from the bag after a time t of permanence in the rumen.

a = soluble fraction that quickly escapes from the bag and is assumed to be completely degradable.

b = nutrient fraction insoluble, but potentially digestible by the rumen microorganisms.

c = degradation rate (% h^{-1}) of fraction b.

a + b = potential degradability (PD) of the substrate after 72 h incubation.

1 - (a + b) = represents the indigestible fraction (IF) of the sample.

The effective degradability (ED) in percent, which define as the material that is actually degraded by the rumen microorganisms, was calculated by the equation:

ED (%) = a + [(b * c) / (c + k)]

where:

a, b, and c = previously defined in the previous equation.

k = flow rate of the rumen particles, established at 2.0 and 5.0% h^{-1} for this study; low and medium milk production, respectively (18).

Statistic analysis

The yield and chemical composition data of the forage were analyzed with the MIXED procedure of SAS 9.1 (29) based on a complete randomized block design; considering the site effect and the interaction site with species as random and the effect of variety or hybrid as fixed.

The comparison of means was made with the least significant difference (LSD), declaring statistical difference when p≤0.05 and tendency between p=0.051-0.100.

The estimation of degradation kinetics parameters was made using the iterative process of the Gauss Newton algorithm, with the NLIN procedure of the statistical package SAS 9.1 (29).

The variables were subjected to an analysis of variance with PROC GLM and comparing means of the parameters obtained for each treatment was done with the Tukey test, declaring statistical difference when p≤0.05 and tendency between p=0.051-0.100.

Results and discussion

Climatic characteristics

In crop development (December 1, 2012 to March 16, 2013; figure 1), the average temperature, relative humidity and cumulative rainfall were 23.2°C, 84.1% and 103.5 mm, respectively. The rainfall was less than 300 mm required by the crop for optimal development; therefore, the potential of the plant to produce biomass could be limited.

There are two critical periods where the crop requires moisture in the soil, the first is between 20 to 25 d post-emergence and the second in the flowering stage (2). Even in this study accumulated rainfall was not optimal, figure 1 shows greater rainfall (47.2%) occurred in February; at the beginning of the flowering stage (62 ± 3 d post-sowing). This contributed to an acceptable panicle development and grain filling in all sorghums.

On the other hand, the corn hybrid A7573 presented small ears, with small grains, this as a result of a higher water stress that crop presented, since it is a species that demands more water (30 to 40% more than sorghum).

Plant density and dry matter yield

The final plants density per hectare presented variation due to the genotype ($p=0.0012$); however, this variation was due to the corn genotype A7573 which presented 61,778 plants ha^{-1}. Among the sorghums, no statistical difference was observed in density, with average of 305,139 plants ha^{-1}, but numerically sorghum DK67 had the highest density (350,972) and sorghum SM350 had the lowest (267,972 plants ha^{-1}).

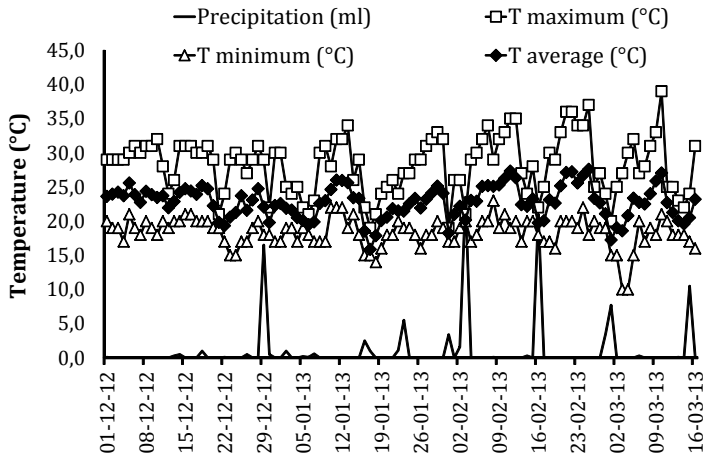

Figure 1. Precipitation and temperatures occurred during the period of crops development in study area.

Figura 1. Precipitación y temperaturas ocurridas durante el periodo de desarrollo de los cultivos en el área de estudio.

At harvest, DM percent was different between treatments (p≤0.05). Grain sorghums presented an average of 27.4%, sorghum SM350 was medium and corn A7573 the lowest level; however, the RBH, SM350 and A7573 materials were statistically equal (table 1).

For the yield of DM ha^{-1}, statistical difference (p=0.0142) was observed between grain sorghums types, bmr SM350 sorghum and corn A7573. At harvest (cut), DM yield in sorghums ranged from 7.2 ± 3.8 to 11.5 ± 2.6 t ha^{-1}. Highest yield was with sorghum DK67 and lowest with SM350, while corn yields 10.6 ± 2.5 t ha^{-1}. The DM yield of silage presented the same behavior among the materials (table 1).

Regarding DM losses during the silage process, these were higher in grain sorghums with average of 7.4%; bmr SM350 lost 2.1%; while corn silage A7573 yielded 5.5% less DM than when freshly harvested.

The forage yield has a relationship to DM content of plant, plant density and plant height. DK67 and Niquel sorghums, due to their higher DM content, high plant density ha^{-1} and plant height, show higher forage yield, medium for corn A7573 and, lower for RBH, RBN and SM350, the latter due to its lower DM content, but also due to the low density and height of the plant (table 1).

The sorghum SM350, had the lowest DM content (24.8 ± 2%). Several studies have reported that bmr sorghums tend to have low DM yields.

The bmr gene present in these sorghums can be the cause of low forage yields in comparison with conventional sorghums (without bmr mutation) (16).

Also, the forage yield is in function of maturity stage at cutting. According to the literature, an average of 14 t ha^{-1} of DM (9.0 to 15.0 t ha^{-1}) is accepted for sorghum harvested in dough grain stage (31). The yields obtained in this study (table 1) coincide with the 11.0 t of DM ha^{-1} (24) and the 11.5 t (27) for conventional sorghum hybrids. In other studies, was reported 16.3 (4) and 14.6 t ha^{-1} (21), values higher than those observed in this study.

However, in these studies, the harvest was made in a more advanced maturity stage (dough grain), with 30.6% DM, higher than the 27.0% observed in this study, which may explain the higher yields obtained in those studies.

Table 1. Means (±SD) of dry matter content and forage yield of cultivars.

Tabla 1. Medias (±DE) del contenido de materia seca y rendimiento de forraje de los cultivos.

Cultivar	DM content at cutting (%)	Forage yield (t ha^{-1} of DM)	
		At cutting	Silage
DK67 (grain)	27.7 ± 0.4 ^{a}	11.5 ± 2.6 ^{a}	10.6 ± 2.4 ^{a}
Niquel (grain)	27.5 ± 0.4 ^{a}	11.3 ± 3.5 ^{a}	10.5 ± 3.4 ^{a}
RBH (grain)	26.7 ± 2.0^{ab}	7.3 ± 3.4 ^{c}	6.8 ± 2.8 ^{b}
RBN (grain)	27.7 ± 0.3 ^{a}	7.8 ± 2.1^{bc}	7.2 ± 2.1 ^{b}
SM350 (bmr)	24.8 ± 2.4 ^{b}	7.2 ± 3.8 ^{c}	7.1 ±3.9 ^{b}
A7573 (corn)	26.7 ± 1.5^{ab}	10.6 ± 2.5^{ab}	10.0 ± 2.5^{a}

abc = Means with the same letter in the same column, are not different (p≤0.05).

abc = Medias con la misma letra en una misma columna, no son diferentes (p≤0,05).

Is important to pointing the following, forages have a wide variation in DM yield when are grown under rainfed conditions; therefore, yields are expected to be different from one crop cycle to another, which is mainly due to the amount and distribution of rainwater throughout the plant's development period.

Regarding the yield of silage, normally in a well prepared silo losses of 5 to 10% of DM occur. In this study, the losses did not exceed 8% (table 1, page 359), which describes the good process followed in the preparation of the silage; in addition to more controlled conditions by the use of minisilos. Thus, the greatest loss occurred in sorghums silages for grain, medium in corn silage A7573 and very low in sorghum SM350 which, with respect to green material, only lost 2.1% in the silage process. This last observation may be another quality that present these sorghums when are used as silage.

Silage pH

During silage process, is important that anaerobic fermentation is reached in a short time to produce the necessary organic acids to increase the acidity to the point that eliminates undesirable microbes and preserve the ensiled material, lactic acid contributes more to the decrease pH in the silo (32). In this study, the pH was equal ($p>0.05$) among silages with an average of 3.6 (table 2), adequate value (27), especially with the use of minisilos.

The average pH of 3.6 obtained in this study was associated with high content of sugar in the forages, mainly starch; explained by the amount of grain present in plant, as well as the conditions controlled by the use of minisilos.

Should be noted that in the final stage of the harvest, sorghums were attacked by birds, with sorghum DK67 being the most affected; this may explain the slight increase in pH observed in this silage, due to the fact that it had a lower amount of fermentable carbohydrates for the production of lactic acid. The average pH of 3.6 is in according with that of 3.9 reported in the literature (4, 9, 21).

Crude protein (CP)

Silage material showed effect ($p=0.0032$) in CP content (table 2).

The sorghums silage Niquel, RBH, RBN and SM350, were equal in CP (10.6%), while sorghum DK67 and corn A7573 silages had the lowest levels (8.4% of DM).

Table 2. Means (±SD) of pH and chemical composition of silages.
Tabla 2. Medias (±DE) del pH y composición química de los ensilados.

Cultivar	pH	Chemical composition of silages			
		% CP	% NDF	% ADF	% ADL
DK67 (grain)	3.7	8.4 ± 0.8^{b}	56.2 ± 4.4^{a}	33.5 ± 3.1^{a}	2.9 ± 0.2^{a}
Niquel (grain)	3.6	10.1 ± 1.1^{a}	55.5 ± 2.5^{a}	33.4 ± 2.8^{a}	3.1 ± 0.3^{a}
RBH (grain)	3.6	10.5 ± 0.9^{a}	54.9 ± 2.7^{a}	32.3 ± 2.1^{a}	3.1 ± 0.3^{a}
RBN (grain)	3.6	11.2 ± 1.1^{a}	54.7 ± 1.9^{a}	32.1 ± 1.7^{a}	2.9 ± 0.2^{a}
SM350 (bmr)	3.6	10.6 ± 1.1^{a}	45.7 ± 4.3^{b}	26.3 ± 2.2^{b}	2.2 ± 0.1^{b}
A7573 (corn)	3.6	8.5 ± 0.9^{b}	51.4 ± 3.0^{a}	28.7 ± 1.6^{b}	1.9 ± 0.3^{b}

ab = Means with the same letter in the same column, are not different ($p\leq0.05$).
ab = Medias con la misma letra en la misma columna, no son diferentes ($p\leq0,05$).

In general, the CP in sorghum silage was higher that other studies, with 9.1 (19) and 9.9% (11), at a level of 28.8 and 27.5% of DM in plant, respectively. Other studies 6.8 (4) and 7.3% (21), at a 30.6% DM and, 8.7 (6) and 8.9% (7) at 32 and 30% of DM in the plant, respectively. These differences in CP content can relate to the percentage of DM content in plant at harvest. On the other hand, the lower content of CP (8.4%) observed in sorghum DK67, could be due to a smaller amount of grain, consequence of the attack of birds. Regarding the corn silage A7573, it showed a CP content according to that reported in the literature (4, 19, 21), a value lower than the rest of the sorghums with the exception of DK67.

The higher concentration of CP present in sorghum silage may be due to the level nitrogen (N) fertilization used. A study reported that above 66,700 plants ha^{-1}, the level N fertilization had significant effect on the CP concentration, with 0.3 units or 4.2% increase (16).

In this research, N fertilization level was 145 kg and phosphorus 77.0 kg ha^{-1}. Higher concentrations are recommended (10) to produce sorghum forages in the tropics; they recommend 200 to 300 kg urea and 100 to 150 kg tripe superphosphate (SFT), equivalent to 92 to 138 and 46 to 69 kg of N and P_2O_5.

Neutral detergent fiber (NDF)

There was a genotype effect ($p<0.05$) on the NDF content. The NDF was similar between grain sorghums silage and corn silage A7573 ($p>0.05$), while silage bmr SM350 had the lowest value (table 2, page 360). Although corn silage A7573 presented intermediate NDF value, statistically it was not different from grain sorghum silages.

The lowest NDF value of SM350 silage was attributed to the lower lignin content, distinctive characteristics present in these sorghum varieties.

Grain sorghums silages presented on average 55.3% of NDF, less than 57.0, 60.7 and 58.1% reported in other studies (11, 19, 21), respectively, but higher than 51.7% observed in other research (4).

The silage bmr SM350 presented 4.7 and 4.5 units less NDF compared to that observed in the literature (4, 21), but was similar to 44.9 and 46.3% reported in other study (22) for sorghum bmr-6 and bmr-12, respectively. In a more recent study (7), with a variety of sorghum for grain, reported an average of 50.7% of NDF in three years of evaluation without observing difference by year effect. For their part (16), they reported 46.6, 50.4 and 50.3% for a variety of corn, a conventional forage sorghum and a bmr sorghum, respectively; where the bmr sorghum presented the highest ($p<0.05$) NDF digestibility.

In this study, a positive relationship between plant density ha^{-1} and NDF content was observed; however, in other works (7, 16), no effect of plant density on the NDF content was detected. Perhaps at higher densities of plants ha^{-1}, as in this study, a significant effect on this variable may occur.

Acid detergent fiber (ADF)

There was a genotype effect on ADF content (table 2, page 360). Grain sorghum silages (DK67, Niquel, RBH and RBN) had similar ADF, with average of 32.8% (table 2, page 360). The sorghum SM350 and corn A7573 silages had the lowest content of ADF, but were equal to each other ($p=0.0945$).

The sorghum SM350 bmr silage presented 24.7 and 9.1% less ADF than the average of grain sorghums and corn silages, respectively. This last observation implies that this type of sorghum (bmr) can equal or exceed corn forage quality under these climatic conditions.

The average 32.8% present in grain sorghums, is lower 34.7, 38.7 and 37.7% observed in the literature (4, 18, 20), respectively, for conventional forage sorghums. In a more recent study (7) made with a grain sorghum variety that was evaluated for three years (2007, 2008 and 2009), a value of 25.7% was found, less than in this study.

In the first three researches, a higher silage DM content (~30.6%) than the one obtained in this study (27.0%) was reported, which implies greater maturity of the plant, which could explain the increase of ADF in those forages. In the fourth study (7), the harvest was made at the same level of DM in plant (30.0%) in the three years of evaluation. In 2007, the grain presented a milky-dough consistency, while in 2008 and 2009 the consistency was milky; this implies that, at the same DM level, plant maturity is different due to the influence of climatic and edaphic factors presents between periods or between soils. This influences the cell walls content; that in this study was 27.9% of ADF in 2007, similar to the one obtained in this study, and 24.6% and 24.3% in 2008 and 2009.

The ADF (26.3%) present in sorghum SM350 bmr silage, is lower than the 28.5 and 33.6% observed in sorghums bmr-18 and bmr-6 (21) and to 36.5% (4). In another study (22) were reported 26.8 and 26.2% for sorghums bmr-12 and bmr-6, values that are similar to the one obtained in this study.

Acid detergent lignin (ADL)

The ADL showed a behavior similar to that of ADF. Thus, the ADL content was equal ($p>0.05$) among grain sorghum silages, with average of 3%; but this average was higher ($p<0.05$) compared with SM350 and corn A7573 silages, that were equal (table 2, page 360). The content of ADL in silage SM350 was 36.4% lower ($p<0.05$) than the average of grain sorghum silages and 13.6% higher ($p>0.05$) than corn A7573 silage.

In grain sorghum silages, ADL coincides with 2.9% observed in the literature in average (7, 21), but it is lower than 5.7, 6.5 and 7.0% reported in other studies (11, 19, 22), respectively. In these last three studies, the highest ADL content can be attributed to maturity stage which sorghums were harvested; for example, in the study (22) the harvest was made in grain full and hard, that is a greater maturity stage to which the sorghums were harvested in this study. This maturity effect, also is observed in the study (7), where the sorghum was harvested in two different stages: milky-dough and milky, with averages of 3.6 and 2.5% ADL, respectively.

In other study (6), report 3.1, 3.5 and 3.8% of ADL for three grain sorghum varieties, being first value more similar to that one observed in this study.

The ADL content in silage sorghum SM350 was 2.2% and was equal ($p>0.05$) to 1.9% observed in corn silage A7573. In this regard (21), they reported 2.3 and 2.5% for sorghums bmr-6, bmr-18, respectively. In other research, reported for the same sorghum of bmr Silo Honey-350 and for Green Giant Sorghum (AgriBio Tech®) 2.5 and 2.1% of ADL, at 24.5 and 25.0% DM level, respectively (8).

In other studies, higher values have been reported (22); however, this higher

ADL concentration is relate to the harvest at the most advanced maturity stage. For example, it has been observed that when the harvest is made in grain milky stage, the ADL is below 3.0% of DM, while in milky-dough it is above this value.

The similar ADL content observed in this study between silage SM350 and corn A7573, make bmr sorghums, a good option to produce good forage quality, especially when are established in dry season of year in the humid tropics. This advantage is attribute to the lower lignin content of these sorghums and their capacity to grow in drought conditions, where the production of corn forage represents a greater risk.

Based on these results, the maturity stage and variety have a marked effect on the fiber and lignin content in the sorghum forage, where structure and relationship of plant components (stem:leaf:panicle) contribute to significant way on forage quality in different genotypes (26). Grain sorghums, due to their own characteristics, have higher fiber content and lower digestibility than conventional forage sorghums and bmr; the latter surpassing even conventional foragers. However, the forage of whole corn plant surpasses in yield and quality any type of sorghum, even under certain critical levels of humidity in the soil, as happened in this study and the Marsalis *et al*. (2009) study.

In situ dry matter disappearance

Parameters degradation of dry matter were affected by forage type ($p<0.05$). Parameters *a*, *b* and *c* were higher in sorghum silage SM350 compared to grain sorghums. Sorghum silage Niquel showed greater degradation of fraction *b* than its homologue DK67 (table 3).

Table 3. Kinetic parameters (%) of *in situ* ruminal disappearance of dry matter of silages.

Tabla 3. Parámetros (%) de la cinética de degradación ruminal *in situ* de la materia seca de los ensilados.

	Silages			
Parameters	**DK67**	**Niquel**	**SM350**	**A7573**
a	32.5 ± 1[c]	30.5 ± 1 [d]	34.3 ± 1[b]	35.2 ± 1 [a]
b	42.0 ± 5 [d]	48.3 ± 5 [c]	49.0 ± 3 [b]	50.5 ± 3 [a]
c (% hour^{-1})	2.4 ± 0.6 [c]	2.0 ± 0.4 [d]	2.7 ± 0.4 [b]	2.8 ± 0.5 [a]
Estimated				
PD (72 h)	75.1 ± 1.9[b]	80.1 ± 1.9[ab]	83.3 ± 1.9[ab]	86.0 ± 1.9[a]
IF	24.9 ± 1.9[a]	19.9 ± 1.9[ab]	16.7 ± 1.9[ab]	14.0 ± 1.9[b]
ED (k_p= 5 % h^{-1})	46.0 ± 0.5[b]	44.6 ± 0.5 [b]	51.5 ± 0.5 [a]	53.5 ± 0.5[a]
ED (k_p= 2 % h^{-1})	55.3 ± 0.6[b]	55.1 ± 0.5 [b]	62.5 ± 0.6 [a]	64.9 ± 0.6[a]

abcd = Means with different letter in the same row, differ significantly ($p\leq0.05$).

a = soluble and rapidly degradable fraction; b = insoluble fraction and slowly degradable; c = degradation rate (% h^{-1}) of fraction b; kp = passage rate through rumen (% h^{-1}); PD = potential degradability (a + b) of substrate at 72 h incubation; IF = indigestible fraction (100 - (a + b)) of substrate; ED = effective degradability with kp at 5 or 2%, for medium and low milk production level (19).

abcd = Medias con diferente letra en el mismo renglón, difieren significativamente ($p\leq0,05$).

a = fracción soluble y rápidamente degradable; b = fracción insoluble y lentamente degradable; c = tasa de degradación (% h^{-1}) de la fracción b; kp = tasa de pasaje por el rumen (% h^{-1}); DP = degradabilidad potencial (a + b) del sustrato a 72 h de incubación; FI = fracción indigestible (100 - (a + b)) del sustrato; DE = degradabilidad efectiva con kp a 5 o 2 %, para media y baja producción de leche (19).

On the other hand, corn silage A7573 presented the highest values ($p<0.05$), this result confirms the nutritional quality maintained by this forage species, even under conditions of water stress.

The potential degradability (PD) at 72 h incubation, was higher in the corn silage A7573, but was not different from SM350 and Niquel silages, while DK67 silage showed the lowest value ($p<0.05$). The indigestible fraction (IF) was similar among sorghums and was lower in corn silage A7573, which showed no difference with SM350 and Niquel silages. The effective degradability (ED) at a Kp of 5 or 2%, showed the same tendency among silages; was higher in corn A7573 and sorghum SM350 silages and lower in grain sorghums silage (table 3, page 363).

The results of degradation kinetics observed in this research are consistent with previous studies (9, 30). This similarity in results may be because sorghums were harvested at a similar stage of maturity and, therefore, the chemical composition was similar. For example, in those studies the soluble fraction was 32.4 and 37.8% in grain sorghum silages, respectively. For their part, in the second study (30) reported 45.6% in *b* fraction, similar to obtained with sorghum Niquel. Other studies there are greater differences in results (5, 12).

The differences in results can be explained by the different conditions under experiments were development. Is knowledge that factors such as soil type, cultivar, climate, maturity stage, plant or seed density, etc., significantly influence the chemical composition forages (15). These factors alter, for example, fiber fractions and their relationship with lignin, which in turn influences enzymatic activity carried out by rumen bacterial on cellulose and hemicellulose polysaccharides, resulting in important changes in dry matter degradability and, finally, affects forage intake and animal performance (17, 22).

Although total precipitation (103.5 mm) occurred in the experiment was low, corn cultivar had good acceptable yield and higher forage quality than grain sorghum silages types. Brown midrib sorghums, such as SM350, because low lignin content, are good option in terms of forage quality in water deficit situations, since corn cultivars cannot be established; however, this sorghum types tends to have lower forage yield (15).

Regarding the grain sorghums silages cultivars, such as DK67 and Niquel, for their adaptation to the prevailing climate in humid tropics, can compete in yield with corn silage; however, their high content of cell walls can limit dry matter intake and animal performance.

Conclusions

In the humid tropics, grain sorghums DK67 and Niquel showed high forage yield, but the silage quality was low. Due to their adaptation to the edaphoclimatic conditions of region, these sorghums are a viable alternative to produce high silage yields comparable to those of corn silage.

The sorghum SM350 bmr, planted in December produce a high quality silage during the dry season, even greater than corn silage. Despite having less soil water availability, corn silage showed similar yield and more digestible nutrients per hectare than sorghums.

Under this scenario, research is required to determine under what level of water deficit sorghum can surpass corn yield and nutritive forage value. Because their low forage yield, RB-Huasteco and RB-Norteño sorghums are not recommended as an option to produce good silages in this region of country.

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Valorization of mulberry (*Rubus glaucus*) by-products: ultrasound-assisted extraction of total anthocyanins

Valorización de subproductos de mora (*Rubus glaucus*): extracción asistida por ultrasonido de antocianinas totales

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Abstract

In this study, the objective was to establish parameters of optimization of the factors such as solvent concentration, extraction time and liquid-to-solid ratio in ultrasound-assisted extraction (UAE) of total anthocyanins from mulberry by-products. The study sample corresponds to mature fruits that did not comply with the size demanded by the market (by-product). Three independent variables including extraction time (6-74 min), liquid-solid ratio (33-66.16 mL/g), and ethanol concentration (63-96%) were investigated. A rotatable central composite design (CCD) and response surface methodology (RSM) was used to investigate the effect of process variables. The optimum conditions of ultrasound-assisted extraction obtained through response surface methodology were as follow: extraction time, 20 min; liquid to solid ratio, 60:1 mL/g, and ethanol concentration, 90%, allowed to obtain a maximum concentration of 259.66 mg/L. To validate the optimized model, the experimental values were compared with the predicted values to check the adequacy of the model. Ultrasound extraction was 13.60% higher than maceration technique and reduces the time from 24h to 20 minutes. The results show that ultrasonic-assisted extraction as a promising agro-industrial process in the recovery of anthocyanins from mulberry by-products.

Keywords
central composite design • response surface methodology • natural colorant • optimization

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Resumen

En este estudio, el objetivo fue establecer los parámetros de optimización de los factores como la concentración de disolvente, el tiempo de extracción y la relación líquido a sólido en la extracción asistida por ultrasonidos (EAU) de antocianinas totales de subproductos de mora. La muestra del estudio corresponde a frutas maduras que no cumplieron con el tamaño exigido por el mercado (subproducto). Se investigaron tres variables independientes, incluido el tiempo de extracción (6-74 min), la relación líquido-sólido (33-66,16 mL/g) y la concentración de etanol (63-96%). Se utilizó un diseño compuesto central rotacional (DCCR) y una metodología de superficie de respuesta (RSM) para investigar el efecto de las variables de proceso. Las condiciones óptimas de extracción asistida por ultrasonido obtenidas a través de la metodología de superficie de respuesta fueron las siguientes: tiempo de extracción, 20 min; relación líquido-sólido, 60:1 mL/g, y concentración de etanol, 90%, permitieron obtener una concentración máxima de 259,66 mg/L. Para validar el modelo optimizado, los valores experimentales se compararon con los valores predichos para verificar la adecuación del modelo. La extracción de ultrasonido fue 13,60% más alta que la técnica de maceración y reduce el tiempo de 24h a 20 minutos. Los resultados muestran que la extracción asistida por ultrasonidos es un prometedor proceso agroindustrial en la recuperación de antocianinas de los subproductos de la mora.

Palabras clave

diseño compuesto central • metodología de superficie de respuesta • colorante natural • optimización

Introduction

The mulberry is of the Rosaceae family, there are between 700 and 750 species distributed in 12 genera, being *Rubus*, the largest species within this family (5, 29). Mulberry is one of the most appreciated fruits in the world market, as this fruit has gained an important position in the food industry, thanks to the presence of anthocyanins (16, 30, 35).

Different research has reported that the health benefits of anthocyanin focus on antioxidant and anti-inflammatory activity, reduced risk of coronary heart disease, stroke, cancer, and aging (1, 16, 30). In addition, the anthocyanins of natural origin are authorized by the European Food Safety Authority (EFSA) and Food and Drug Administration (FDA) to produce colorants in the food industry (6).

The growing interest of the consumer and industry, due to the health benefits and coloring properties of natural anthocyanins, has encouraged the search for new sources of anthocyanins.

Ayala-Zavala *et al.* (2011) reports, that fruit by-products are an important source of natural pigments, as these residues are produced in high volumes and are available at a low cost.

Different fruit by-products are sources of anthocyanins, among them banana (28), mulberry (38), black chokeberry (10), grape (3, 9, 15), coffee exocarp (31) blackberry pomace (13, 27).

Ghafoor *et al* (2009), Ghafoor *et al* (2011), and Tao *et al.* (2015) commented that extraction is an important stage in the processes of obtaining the phenolic compounds; also indicate that the time, temperature, solvent/sample ratio and solvent concentration are important factors to be optimized.

Bioactive compounds in plant matrices can be obtained with conventional techniques such as maceration, Soxhlet and cold compression, also with emerging techniques such as supercritical fluid, extraction by electric pulses, high pressure extraction and ultrasonic assisted extraction (14).

The emerging technology of ultrasound is a valid technique in obtaining bioactive compound on natural matrices due to the high yields, simplicity of management and reduced costs in the extraction of such compounds (7, 8).

Dranca and Oroian (2016) indicate that the ultrasound process exerts a mechanical effect, which breaks plant cell walls, improving mass transfer by increasing the contact surface between the solvent and the plant material.

Ultrasonic assisted extraction (UAE) of anthocyanins were reported in plant materials by-product such as saffron, eggplant, mulberry, grape, blueberry, and jabuticaba (11, 14, 16, 17, 19, 20, 22, 32). Although studies have been carried out for the extraction of anthocyanins from different plant matrices, the scientific literature does not report any investigations on the optimization UAE of anthocyanins from mulberry (*Rubus glaucus*) by-products. In addition, this emerging technology can be an alternative of valorization of the mulberry by-products produced in the commercialization of this fruit, since this residue is an important source of anthocyanins, appreciated by the food, pharmaceutical and cosmetic industry as natural pigments. Therefore, the objective of the present investigation was to establish parameters of optimization of the factors such as solvent concentration, extraction time and liquid-to-solid ratio in UAE of total anthocyanins from mulberry by-products.

Materials and methods

Materials

Fresh mature mulberry (R*ubus glaucus*) fruits were obtained from a commercial crop, located in the rural area of the municipal of Cali, Valle del Cauca.

The study sample corresponds to mature fruits that did not comply with the size demanded by the market (by-product), according to what is established by the Colombian Technical Standard NTC 1406 (1997). The material was left to dry at room temperature for 3 hours, pre-cooled to 3°C and immediately frozen for 12 hours at -80°C.

Finally, the material went through lyophilization (FreeZone 4.5 freeze-dryer). The lyophilization of the samples was conducted at a temperature of less than -55°C with a condenser surface of 13.6 dm^2 and a pressure of 0.12 mbar for 24 hours.

The lyophilized pod was hammer milled (IKA WERKE M20), screened with a 60 mesh sieve (particle size of 246 μm), packed in glass tubes with plastic caps, and covered with aluminum foil.

The samples were kept refrigerated at 4°C until the various analyses were performed. All of the chemicals used were of analytical grade, and they were purchased from were obtained from Merck.

Ultrasound-assisted extraction of the total anthocyanins in mulberry by-products

The extraction was assisted by an ultrasonic cleaning bath using a Branson 2510R-DTH ultrasonic cleaner (Branson Ultrasonics Corp. Danbury, USA), which operates at a frequency of 40 kHz with a power intensity of 130 W and a tank capacity of 2.81 L (internal dimensions: 241.3 x 139.7 x 102.6 mm), equipped with a digital timer and a temperature controller.

The temperature was controlled and maintained at 30 ± 2°C by circulating external water from a thermostatic water bath. The power of the ultrasonic bath during the experiments was 90-110 W, this parameter was determined following the calorimetric method described by Kiani *et al.* (2012). Samples of the powder of mulberry by-products were transferred into a volumetric flask (10 mL), and the extraction procedure was conducted, according to the experimental design conditions under study.

The extractor solution used in the present study corresponds to ethanol: water solution acidified at pH 1 with HCl, as recommended by Rodrigues *et al*. (2015).

The volumetric flask with the solution was immersed into water in the ultrasonic device, and irradiated for the predetermined extraction time.

After the extraction process, the extracts were filtered with Whatman Grade 4 filter paper and filtered through a 0.20 µm nylon syringe filter, and the filtered material was used to quantify the total anthocyanin. Maceration was carried out as a control for comparison with UAE. Samples were extracted at room temperature in the dark for 24 h, using liquid to solid ratio, 60:1 mL/g, and ethanol concentration 90%.

Determination of total anthocyanin concentration

The total anthocyanin content in mulberry by-products extract was determined using the pH differential method, previously described by Lee *et al.* (2005). The extracts were separately mixed with potassium chloride buffer (KCl, 0.025 M, pH 1.0) and sodium acetate buffer (CH3COONa, 0.4 M, pH 4.5) and the absorbance was measured at 520 nm and 700 nm, against a blank consisting of distilled water, using a spectrophotometer Jenway 6320D.

The concentration of anthocyanins in the extract was expressed as cyanidin -3-O-glucoside equivalent according to Equation 1.

$$Total\ anthocyanin\left(\frac{mg}{L}\right) = \frac{AxMWxDFx1000}{\epsilon \text{ x L}} \quad (1)$$

where:

$A = (A520\text{nm} - A700\text{nm})_{pH1.0} - (A520\text{nm} - A700\text{nm})_{pH\ 4.5}$

MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu)

DF = dilution factor

L = path length (1 cm)

ε = 26 900 molar extinction coefficient, in L x mol^{-1} x cm^{-1}, for cyd-3-glu

1000 = factor for conversion from g to mg.

Experimental design

Following the recommendation of Bezerra *et al.* (2008), preliminary tests of one factor were carried out, which allowed the identification of the three main factors, with greater effect in the extraction of the total anthocyanins presents in the mulberry by-products.

The effects of extraction time (20, 30, 40, 50, and 60 min), liquid-solid ratio (30:1, 40:1, 50:1, 60:1, and 70:1 mL/g), and ethanol concentration (50, 60, 70, 80, and 90%) were investigated separately on the basis of extraction efficiency.

The optimal levels were selected as center points in the designed experiment. A 2K rotatable central composite design (CCD) with 8 factorial points, 6 axial points and 6 center points was used to obtain the best combination of process variables that optimizes the ultrasound-assisted extraction of the total phenols from the mulberry by-products.

The model selection criteria included coefficient of determination (R2) and Lack or Fit, and analysis of variance (ANOVA) was used to assess the significance of each factor, their quadratic effects and interactions.

The experiments were carried out in triplicate, and the standard deviation was used to express the results.

Table 1, shows the independent variables used in the CCD.

A regression analysis was performed on the results obtained with regard to the implementation of the independent variables, and it was adjusted to an empirical second order polynomial model as shown in general equation 2 for the total anthocyanins.

Design Expert 11 statistical software was used to optimize the extraction process of anthocyanins in the mulberry by-products.

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

where:
Y_i = response variable
β_0 = constant
β_1, β_2 and β_3 = regression coefficients for the linear effect
β_{11}, β_{22} and β_{33} = coefficients for the quadratic effects
β_{12}, β_{13}, and β_{23} = coefficients for interactions X_1, X_2 and X_3 = independent variables (time extraction, ethanol concentration, and liquid-to-solid ratio, respectively).

Results and discussion

Effect of independent variables on ultrasound extraction of anthocyanin

Figure 1(A) (page 372), shows the effect of extraction time on the extraction of total anthocyanins. The yield significantly increased from 212.85 to 254.88 mg/L as the extraction time increased from 20 to 40 min, then began to decrease as the extraction time increased from 40 to 60 min.

Table 1. Independent variables and their levels used for in a central composite rotatable design.

Tabla 1. Variables independientes y sus niveles utilizados en un diseño central compuesto rotacional.

Independent variables	Coded level				
	-α (-1.68179)	-1	0	1	+α (+1.68179)
	Natural levels				
Extraction time (X_1)	6	20	40	60	74
Ethanol concentration (X_2)	63	70	80	90	96
Liquid-to-solid ratio (X_3)	33	40	50	60	66.16

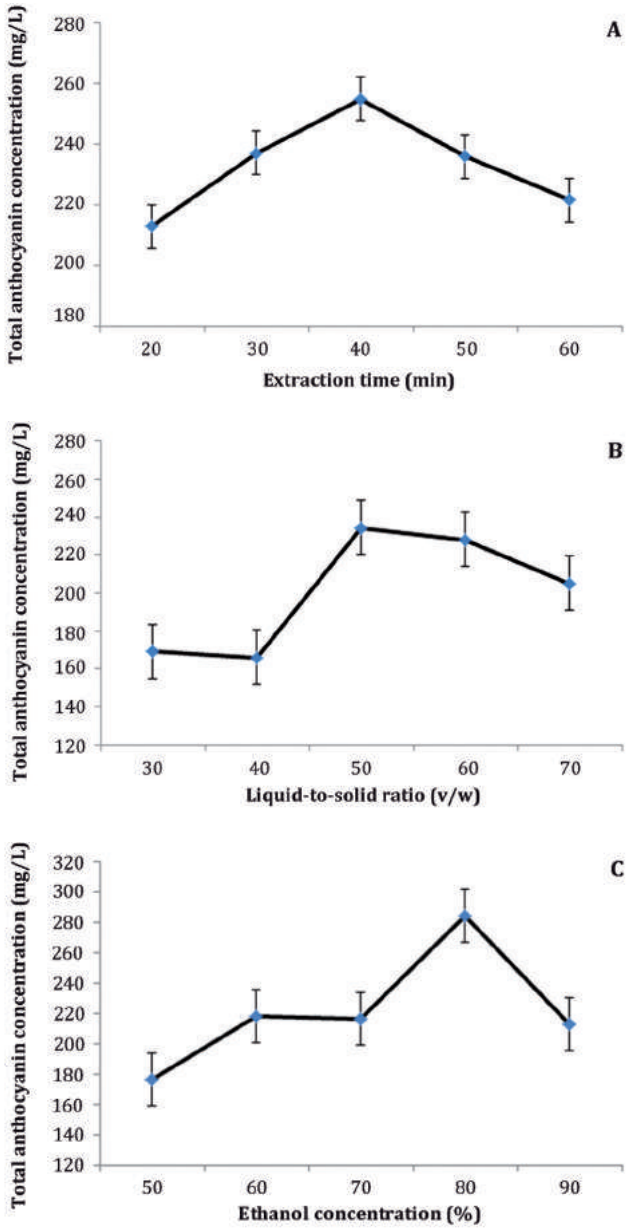

Figure 1. The effects of extraction parameters on total anthocyanin concentration. (A) Effect of time on the extraction, (B) Effect of liquid-to-solid ratio on the extraction, (C) Effect of ethanol concentration on the extraction.

Figura 1. Los efectos de los parámetros de extracción en la concentración total de antocianinas. (A) Efecto del tiempo en la extracción, (B) Efecto de la relación líquido-sólido en la extracción, (C) Efecto de la concentración de etanol en la extracción.

According to the results, 40 min was selected as the central point for this treatment. Tao and Da-Wen (2015) reports that the extraction efficiency depends directly on duration of ultrasound-assisted extraction (UAE).

Explanation for this increase is due to the action of the cavitation phenomena that affect the cell wall, allowing the diffusion of the pigment to the solvent (37). Reduction in the extraction of total anthocyanins could be explained that, within a certain time the internal and external pressure of the cell reached the equilibrium, with the extraction time prolonged, generated the reactions of oxidation and polymerization, responsible for the degradation of anthocyanins (23, 37).

The extraction of total anthocyanins by different liquid-to-solid ratio to material is shown in figure 1B (page 372).

The results indicate a greater extraction of total anthocyanins from 165.98 to 234.20 mg/L, with the liquid-to-solid ratio increasing from 40:1 to 50:1 mL/g. According to the results, 50:1 liquid-to-solid ratio is recommended as the center points in the optimization experiment. Zou *et al.* (2011) reports that liquid-to-solid ratio is other independent variables to be studied, in order to avoid solvent losses or lower yields of the compound of interest. This result was consistent with the principle of mass transfer, where the concentration gradient between the material and the bulky solvent is the driving force of mass transfer (34). However, increasing the solvent does not increase the extraction yield of the total anthocyanins as shown in figure 1B (page 372). Lou *et al.* (2010) reports that the mass transfer is more restricted to the solid interior, therefore, larger amount of solvent would not change the driving force.

Other possible explanations for the reduction in the extraction of total anthocyanins are the extractions of impurities such as polysaccharides and proteins were dissolved out, affecting the dissolution of total anthocyanins (37). As shown in figure 1C (page 372), the extraction of total anthocyanins were increased to increase the ethanol concentration from 50 to 80%, and decreased when the ethanol concentration was higher than 80%.

According to the results, ethanol solution 80% is recommended as the center points in the optimization experiment. The increase and decrease of the extraction of total anthocyanins in the samples can be explained by the polarity between the solvent and the solute, as previously explained by Yang *et al*. (2010) authors, indicate that as there are similar polarities between the solvent and solute, the bioactive compound of interest is easily dissolved from plant cells, and Roselló-Soto *et al.* (2015) reports that polyphenols are easily solubilized in hydroalcoholic mixtures (polar protic mediums), where fractions can be recovered based on polarity by varying alcohol concentration.

Solvent type is an important criterion for ultrasonic assisted extraction, since the physical properties of solvent (polarity, viscosity, surface tension, density, diffusivity and vapor pressure) are correlated with its molecular affinity with the components of interest and its diffusion into biological matrix (17). Yang *et al.* (2010) and Chemat *et al.* (2012) reports that ethanol is one of the most used solvents in the extraction of phenolic compounds in the plant matrix, it is a bio-solvent, completely biodegradable, non-toxic, and economical.

Response surface optimization

Table 1 (page 371), presents the experiment design and corresponding response data for the total anthocyanins extraction.

The range of total anthocyanin extracted mulberry by-products, experimental values between 104.64 - 262.17 mg/L (table 2).

Rodrigues *et al.* (2015) obtained values of total anthocyanin in jabuticaba peel (373.72 mg/L) that exceed those recorded in the present study; extraction conditions used by researchers were 150W ultrasonic power, ethanol concentration of 38% on 30°C, with an extraction time of 60 min and solvent to ratio 1:20 w/v. By contrast, our results exceed the values reported by Demirdöven *et al.* (2015) in red cabbage, where the concentration of total anthocyanins were 28.65 to 58.67 mg/L, which was obtained using 150W ultrasonic power, ethanol concentration of 42.39% on 40°C, with an extraction time of 75 min and solid–liquid ratio 1:3 w/v.

Zhao *et al.* (2011) commented that these differences in total anthocyanins concentration are due to genetic differences between species, which affect the type of structures and solubility of anthocyanins in the plant matrix.

Table 2. Central composite rotatable design matrix and response values for total anthocyanin.

Tabla2. Matriz del diseño central compuesto rotacional y valores de respuesta para antocianina total.

Experimental number	Extraction conditions			Analytical results total anthocyanin (mg/L)
	X_1, Extraction time (min)	X_2, Ethanol concentration (%)	X_3, Liquid-to-solid ratio (mL/g)	
1	40(0)	80(0)	50(0)	127.33 ± 15.94
2	20(-1)	90(1)	60(1)	262.17 ±13.60
3	6(-1.68179)	80(0)	50(0)	248.26±14.97
4	60(1)	70(-1)	40(-1)	219.31 ± 5.84
5	40(0)	80(0)	50(0)	138.60 ± 5.47
6	74(+1.68179)	80(0)	50(0)	230.72 ± 25.05
7	60(1)	90(1)	60(1)	239.46 ± 25.92
8	40(0)	80(0)	66.16(+1.68179)	222.50 ± 33.10
9	20(-1)	70(-1)	40(-1)	242.47 ± 38.76
10	40(0)	80(0)	50(0)	113.83 ± 19.12
11	40(0)	96(+1.68179)	50(0)	208.74 ± 19.26
12	40(0)	63(-1.68179)	50(0)	153.91 ± 20.70
13	40(0)	80(0)	33(-1.68179)	234.38 ± 11.77
14	60(1)	70(-1)	60(1)	205.06 ± 25.94
15	40(0)	80(0)	50(0)	107.98 ±6.75
16	40(0)	80(0)	50(0)	104.64 ± 5.43
17	20(-1)	90(1)	40(-1)	212.63 ± 8.24
18	40(0)	30(0)	050(0)	109.40 ± 8.24
19	20(-1)	70(-1)	60(1)	239.13 ± 15.30
20	60(1)	90(1)	40(-1)	192.82 ± 4.74

The analyses of the ANOVA of the experiment are presented in table 3. The model allows monitoring the optimization of the extraction stage, since it presented a level of significance ($p < 0.05$).

The interaction ethanol concentration and liquid-to-solid ratio, and quadratic effect of factors statistically influenced on extraction of total anthocyanins (table 3).

Lack of fit was not significant ($p > 0.05$) in the proposed mathematical model guarantees that it is possible to predict the variations within the extraction process (table 3).

The experimental values showed a good fit with the empirical regression equation $Y = 116.83 - 9.46X_1 + 6.83X_2 + 4.29X_3 + 1.84 X_1X_2 - 1.73X_1X_3 + 14.22X_2X_3 + 44.20X^2_1 + 23.64X^2_2 + 40.30 X^2_3$, as they presented the value of the coefficient determination r^2 was 0.9550 and $r^2_{adj} = 0.9144$ (table 3). Therefore, the model adequately represents the real relationship between the chosen parameters.

Response surface and contour plots

The response surface is plotted to evaluate the interaction of the independent variables and to estimate the optimal level of each variable to obtain a maximum extraction of total anthocyanins.

Figure 2A (page 376), the response surface generated by the effect of ethanol extraction and extraction time of total anthocyanin extraction is presented. As was shown in figure 2A (page 376), a positive quadratic effect of ethanol concentration and extraction time on extraction of total anthocyanins were illustrated as a parabolic shaped surface plot where initially reached its minimum at 80% and 40 minutes followed by a marked increase.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters.

Tabla 3. Análisis de varianza (ANAVA) para el modelo polinomial cuadrático ajustado en la optimización de los parámetros de extracción.

Source	Sum of squares	Degree of freedom	Mean squares	F value	p > F	Significance[a]
Model	54463.74	9	6051.53	23.54	< 0.0001	***
Extraction time (X_1)	1222.98	1	1222.98	4.76	0.0541	NS
Ethanol concentration (X_2)	637.71	1	637.71	2.48	0.1463	NS
Liquid-to-solid ratio (X_3)	251.51	1	251.51	0.98	0.3459	NS
$X_1 X_2$	26.99	1	26.99	0.11	0.7526	NS
$X_1 X_3$	23.82	1	23.82	0.093	0.7670	NS
$X_2 X_3$	1618.10	1	1618.10	6.30	0.0310	*
X_1^2	28157.51	1	28157.51	109.55	< 0.0001	***
X_2^2	8051.68	1	8051.68	31.33	0.0002	***
X_3^2	23401.34	1	23401.34	91.04	< 0.0001	***
Residual	2570.37	10	257.04			
Lack of Fit	1695.09	5	339.02	1.94	0.2428	NS
Pure error	875.28	5	175.06			
Cor total	57034.10	19				

$r^2 = 0.9550$. $r^2_{Adj} = 0.9144$, [a]*($p < 0.05$); **($p < 0.01$) and ***($p < 0.001$), and NS = Not significant. / No significativa.

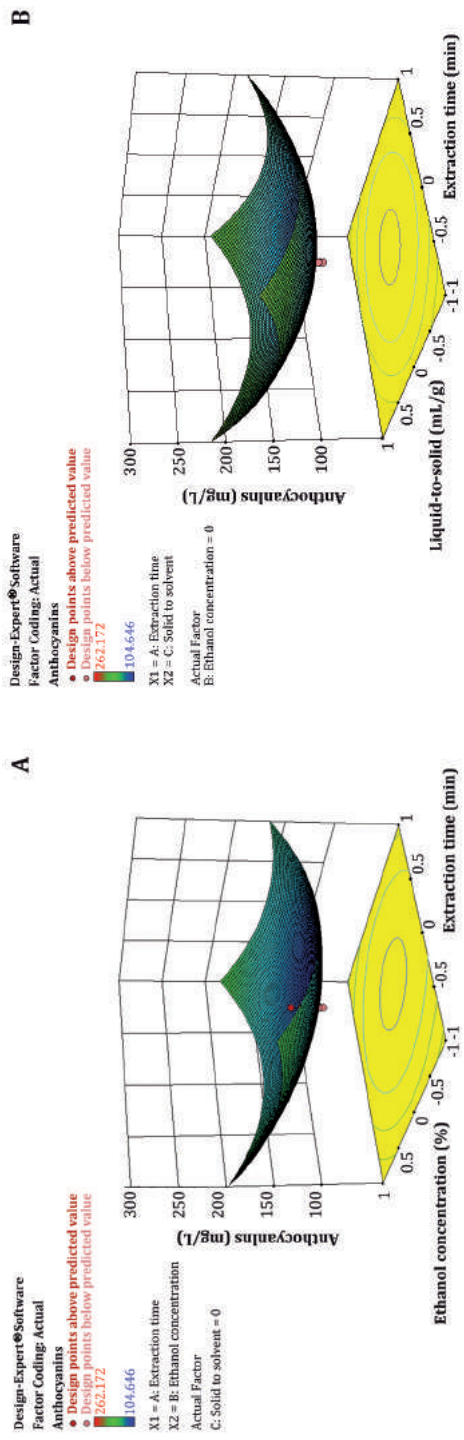

Figure 2. Response surface plots showing the interaction of the independent variables. (A) Effect of ethanol concentration and extraction time on extraction of total anthocyanin. (B) Effect of liquid-to-solid and extraction time on extraction of total anthocyanin. (C) Effect of liquid-to-solid and ethanol concentration on extraction of total anthocyanin.

Figura 2. Diagramas de superficies de respuesta que muestran la interacción de las variables independientes. (A) Efecto de la concentración de etanol y el tiempo de extracción en la extracción de antocianina total. (B) Efecto de líquido-sólido y tiempo de extracción en la extracción de antocianina total. (C) Efecto de la concentración de líquido-sólido y etanol en la extracción de antocianina total.

Figure 2B (page 376), a significant effect in liquid to solid ratio and extraction time to total anthocyanin extraction is observed, a positive quadratic effect of liquid to solid ratio and extraction time on extraction of total anthocyanins were illustrated as a parabolic shaped surface plot where initially reached its minimum at 50 and 40 minutes followed by a marked increase.

Figure 2C (page 376), confirm the effect of ethanol concentration and solid to solvent ratio of the level of total anthocyanins extraction.

The incidence of the quadratic effect of liquid to solid ratio and ethanol concentration also is observed (figure 2C, page 376). Zou *et al.* (2011) and Demirdöven *et al.* (2015) observed that the liquid-solid ratio, solvent concentration and extraction time have a quadratic effect positive on the extraction of total anthocyanins from mulberry and red cabbage.

The parabolic form of the response surface of the total anthocyanins concentration may be associated with differences in pigment solubility in the extraction solvent, Teng *et al.* (2014) report that solubility is affected by the molecular structures of anthocyanin, as cyanidins include two hydroxyl groups and one hydrogen group at different bound positions, resulting in hydrophilism and dissolvability.

Optimum conditions for maximum extraction

The optimal condition obtained using response surface methodology (RSM) corresponded to a extraction time, 20 min; liquid to solid ratio, 60:1 mL/g, and ethanol concentration, 90%, allowed to obtain a maximum concentration of 259.66 mg/L.

The values of optimization extraction were validated experimentally obtaining total anthocyanins concentration of 265.81 ± 22.33 mg/L, which showed no significant difference ($p > 0.05$) with the value recorded in the optimization.

Moreover, ultrasound extraction was compared with maceration techniques, considered conventional methods of extraction of anthocyanin. Total anthocyanin maceration extraction was 230.44 ± 8.60 mg/L. These results show that the ultrasound technique is effective for extraction of total anthocyanin in the mulberry by-product, compared to extraction with maceration; ultrasound exceeds performance by 13% and reduces the time from 24h to 20 min.

The efficient extraction of total anthocyanin in mulberry by-products by ultrasound is the result of the mechanical fracture of the cell wall, which allows the solvent to enter the cell interior, increasing the mass transfer (14). Ghafoor *et al.* (2009) reports that the efficiency of ultrasonic extraction has its explanation in that ultrasound simultaneously increased the process of hydration and fragmentation process facilitating the mass transfer of solutes to the extraction solvent. Author also comments that mass transfer is positively affected by changes in the diffusion coefficients induced by the liquid-solid ratio, solvent concentration and extraction time.

Conclusions

In the present work, ultrasonic-assisted extraction process for maximum total anthocyanins from mulberry by-products was investigated by RSM.

The response surface methodology using the Composite Central Design allows the optimization of the extraction conditions for important valorization of

the total anthocyanins from mulberry by-product. The optimum conditions of UEA obtained through RSM were as follow: extraction time, 20 min; liquid to solid ratio, 60:1 mL/g, and ethanol concentration, 90%, allowed to obtain a maximum concentration of 259.66 mg/L.

The extraction time, the liquid-solid ratio and the ethanol concentration showed an important quadratic effect on the extraction yield of total anthocyanins. The results show that ultrasonic-assisted extraction as a promising agro-industrial process in the recovery of anthocyanins from mulberry by-products.

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Propuesta metodológica para la obtención de indicadores de sustentabilidad de agroecosistemas desde un enfoque multidimensional y sistémico

Methodological proposal to obtain agroecosystem sustainability indicators from a multidimensional and systemic approach

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Resumen

Los agroecosistemas son ecosistemas construidos por los seres humanos para obtener productos y servicios de su interés. De este modo, los ecosistemas adquieren la forma de socioecosistemas. Por ello, para poder describirlos, comprenderlos o intervenir sobre los mismos, resulta necesario observar propiedades emergentes de tipo biológicas y atributos relacionados con aspectos sociales, económicos, culturales e institucionales. A su vez, si se pretende analizar dichos agroecosistemas en clave de sustentabilidad, además de contemplar las diferentes dimensiones que la integran, se deberá tener un enfoque sistémico en las construcciones conceptuales y operativas de tal modo que sea posible obtener indicadores de sustentabilidad. El presente trabajo repasa y clasifica las diferentes formas expuestas en la bibliografía para obtener indicadores y muestra la escasez de propuestas que operen con un enfoque sistémico para resolver la tensión entre producción y conservación que gira en torno a la idea de sustentabilidad. Por ello, se presenta una propuesta metodológica alternativa para la obtención de indicadores de sustentabilidad de agroecosistemas que opera conceptual y empíricamente desde un enfoque multidimensional y sistémico, mediante la contraposición del estado del agroecosistema, en cada una de sus dimensiones, con las exigencias que dichas dimensiones de la sustentabilidad establece. Finalmente se vierten consideraciones metodológicas sobre su uso y se destacan fortalezas y limitaciones de su aplicación.

Palabras claves
metodología • interacción • enfoques

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Abstract

Agroecosystems are human-made ecosystems to obtain products and services of their own interest. In this manner, the ecosystems come into being agroecosystems. Therefore, in order to describe, understand or intervene on them, it is necessary to observe emerging biological properties and attributes related to social, economic, cultural and institutional aspects. At the same time, if these agroecosystems are to be analyzed in terms of sustainability, in addition to contemplating the different dimensions that integrate it, a systemic perspective must be taken into account in the conceptual and operational constructions that allow sustainability indicators to be obtained. In this sense, the present work reviews and classifies the different forms exposed in the bibliography to obtain indicators and shows the scarcity of proposals that operate with a systemic perspective in order to resolve the tension between production and conservation which swings around the idea of sustainability. Taking all this into account, a methodological proposal alternative is presented to obtaining indicators of aroecosystems sustainability, which operates conceptually and empirically from a multidimensional and systemic perspective by contrasting the state of the agroecosystem, in each of its dimensions, with the demands established by the sustainability of these dimensions. Finally methodological considerations about its use are made and advantages and limitations of its application are highlighted.

Keywords
methodology • interaction • approaches

Introducción

Los agroecosistemas son ecosistemas modificados por los seres humanos con el fin de producir alimentos, fibras y, más recientemente, combustibles, entre otros servicios ambientales. Poseen una estructura dada por sus componentes biofísicos y socioeconómicos, y de la interacción de los mismos surgen propiedades emergentes como la resiliencia, la diversidad y la autonomía, entre otras.

Entre las características principales de los agroecosistemas, Odum (1983) manifiesta que: a- requieren fuentes auxiliares de energía (humana, animal y combustible) para aumentar la productividad de organismos específicos; b- la diversidad puede ser muy reducida en comparación con la de otros ecosistemas; c- los animales y plantas que dominan son seleccionados mediante el interés humano y no por selección natural y d- los controles del sistema son en su mayoría externos y no internos. En forma complementaria, se concibe que los componentes, las propiedades y el propio funcionamiento de los agroecosistemas encuentran grandes relaciones con los actores sociales y, en particular, con los tipos sociales agrarios (agricultores capitalizados, agricultores familiares, agricultores *part time*, etc.) encargados de conducirlo. De este modo, los agroecosistemas son socioecosistemas (32, 39) que surgen y se modifican en función de las relaciones sociales y de la relación sociedad-naturaleza. En este sentido, son los agentes sociales, condicionados por su trayectoria histórica y por un determinado contexto socioeconómico y político, los que le dan las características finales y particulares a los agroecosistemas.

Los agroecosistemas pueden pensarse en forma reducida, observando al cultivo y lo que afecta al mismo (plagas, enfermedades, malezas o nutrientes) (11, 12, 24, 25) o como un sistema complejo donde interactúa el ecosistema propiamente dicho, el tecnosistema (tecnología aplicada) y el sociosistema (actores sociales involucrados, propósitos y contexto socio económico, cultural, político) (32). Dentro de esta última forma de pensamiento, el análisis de cada sistema por separado puede constituir un primer paso, pero para comprender la dinámica de estos en forma integral y global, resulta necesario indagar sobre las relaciones entre los mismos. Esta visión global e interdisciplinaria, que pone en interjuego los procesos que suceden a nivel de cultivo, a nivel de agroecosistema y a nivel de territorio, preferentemente bajo una óptica diacrónica (conjunto de atributos observados en más de un tiempo), permite conocer tanto los condicionantes como los determinantes que actúan sobre el agroecosistema y permite sacar a superficie ciertas problemáticas no visualizadas. Así, proceder de este modo otorga una clara y completa conceptualización y comprensión de los agroecosistemas y con ello la posibilidad de lograr un óptimo diagnóstico que permita realizar una intervención técnica y social acertada.

La evaluación de los agroecosistemas ha sido un tema de interés para diferentes actores, quienes han aplicado mayoritariamente herramientas provenientes de la disciplina económica. Pero, desde la emergencia del concepto de sustentabilidad se ha buscado obtener indicadores incorporando herramientas de la economía ambiental y, en menor medida, de la economía ecológica (20, 40).

La sustentabilidad es temática de estudio de diversas instituciones a nivel mundial, nacional y local, y se han logrado valiosos aportes teóricos (7, 9, 10, 13, 23, 27, 31, 33), pero estas publicaciones se destacan por sus enunciados o por la enumeración de cualidades que debería tener un agroecosistema para lograr ser sustentable, y no así por su forma de medición (3, 4, 21, 41). En esta línea, se puede afirmar que los intentos por medir o proponer metodologías para evaluar la sustentabilidad de los agroecosistemas están en desarrollo, observándose que las propuestas para la construcción de indicadores han sido predominantemente fragmentadas (operan por dimensión de análisis) o integrales (adición de dimensiones) pero no sistémicas, limitando por ello la capacidad explicativa de la implícita tensión entre el logro de objetivos productivos y de objetivos de conservación que acompañan los procesos de sustentabilidad en los cuales están insertos los agroecosistemas.

Por ello, en el presente escrito se propone: 1- debatir y clasificar las propuestas para obtener indicadores de sustentabilidad y 2- proponer una metodología multidimensional y sistémica para obtener indicadores de sustentabilidad útiles en la evaluación de agroecosistemas. El enfoque multidimensional asume que un proceso productivo está conformado por un conjunto necesario e imprescindible de dimensiones que deben ser consideradas en su realización. El enfoque sistémico toma conceptos de la teoría de sistemas y los aplica a los procesos productivos postulando que cualquier práctica realizada sobre este repercutirá en mayor o menor medida en cada una de las partes que conforman ese proceso productivo.

En este sentido, se presenta, en primera instancia, una revisión y una clasificación de las formas de obtener indicadores de sustentabilidad que expone la bibliografía sobre el tema; en segunda instancia, se desarrolla una propuesta metodológica

alternativa para obtener indicadores desde un enfoque multidimensional y sistémico, y, en tercera instancia, se vierten sugerencias metodológicas sobre su uso. Finalmente, se destacan las principales ventajas y limitaciones de la metodología propuesta.

Formas de obtener indicadores de sustentabilidad. Análisis bibliográfico

Se entiende por obtención, al proceso por el cual se puede construir un indicador o en caso de que este ya exista, se seleccione bajo criterios establecidos.

El espíritu general del informe Nuestro Futuro Común que elaboró la Comisión Mundial para el Medio Ambiente y el Desarrollo (49), o Comisión Brundtland, contempla la necesidad de pensar los procesos sociales y productivos a largo plazo sin tener que detener el crecimiento económico y sin agotar los recursos naturales. Para ello se propone trabajar en forma integral y sistémica las dimensiones que puede contener un determinado proceso como medio para equiparar los objetivos entre las dimensiones. Dicha declaración ha tenido múltiples usos y, en ocasiones, han sido contrapuestos. Tal es así que algunos autores postulan que dicho enunciado ha ido perdiendo fuerza con el tiempo debido a su sobreuso (31) y otros autores sostienen que la sustentabilidad no existe (33).

En el marco del desarrollo conceptual de la sustentabilidad se desprende la idea de agricultura sustentable. La misma centra su atención y crítica en la orientación y desarrollo de la agricultura convencional a nivel mundial para postular la idea fuerza de mantener o aumentar los niveles de producción agrícola, sin sobreexplotar los recursos naturales y sociales volcados al acto productivo. Es decir, una agricultura sustentable busca incorporar, de forma integrada e igualitaria, aspectos económicos, ambientales y sociales. En este sentido, Altieri (2011) la define del siguiente modo: "Es aquella agricultura que intenta proporcionar rendimientos sostenidos a largo plazo, mediante el uso de tecnología y prácticas de manejo que mejoren la eficiencia biológica del sistema. Además, busca una distribución justa y equitativa de los costos y beneficios asociados con la producción agrícola; se preocupa por el rescate crítico de las prácticas de manejo utilizadas por diferentes etnias y culturas, y busca reducir las desigualdades actuales en el acceso a recursos productivos; intenta así mismo desarrollar tecnologías y sistemas de manejo adaptados a la diversidad de condiciones ecológicas, sociales y económicas locales; finalmente la agricultura sustentable trata de ser rentable económicamente, sin dejarse llevar por una lógica económica de corto plazo".

Desde la promulgación del concepto de sustentabilidad se estableció la necesidad de obtener una medición de la misma que ayudara a hacer más tangible el concepto. Producto de esto se han desarrollado diversas formas de obtener indicadores de sustentabilidad que expresen de modo simple la condición de sustentabilidad de esos sistemas productivos. Padua (1979) manifiesta que "la identificación y selección apropiada de indicadores simples o el desarrollo de indicadores complejos requiere de mucho cuidado y experiencia, además una aguda intuición y también de sólidos conocimientos sobre el tema a investigar, pero sobre todo, mucha apertura a recibir sugerencias".

La bibliografía consultada sobre indicadores de sustentabilidad (14, 26, 41b, 43a, 44b, 45c, 46, 47) menciona que lo determinante y que cualifica cualquier forma de medición de la sustentabilidad es el proceso de obtención de los indicadores. Proceso que, llevado con rigurosidad, permitirá obtener los argumentos y fundamentos de los indicadores, así como la validez y solidez de los mismos, además de la capacidad heurística de los resultados.

En sintonía con Massera *et al.* (1999), Sarandón (2003b) y Gallopín (2006), se puede afirmar que no hay indicadores de uso universal ni con una legitimidad global, y que no existe una sola forma de obtener indicadores de sustentabilidad. En este sentido, la bibliografía analizada (4, 8, 14, 38, 41, 43a, 44b) muestra varias alternativas para la obtención de indicadores que, en términos generales, contienen cuatro grandes partes: a- estructura conceptual sobre cómo obtener los indicadores (definiciones y conptualizaciones); b- criterios para la selección de indicadores (ej. confiabilidad, validez, simpleza, etc.); c- fundamentación y conceptualización de los indicadores y d- operacionalización de los mismos (mecanismos matemáticos). Las alternativas para obtener indicadores pueden ser clasificadas según la estructura conceptual implementada en al menos tres grupos, que representan distintos niveles de complejidad conceptual y empírica: a- selección arbitraria de indicadores por especialistas o actores involucrados; b- indicadores desprendidos de una definición u objetivo y c- indicadores obtenidos con un enfoque sistémico. A su vez, esta última alternativa contiene dos formas: una desprende los indicadores desde los atributos o propiedades emergentes de los agroecosistemas y la otra, los desprende desde las interacciones entre las dimensiones de análisis.

Selección arbitraria de indicadores por especialistas o actores involucrados

Esta alternativa de obtener indicadores se visualiza en los primeros trabajos (CIAT, 1998; MIDEPLAN, 1998; UNDSD, 2001; IISD, 2002 y Spangenberg *et al.*, 2002; citados en 20) que han practicado la operacionalización del concepto de sustentabilidad. Como el nombre de este grupo indica, consiste en que especialistas, o actores involucrados en la problemática a abordar, realizan una selección de indicadores que muestren el estado, las tendencias o las transformaciones en términos de sustentabilidad de los objetos de estudio. Para ello, estos actores utilizan mayores o menores criterios de pertinencia que, en algunos casos, no son totalmente explícitos. Asimismo, se observa en muchos de estos trabajos (14, 17, 18) que predominan indicadores de aspectos ambientales y económicos sobre los de las dimensiones sociales, culturales e institucionales. Además, algunos indicadores presentan argumentos y fundamentos poco desarrollados, o carecen de un análisis sobre la validez del mismo (se observa que son pocos los trabajos que han sometido su selección de indicadores a otros especialistas y/o actores involucrados en la problemática, como forma de dar validación a los mismos). De este modo, los indicadores propuestos aparecen como una libre elección que no garantiza la ausencia de intereses en esa selección (confiabilidad), su relevancia ni su ajuste a las condiciones socioeconómicas y ambientales de los objetos de estudio; como sí lo harían los indicadores que resultasen de un proceso ordenado, riguroso y consistente teóricamente de selección de variables que respondan a la problemática de la sustentabilidad.

Indicadores desprendidos de una definición u objetivo

En esta alternativa se incluye a aquellos trabajos que operacionalizan el concepto de sustentabilidad a partir de una definición de la misma (FESLM, 1994; Stocke *et al.*, 1994; PICABUE, 1995; MARPS, 1997; Lewandoski *et al.* 1999 y CIFOR, 1999 citados en 19, 41b); y, según su exigencia y criterio, desprenden los indicadores por dimensión de análisis y/o propósitos contemplados en dicha definición.

También, y sin hacer uso del concepto de sustentabilidad, dentro de esta orientación existen formas de obtención de indicadores que se proponen objetivos o definiciones que reflejan los deseos, las aspiraciones o las expectativas (objetivos) que deben satisfacer los agroecosistemas y desde estos desprenden los indicadores (1, 2, 15, 41, 42). Se considera que este proceso es un esfuerzo por superar la primera alternativa. Es decir, con el objeto de lograr una mayor claridad, coherencia y objetividad, los autores que trabajan desde esta perspectiva explicitan una definición u objetivo (marco teórico) y se plantean desde ella una secuencia de pasos coherentes y necesarios entre sí, conducente a obtener indicadores de cuya medición se alcancen valores consistentes.

No obstante, estas propuestas metodológicas no logran resolver la arbitrariedad en la selección de indicadores y, sobre todo, omiten que entre las dimensiones de análisis hay relaciones tanto determinantes como condicionantes de la sustentabilidad que se pretende medir, como, por ejemplo, que el logro de una productividad alta y sostenida en el tiempo puede condicionar la conservación de materia orgánica y de los nutrientes del suelo.

Indicadores obtenidos con un enfoque sistémico

Los métodos que abordan la obtención de indicadores de sustentabilidad desde una mirada sistémica asumen que lo sucedido en una dimensión de análisis puede afectar a otras dimensiones y se centran en las interacciones entre las partes que conforman esos agroecosistemas. Como ya se mencionó, dentro de esta alternativa de obtención pueden distinguirse al menos dos formas: una de ellas desprende los indicadores desde atributos de los agroecosistemas y la otra desde la interacción entre las dimensiones de análisis contempladas. La primera de ellas trabaja con el postulado implícito de que, si el propósito de realizar una evaluación es lograr una contribución para mantener o mejorar el sistema, los puntos de observación tendrán que ver con aspectos, propiedades o atributos funcionales del sistema. Massera (1999) propone los siguientes atributos: productividad, equidad, estabilidad, resiliencia, confiabilidad, adaptabilidad y autodependencia), cuyos mejores o peores comportamientos permitirá una mejor comprensión sobre la continuidad en el tiempo del sistema (4, PER, 1993; SEAN, 1997; Evaluación de satisfactores,1999; Manejo de la resiliencia, 2002 y AMESH, 2005 citados en 20). De este modo, estos trabajos siguen avanzando en la construcción de una secuencia de pasos coherentes y necesarios entre sí, conducente a obtener indicadores, pero en este caso se desprenderán desde atributos sistémicos y reflejarán el desempeño del sistema.

No obstante, resulta interesante mencionar que la predeterminación de ciertos atributos puede limitar u ocultar a otros atributos, y con ello, a indicadores que pueden resultar de interés. Por su parte, la segunda forma contiene a la anterior pero centra sus esfuerzos en la obtención de indicadores que reflejen las interacciones entre los diferentes aspectos o dimensiones que conforman un agroecosistema. Es decir, en los trabajos se desprenden los indicadores desde las interacciones determinantes y condicionantes que hacen a la sustentabilidad del sistema (16, 30, 50).

Bajo cualquiera de las dos formas se aportan criterios que avanzan en la objetividad para la selección de indicadores y en la fundamentación y argumentación de los mismos. Asimismo aportan a la homogeneidad metodológica y también al poder comparativo.

En este punto resulta oportuno expresar que las alternativas mencionadas para derivar indicadores son diferentes a los protocolos para establecer la sustentabilidad, como también a los marcos de evaluación de agroecosistemas. Los primeros consisten en un conjunto de indicadores ya seleccionados que permiten testear el estado o tendencia de la sustentabilidad de un agroecosistema (Agroecoindex©) (6, 38, 51). Los segundos son propuestas metodológicas flexibles que permiten guiar a un actor social determinado en el proceso de evaluación (ej. MESMIS) (34, 41b). Dichos marcos contienen diferentes etapas o pasos que son aplicables en disímiles situaciones y sistemas de manejo, y permiten lograr una comprensión de la estructura y de la dinámica de los agroecosistemas (46). De este modo, los marcos de evaluación contienen alguna de las formas mencionadas sobre cómo obtener indicadores de sustentabilidad.

Dentro de las alternativas mencionadas, las que obtienen indicadores mediante un enfoque sistémico han tomado las críticas a las anteriores y aportan en el sentido de lograr mayor robustez conceptual y práctica. A su vez, se observa que la alternativa que trabaja contemplando las interacciones de las dimensiones que contiene la sustentabilidad es la que menor desarrollo ha tenido y presenta un desigual grado de conceptualización y rigurosidad. En este sentido, el presente artículo aporta una propuesta metodológica para la obtención de indicadores de sustentabilidad desde un enfoque multidimensional y sistémico, mediante la contraposición del estado del agroecosistema, en cada una de sus dimensiones, con las exigencias que la sustentabilidad de dichas dimensiones establece.

Metodología

Propuesta metodológica multidimensional y sistémica para la obtención de indicadores de sustentabilidad

La propuesta que se desarrolla a continuación ha sido construida contemplando los aportes de Kaplan y Norton (2008), Durán *et al.* (2009) y Wehbe *et al.* (2015) y se ha trabajado en la cualificación del proceso de obtención de indicadores desde la interacción entre las dimensiones de análisis y en el ajuste de la misma a los agroecosistemas.

La metodología consiste en construir un tablero de contraste (figura 1) donde se confronta el estado del agroecosistema en cada una de sus dimensiones (columnas) con las exigencias que la sustentabilidad de dichas dimensiones establece (filas).

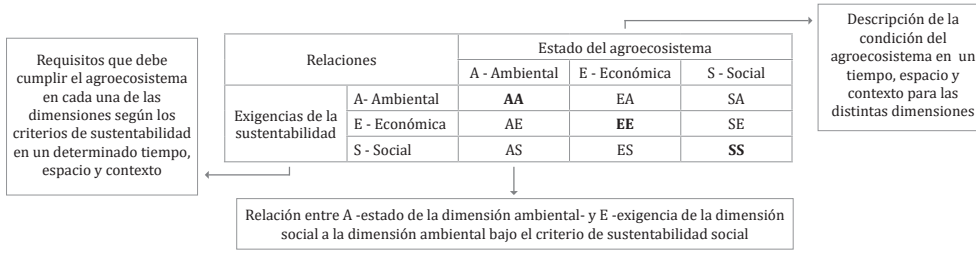


Figura 1. Estructura del tablero de contraste (estado/exigencia) entre dimensiones de análisis para la obtención de indicadores de sustentabilidad de agroecosistemas.

Figure 1. Structure of the contrast panel (state/requirement) between dimensions of analysis to obtain sustainability indicators of agroecosystems.

La sustentabilidad ambiental está vinculada con los procesos biofísicos y con la continuidad de la productividad y funcionamiento biológico de los agroecosistemas. El propósito de la misma, y por ende el criterio directriz en la dimensión Ambiental, será la obtención de una producción constante o en ascenso a través del tiempo, bajo la condición de mantener la cantidad y calidad de recursos naturales (suelo, agua y biodiversidad) volcados al acto productivo (47).

La sustentabilidad económica está vinculada con la apropiación, combinación e interacción de los factores de producción. Su propósito, y por ende el criterio directriz en la dimensión Económica, será obtener a lo largo del tiempo un ingreso que permita a los actores sociales involucrados en el agroecosistema, mantenerse o escalar en el campo económico del que participan, así como eficientizar económicamente el proceso productivo y promover la distribución equitativa de los factores de producción del agroecosistema y de los beneficios de su puesta en funcionamiento (47). Este conjunto de propósitos que contribuye, a su vez, a garantizar el traspaso de los factores de producción de generación en generación.

La sustentabilidad social está vinculada con las relaciones sociales y con el mantenimiento del capital social. Su propósito, y por ende el criterio directriz en la dimensión Social, será desarrollar un modo de producción que a través del tiempo otorgue beneficios constantes o en aumento para reproducir en forma ampliada el capital social puesto en funcionamiento bajo condiciones dignas de trabajo, además de contemplar el criterio de equidad en la búsqueda de prosperidad y oportunidades sociales. Adicionalmente, una parte de la sustentabilidad social estará indefectiblemente relacionada con los aspectos institucionales, ya que estos aportan capacidad de adaptación (habilidad de gestionar tareas y procesos en forma rápida y confiable), menor vulnerabilidad y mayor resiliencia (45).

Desde dichos criterios se deberán seleccionar los componentes, construir los indicadores y elegir las variables a considerar en el tablero para el agroecosistema donde se aplique.

Operativamente, se organizan las dimensiones de análisis: ambiental, económica y social, tanto en columnas como en filas del mencionado tablero (se toman estas tres dimensiones por ser las mayormente consideradas en las diversas definiciones consultadas (2, 4, 9, 13)). De este modo, en cada columna se considerará la descripción de la condición ("estado") que reviste el agroecosistema según la dimensión abordada para un determinado tiempo, espacio y contexto. Por su parte, en cada fila se establecerán los requisitos que debe cumplir el agroecosistema en cada una de las dimensiones según los criterios de sustentabilidad en un determinado tiempo, espacio y contexto.

En la figura 1 (page 387) se muestra que se ha asignado a cada dimensión una letra (A. Ambiental; E. Económica, S. Social) y se identifica la relación entre el estado y la exigencia de dos dimensiones mediante la combinación de ambas letras. Así el cuadrante AS, representa la relación entre A -estado de la dimensión ambiental- y E -exigencia de la dimensión social a la dimensión ambiental bajo el criterio de sustentabilidad social.

El hecho de conceptualizar el estado y la exigencia en clave de relación, otorga la posibilidad de describir aquellos aspectos elementales de cada una de las dimensiones de un agroecosistema (estado) y la respuesta de esos aspectos a las exigencias de las mismas dimensiones en términos de sustentabilidad, explicitando así, el abordaje sistémico de la propuesta metodológica.

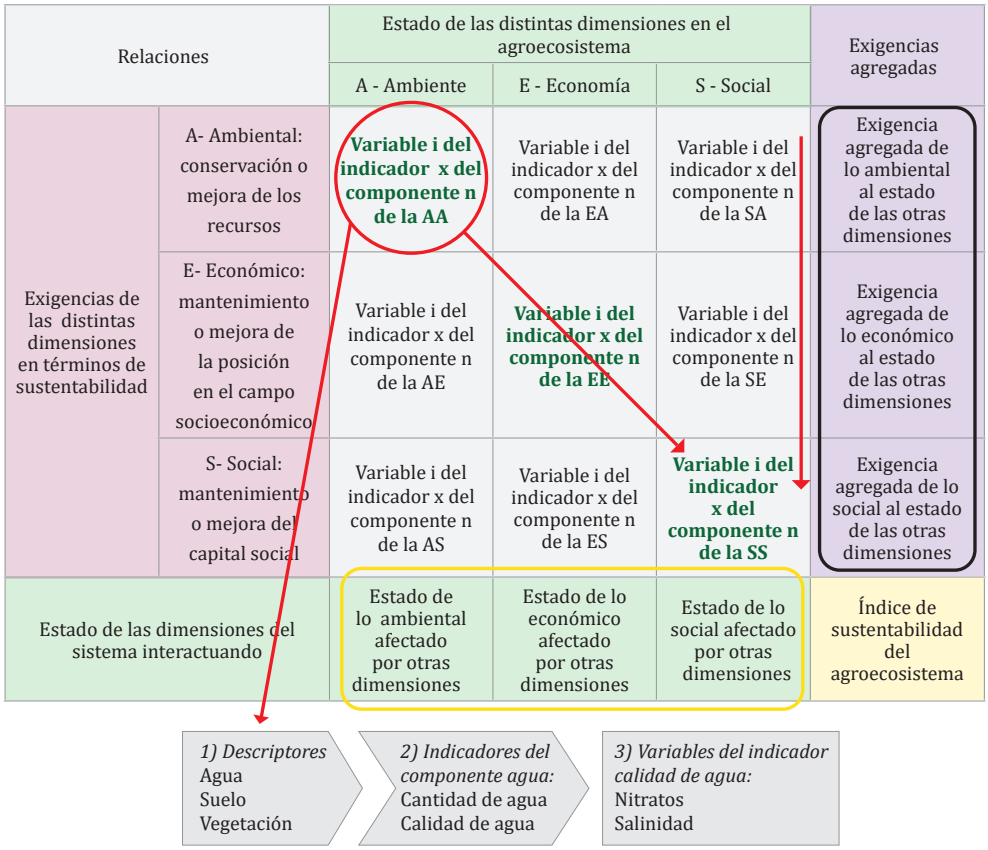
En la figura 2 (page 390) se muestra en el tablero de contraste que cada relación se materializa mediante componentes y/o descriptores, luego como indicadores para cada componente/descriptor considerado y por último como variable/s que integra/n los indicadores, según sea el caso (Variable/s "i" del indicador "x" del componente "n" de la relación EA).

A modo de ejemplo de lo relatado, una posibilidad es que los componentes del cuadrante AA (estado de la dimensión ambiental y exigencia de dicha dimensión en clave de sustentabilidad) sean: suelo, agua, aire y vegetación como categorías descriptivas; o biomasa, productividad primaria neta, resiliencia y resistencia como descriptores de los atributos o propiedades emergentes de los agroecosistemas, ya que dichas categorías o atributos se vinculan con el criterio directriz de la dimensión ambiental de obtener una producción constante o en ascenso a través del tiempo, bajo la condición de mantener la cantidad y calidad de recursos naturales (suelo, agua y biodiversidad) volcados al acto productivo. Luego como indicadores del componente agua: se podría tomar calidad y cantidad de agua y como variables del indicador calidad de agua: salinidad y nitratos.

Los componentes y/o descriptores se eligen respondiendo primero a la pregunta ¿qué componentes o descriptores describen el estado de la dimensión X en el agroecosistema? (ej. agua, suelo, vegetación para la dimensión ambiental). Luego ¿qué componentes o descriptores describen la exigencia en términos de sustentabilidad de la dimensión X en el agroecosistema? (ej. agua, suelo, vegetación y aire para la dimensión ambiental). Producto de la respuesta a estas preguntas surge un conjunto de componentes o descriptores que aportan a la descripción del estado y de la exigencia. Lo óptimo sería que los componentes elegidos representen en forma simultánea la descripción del estado y la exigencia propuesta.

Tanto la construcción o selección de componentes como de indicadores y de variables de un cuadrante se realiza contrastando estado (columna) y exigencia (fila), según los criterios de sustentabilidad general y particular de la interacción elegida. El procedimiento sugerido es tomar en primera instancia las relaciones entre estado y exigencia para la misma dimensión, completando así la diagonal del tablero respecto de componentes y/o descriptores (indicado en la figura 2, pág 390 con una flecha de borde rojo). Luego trabajar por columna, es decir las relaciones entre el estado de una dimensión según las exigencias de las otras dimensiones (indicado en la figura 2 con una flecha de borde rojo). Este procedimiento permite: a- ordenar el proceso desde relaciones de mayor condicionamiento o estructurantes (diagonal) hacia relaciones de menor condicionamiento; b- eliminar componentes y/o descriptores repetidos o que observen aspectos similares (permite detectar sobrevaloraciones no intencionadas de un aspecto) y c- reubicar componentes en una posición del tablero donde otorguen mayor poder heurístico.

El mismo procedimiento descripto para los componentes o descriptores se realiza para los indicadores y luego para las variables, logrando las mismas ventajas descriptas para los componentes. Posteriormente se sugiere aplicar un análisis jerárquico de las variables e indicadores y finalmente aplicar un análisis de validación de los mismos. Un análisis jerárquico consiste en establecer qué indicadores y qué variables tienen mayor poder heurístico, cuáles tienen una característica determinante o condicionante y cuáles contemplan a otros indicadores y variables.



1) Se propone trabajar con componentes y/o descriptores, ya que en ambos casos se trata de establecer los aspectos elementales o esenciales de una dimensión, tanto para su descripción (estado) como para su observación frente a exigencias.

2) Un indicador es una característica observable o un aspecto discernible en un objeto de estudio que puede adoptar diferentes valores o expresarse en varias categorías, al menos dentro de ciertos límites, en una escala continua. La definición operacional de los indicadores o transformación de conceptos a indicadores o a índices parte de la definición teórica de los mismos, así como del conocimiento y la experiencia sobre el tema. Los indicadores complejos o integradores (incluyendo los no físicos o cualitativos que no pueden medirse con escalas lineales) son construidos a partir de sus múltiples dimensiones e isomorfismo (semejanza de los rasgos estructurales de un concepto teórico con su definición operacional (28, 37), sustentados en los principios lógico-matemáticos de las escalas de medición apropiadas: nominal, ordinal, de intervalo o de razón (29). Los principales criterios en el uso de indicadores son: 1- seleccionar indicadores de fácil obtención y que muestren alta confiabilidad de la información y accesibilidad; 2- contener el menor número de variables que sean confiables, representativas y fáciles de medir y 3- contar con procedimientos específicos de medición para cada indicador y variable.

3) Variable: Una variable designa cualquier característica de la realidad que puede ser determinada por observación y que puede mostrar diferentes valores de una unidad de observación a otra. Se llaman así porque varían, y esa variación es observable y medible. Las variables pueden ser cuantitativas (se expresan en números) o cualitativas (expresan cualidades).

Figura 2. Tablero de contraste para el análisis multidimensional y sistémico de la sustentabilidad.

Figure 2. Contrast board for multidimensional and systemic analysis of sustainability.

Este análisis permitirá a *posteriori* seleccionar indicadores de fácil obtención y que muestren alta confiabilidad de la información. Vale destacar que estos últimos criterios dependerán del nivel de precisión a que se quiera llegar, de la disponibilidad económica y de los recursos humanos que se posean. La validación es un procedimiento por el cual un indicador puede resultar válido o no válido. Existen diversas formas de lograr la validación de un indicador, una de ellas es someterlo al análisis de paneles de expertos (12) y otra es el procedimiento detallado por Bockstallera y Girardin (2003). Dicho procedimiento consiste en realizar una validación del diseño (fundamentación científica), una validación de fiabilidad (solidez en su uso) y una validación del uso final (útil como herramienta de decisión).

En la figura 2 (pág. 390) se puede observar que cada columna (estado de una dimensión) puede responder a una función específica cuya resultante (cuadrantes de la última fila, indicado con un óvalo de contorno amarillo) refleja el estado de cada dimensión afectado por las exigencias de ella y del resto de las dimensiones. En forma análoga, cada fila (exigencia de una dimensión) puede responder a una función específica, cuya resultante (cuadrantes de la última columna, indicado con un óvalo de contorno negro) refleja la exigencia agregada de una dimensión hacia el resto de los estados del agroecosistema.

Finalmente, el conjunto de relaciones con sus correspondientes componentes, indicadores y variables permite obtener un índice de sustentabilidad de agroecosistemas (indicado con letras verdes). Dicho índice consiste en una función agregada, ponderada o no, de los indicadores observados. La ponderación es un procedimiento matemático para asignar mayor valor un aspecto que a otro, amplificando las diferencias entre estos. Es una herramienta que el usuario de esta propuesta deberá evaluar en función de las características del caso en que se esté trabajando.

Resulta oportuno mencionar que en general los índices agregados brindan escasa información sobre cómo van cambiando los valores de los indicadores que integran el índice (ej. puede suceder que el valor del índice se mantenga a través del tiempo, pero que los indicadores que lo forman cambien y que alguno de ellos, de condición determinante, pueda estar quedando fuera del rango deseado y ser motivo de insustentabilidad).

No obstante, dicha situación puede ser atendida mediante la observación de cada uno de los componentes de las interacciones entre dimensiones, donde se podrán identificar los valores que comprometen algún aspecto de la sustentabilidad y que requieren de intervención. Otro elemento que mitiga dicha falencia de los índices, consiste en una construcción sistémica de los indicadores, ya que el mismo proceso de construcción de los indicadores contempla las tensiones entre las dimensiones.

En forma complementaria a lo que se viene relatando, se debe abordar la temática del indicador, que además de las características citadas y por la bibliografía (14, 35, 41b, 44b, 45c, 48), debe estar ajustado a las condiciones y características del agroecosistema a evaluar, ser sensible a los cambios y responder a las preguntas que orientan el trabajo.

Consideraciones metodológicas complementarias

Trayecto de variables a relaciones entre estado y exigencia

A partir de la determinación de la unidad de observación y la de análisis, se debe proceder a la obtención de datos mediante el relevamiento de las variables en estas unidades de observación o mediante las fuentes secundarias de datos.

La información obtenida se puede organizar en una tabla de datos: unidades de observación por características, siendo estas las variables integrantes de los indicadores en los componentes de cada dimensión de la sustentabilidad. Luego, calculando el promedio de las variables se obtiene el valor del indicador. Del mismo modo, se calculan los componentes y el valor de cada una de las relaciones.

Homogeneización / estandarización

La estandarización es un procedimiento necesario para homogeneizar información de diversa índole y consiste en la construcción de una escala arbitraria que permita relacionar el valor real -observado para cada variable o indicador existente en cada dimensión- con valores adimensionales (6). De este modo se garantiza una correcta y comparable expresión de la sensibilidad de cada variable.

Comúnmente las variables tienen una relación conceptual directa o indirecta (41) con el proceso que se está observando, pero aparecen otros casos donde la relación puede ser de tipo umbral, de saturación u óptima. En ejemplo de respuesta umbral se presenta en el caso del indicador, existencias ganaderas, en el sentido de que una mayor cantidad de existencias ganaderas les otorga a los campesinos la posibilidad de cubrir las necesidades de autoabastecimiento y les deja margen para la comercialización como forma de lograr un ingreso monetario (47). No obstante dicha lógica de comportamiento es válida hasta un valor umbral, donde por encima del mismo la potencialidad productiva puede verse afectada a causa de una disminución en la vegetación que abastece de alimento a los animales involucrados.

Debido a esto, se debe estudiar el comportamiento de cada variable y establecer un procedimiento matemático de homogeneización acorde. Así, en caso de que la variable/indicador tenga una relación conceptual directa y lineal, es decir, a mayor valor mejor desempeño, se implementa la siguiente fórmula:

$$x_{klm} = \frac{x_{kl}real - x_{kl}mín}{x_{kl}máx - x_{kl}mín}$$

donde:

X_{kl} = real es el valor observado para la variable l del indicador k, en el relevamiento

X_{kl} mín = valor mínimo de referencia (regional, nacional, de cita bibliográfica) u observado (el menor de los valores medidos para el caso en estudio) para la variable l del indicador k

X_{kl} máx = valor máximo de referencia u observado para la variable l del indicador k (los valores de referencia mínimo y máximo se determinan para cada variable de cada componente).

Así X_{klm} toma una valor adimensional que varía entre 0 y 1 y es el valor homogéneo de la variable l en el indicador k observada en la unidad de observación m del agroecosistema analizado.

Ejemplo: el indicador Riqueza Productiva (indicador económico) observa el número total de actividades productivas que se realizan en el agroecosistema analizado. Se conceptualiza que una mayor cantidad de actividades productivas desarrolladas en el predio aportan una mayor y diversa cantidad de productos que pueden ser destinados al autoabastecimiento de la unidad productiva y también a la comercialización u otra forma de intercambio para obtener otros bienes no producidos en el predio. Se interpreta que una mayor riqueza productiva puede otorgar mayor estabilidad de productos destinados al autoabastecimiento y al intercambio, hasta un óptimo que estará en relación con la disponibilidad de mano de obra familiar para ejercer esas actividades productivas y las necesidades de autoabastecimiento relativas.

Para el cálculo del valor estandarizado se considera como valor mínimo una actividad (ya que si fuese cero, no sería una unidad productiva, pueden presentarse casos donde el valor mínimo de una determinado indicador sea "cero". Por ello, según sea el indicador el valor X_{gm} mín puede ser 0, 1 u otro valor) y como máximo, el mayor valor observado (ya que este valor permite relativizar con los valores reales de la zona y ubica por debajo de un valor umbral). Así la unidad de análisis que obtuvo el mayor valor fue la unidad "x" con un valor real de 10 y la unidad de análisis "y" fue de 7. Aplicando la fórmula el valor estandarizado de la unidad de análisis "y" es 0,67.

Cálculo del valor estándar para la unidad de análisis:

$$y = \frac{7-1}{10-9} \quad \text{entonces} \quad y = \frac{6}{9}$$

por ello "y" es igual a 0,67.

En caso de que la variable/indicador tenga una relación conceptual indirecta y lineal, es decir a mayor valor, peor desempeño, se implementa la siguiente fórmula con las mismas consideraciones que en el caso anterior:

$$x_{glm} = \frac{x_{gl}real - x_{gl}máx}{x_{gm}mín - x_{gl}máx}$$

Cálculo del estado agregado y de la exigencia agregada por dimensión de sustentabilidad

El cálculo del promedio de los valores de las relaciones presentes por columna del tablero de contraste, permite obtener el estado de una dimensión afectado por las exigencias de esa misma dimensión y de las restantes (cuadrantes de la última fila). Con idéntico procedimiento pero actuando por filas del tablero de contraste, se puede obtener la exigencia de cada dimensión sobre el estado de la misma dimensión y de las restantes (cuadrantes de la última columna). En ambos casos y debido a que se trabaja con valores homogéneos/estandarizados, se alcanzaran valores entre 0 y 1.

Obtención del índice de sustentabilidad del agroecosistema e interpretación del mismo

El índice de sustentabilidad surge del promedio de los valores de todos los indicadores obtenidos mediante el tablero de contraste y variará entre 0 y 1. Este índice otorga una estimación de la situación del agroecosistema, cuya precisión dependerá de la calidad de los indicadores y de la información recabada, así como de los parámetros contextuales.

Es importante marcar que este índice es implícitamente comparativo y solo describe un comportamiento que deberá ser sometido a una interpretación y que requerirá de la mayor cautela posible para no establecer relaciones directas entre, por ejemplo, manejo convencional y nivel de escolarización; ni establecer conclusiones no situadas en el tiempo, el espacio y el contexto socioeconómico en el que se encuentre el o los agroecosistemas en estudio. Además, se sugiere que la interpretación del índice de sustentabilidad sea acompañado de un análisis e interpretación de los indicadores que muestran un comportamiento destacado, además de una correcta vinculación con el marco teórico tomado.

Caso de estudio: el proceso de construcción de indicadores de sustentabilidad para la Reserva Provicial Bosques Telteca

Un equipo interdisciplinario de investigadores de la Universidad Nacional de Cuyo y del CONICET (CCT-Mendoza) se propuso construir indicadores que ayudaran a describir la situación de sustentabilidad de las unidades productivas que se ubican en la Reserva Provincial Bosques Teltelca y sus alrededores en el Departamento de Lavalle, Mendoza. Dicho

equipo contaba con años de trabajo científico en el mencionado territorio y la posibilidad de realizar algunas observaciones de campo complementarias para alcanzar el objetivo mencionado. Para organizar la discusión entre investigadores de distintas disciplinas y que la misma fuese conducente a lograr indicadores consensuados que reflejaran las descripciones de las condiciones sociales, económicas y ambientales y que a su vez contemplaran la idea sustentabilidad, es decir, lograr una mirada a la posibilidad de que estas unidades productivas y sus recursos asociados se mantuviesen en el tiempo, se optó por implementar el tablero de contraste desarrollado en este artículo. El resultado parcial de este trabajo se presenta en la tabla 1 (pág. 395).

Para la obtención de los indicadores presentados en la tabla 1 (pág. 395) los investigadores involucrados debatieron en primera instancia sobre cuáles eran los componentes o descriptores necesarios de contemplar en cada una de las dimensiones, luego, para cada componente, cuáles eran los indicadores que mejor reflejaban el estado de las unidades productivas y que a la vez permitieran representar las exigencias de sustentabilidad. Sobre esos indicadores se realizó un proceso de selección jerárquica y de validez obteniendo una lista de indicadores posibles de observar. Sobre la base de ese listado de indicadores, y por la dimensión territorial del trabajo, se eligieron observar los indicadores listados en la tabla 1 (pág. 395).

Cabe destacar que se presenta un listado parcial, producto de la disponibilidad de datos y de la posibilidad de medirlos, fueron efectivamente observados.

Como oportunamente se mencionó, la selección y/o construcción de indicadores mediante el tablero de contraste permite abordar la tensión entre propósitos de conservación y de producción de forma multidisciplinaria y sistémica, logrando indicadores con mayor ajuste para medir lo que se desea observar y con una completa conceptualización y argumentación que luego dará herramientas para explicar los resultados obtenidos.

Dado que no es objetivo de este artículo presentar indicadores para las unidades productivas de la Reserva Provincial Bosque Telteca, se menciona y desarrolla uno de ellos para ejemplificar el producto del trabajo con el tablero de contraste.

Indicador Existencias ganaderas: Se refiere a la cantidad de caprinos, vacunos, ovinos y equinos presentes en la unidad doméstica de producción como expresión de potencialidad de producción pecuaria en un ambiente dado. Esta categoría representa el número de animales presentes en cada UDP en valores de equivalente vaca. Para su cálculo se dividió por seis las existencias caprinas y ovinas y a ello se les sumaron las existencias vacunas y equinas, para ser expresada como unidades de equivalente vaca. Se conceptualiza que la producción pecuaria es la actividad que estructura al resto de las actividades realizadas en el predio y otorga bienes pasibles de ser usados tanto para el autoabastecimiento como para el intercambio. Se interpreta que esta categoría contribuye a la sustentabilidad en el sentido de que una mayor cantidad de existencias ganaderas les otorga la posibilidad de cubrir las necesidades de autoabastecimiento y les deja margen para la comercialización como forma de lograr un ingreso monetario. No obstante, dicha lógica de comportamiento es válida hasta un valor umbral, donde por encima del mismo la potencialidad productiva puede verse afectada a causa de una disminución en la vegetación que abastecen a los animales involucrados.

Tabla 1. Resultado parcial de los indicadores de sustentabilidad seleccionados o construidos para las unidades productivas de la Reserva Provincial Bosques Telteca.

Table 1. Partial result of selected or built sustainability indicators for the productive units of the Provincial Reserve of Bosques Telteca.

Relaciones		Estado de las unidades productivas de la Reserva Provincial Bosques Telteca		
		A- Ambiental	**B- Económica**	**3- Social**
Exigencias en términos de sustentabilidad	**A- Ambiental**	Cobertura vegetal total Cobertura vegetal forrajera SATVI	Existencia ganadera Manejo técnico	
	E- Económico	Disponibilidad maderable Cantidad de agua para la producción Calidad de agua para la producción	Riqueza productiva Destino de la producción	
	S- Social	Cantidad de agua para consumo humano Calidad de agua para consumo humano	Fuentes de ingresos monetarios Calidad de Ingresos monetarios Inserción laboral	Demografía Educación

Para el establecimiento del valor umbral, se establecieron 24 intervalos de 15 Equivalentes Vaca (EV) y se ubicaron dentro de cada uno de ellos las observaciones realizadas. De este modo se pudo obtener el intervalo de mayor frecuencia y bajo el criterio de que dicho intervalo representa la mejor aproximación al valor de existencias ganaderas con mejor ajuste a la zona a lo largo del tiempo. Se resolvió asignar un valor de 0,9 (en una escala entre cero y uno) y establecer a los intervalos ubicado por debajo y por arriba, valores proporcionales hasta llegar a cero.

Conclusiones

Aspectos relevantes del tablero de contraste

El tablero de contraste para la obtención de indicadores de sustentabilidad que en este artículo se ha desarrollado presenta las siguientes características relevantes.

El tablero permite obtener indicadores de forma ordenada y coherente en el plano teórico y operativo. Además, propone el sometimiento de los mismos a un análisis jerárquico y de validación para evitar que los indicadores sean interdependientes o que se observen dos o más veces la misma variable o indicador para aspectos diferentes sin ser advertidos ni pensados, si corresponde o no, su reiteración.

Un aspecto importante es que este tablero puede, o no, otorgar nuevos indicadores de sustentabilidad. Pero, y a diferencia de otras propuestas metodológicas, el procedimiento de esta metodología permite avanzar en argumentos, fundamentos, capacidad heurística, solidez y validez en cada uno de los indicadores construidos o elegidos y también en el índice de sustentabilidad. Siendo este conjunto de aspectos, elementos de cualificación de cualquier medición e interpretación y, en particular, aquellas relacionadas con los procesos de sustentabilidad, como lo expresan Garibaldi *et al.* (2007) y Tonolli (2018).

La propuesta contempla una mirada integral y multidimensional de la sustentabilidad pero la complementa con un enfoque sistémico tanto en la conceptualización (aborda las relaciones conceptuales entre las dimensiones) como en su parte operativa (el desempeño de una y otra variable se auto-condicionan en el mismo tablero), que permite contrastar el estado de un agroecosistema dado con las exigencias que las dimensiones de la sustentabilidad establece para comprender la dinámica entre estas relaciones. Además, permite complementar el análisis integral y multidimensional con uno de tipo particular, ya que posibilita el análisis de los valores agregados de estado o exigencias por dimensión (este tipo de análisis se enmarca en la concepción de sustentabilidad fuerte (38), ya que identifica los capitales puestos en producción y permite observar si hay complementación o reemplazo entre ellos) y también de cada unos de los indicadores que conforma el índice. Estas características otorgan mayores argumentos para comprender la dinámica del o los agroecosistemas observados y los problemas emergentes.

Por ultimo, otra bondad es que esta propuesta puede ser implementada, con los recaudos correspondientes, como herramienta metodológica de investigación científica o para la elaboración de diagnósticos en procesos de intervención técnica. Asimismo puede ser acoplada a cualquiera de los marcos de evaluación de agroecosistemas presentes en la bibliografía, ya que este dista de ser excluyente de ellos y resulta virtuoso indagar en situaciones de complementación.

En contraposición a las bondades citadas, se puede mencionar, como principal limitante, que los usuarios de esta propuesta requieren de un nivel de formación educativa que les otorgue el conocimiento teórico necesario para conceptualizar las dimensiones de análisis y las interacciones presentes entre ellas. Asimismo, les debe permitir resolver los procesamientos matemáticos necesarios.

Por ello, se espera que esta herramienta sea mayoritariamente apropiada para técnicos, profesionales o investigadores y en menor medida, para otros actores sociales.

Otra limitante se puede presentar cuando no se disponga de tiempo suficiente para trabajar en el tránsito desde la parte conceptual hasta la parte empírica, debido a que esta propuesta requiere de procesos de incorporación de conocimiento, de reflexión y de confrontación permanente entre lo que se va proponiendo a nivel de componentes, indicadores y variables, y lo que en definitiva va quedando, lo cual alarga el proceso de construcción, selección, medición y procesamiento de indicadores. Cabe aclarar que las limitantes mencionadas dependerán del nivel de precisión al que se quiera llegar, de la disponibilidad económica para realizar la evaluación y de los recursos humanos que se posean.

A modo de cierre

Desde la declaración del informe Bruntland, resulta imprescindible partir de un conjunto de principios básicos sobre el comportamiento de los sistemas que incorpore aspectos ambientales, sociales y económicos. Por ello, es imperativo adoptar una perspectiva interdisciplinaria e impulsar una mayor participación por parte de los diferentes sectores involucrados en el manejo de los agroecosistemas, que coloquen sobre la balanza las necesidades de corto plazo contra los beneficios y perspectivas de largo alcance. En este sentido, resulta necesario disponer de una conceptualización y comprensión integral y global de los agroecosistemas que permita producir conocimientos y diagnósticos para alcanzar una intervención técnica y social acertada.

En el presente trabajo se ha pasado revista a diversas propuestas de construcción de indicadores y se pudo distinguir tres formas de derivar indicadores de sustentabilidad según la estructura conceptual con que se trabajan las metodologías, pero no se descartan otras posibles formas de hacerlo, ni otras posibles formas de clasificación de los aportes en la temática presente. Asimismo se ha demostrado que las propuestas para la construcción de indicadores consultadas han sido predominantemente fragmentadas (por dimensión de análisis o por tipo de indicadores) o integrales (adición de dimensiones) pero no sistémicas y que esto limita intrínsecamente la capacidad explicativa de la tensión entre el logro de objetivos productivos y de objetivos de conservación, que están implícitos en los procesos de sustentabilidad en los que están insertos los agroecosistemas.

La propuesta metodológica desarrollada en este artículo aporta en dicho sentido y también al debate sobre la sustentabilidad, propone elementos sustantivos y prácticos para alcanzar acciones concretas. En este sentido, el tablero de contraste desarrollado en este trabajo se propone como una herramienta multidimensional, sistémica e interdisciplinaria, que pone en interjuego las dimensiones y los procesos que suceden a nivel de agroecosistema, para construir indicadores de sustentabilidad. La implementación de este tablero contribuye que los indicadores seleccionados o construidos tengan una sólida fundamentación y argumentación, condiciones necesarias para conocer con mayor certeza los condicionantes y los determinantes que actúan sobre un agroecosistema, así como para caracterizar u observar el comportamiento de sus aspectos claves y sacar a superficie ciertas problemáticas no visualizadas o proponer soluciones plausibles y ajustadas a la escala.

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Instituto de Biología Agrícola de Mendoza (IBAM). The tenth birthday of a research envision with international impact but strongly involved with the territory

At the beginning of the 2000' a small group of researchers belonging to the Facultad de Ciencias Agrarias (FCA), began meeting regularly with colleagues from other institutions in a sort of "scientific club" then called Centro de Biología Vegetal de Mendoza (CEBIVEM). The aim of this group was to carry on seminars to discuss the advances either of the CEBIVEM members and/or the state of arts in some specific subjects related to plant biology and associated organisms. From the beginning, the holistic vision of plants and their environment was clear.

Nonetheless, the gathering led to a more ambitious scope, and so the project of a research institute was proposed. The idea was, at first, advancing with timidity, but with the support of the local Universidad Nacional de Cuyo (UNCuyo) authorities and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), representatives in Mendoza, the proposal strengthened. Eventually, on November 5th 2009, the Institute of Agricultural Biology of Mendoza, Instituto de Biología Agrícola de Mendoza (IBAM), was formally inaugurated.

The foundational objetives declared were: i) to promote basic and applied research in the field of agro-biotechnology, taking in consideration the hypothesis of the disciplines and sub-disciplines related with the agronomical sciences, plant biology and biotechnology; ii) to contribute to the field of the Institute's expertise in generating knowledge for the development of technologies, primarily having in mind the regional demand, as well as projects of national relevance; iii) to train qualified personnel in high education, in number and quality, according to the necessities of the region and the country; iv) to collaborate with the productive sector related with agribusiness, in projects of technical assistance and technological transfer, aiming to a tied interrelationship with the local community.

From the beginning the IBAM's scope was of course to develop knowledge at the best scientific level, on condition that the generated information should be linked and transferred to the society that support the activities, especially regarding plant productivity and their applications. It was in this way that a collaborative process started with local firms even before the creation of the institute. The collaboration between the academy and the private industry was gaining momentum as time went by. Today, after 10 years, more than 90% of the research developed at IBAM is in collaborative association with different companies, mainly from the viticulture and winemaking area, although production of vegetables of local importance like tomato, olive, garlic, onion and potato is also included. As well, the search of knowledge has been carried out in collaboration among the different scientific groups of the institute, other scholars of FCA, UNCuyo, other Argentinean institutions and scientists from all over the world. More precise data on the matter are provided by an article in this issue.

The Institute was a collective construction. Those that opposed and/or presented hard criticisms were a valuable contribution for mending pathways or simply reinforcing the will to go forward with the enterprise. But people that contributed positively were many more, starting with the political willingness of authorities in charge, both from CONICET as well as from UNCuyo, but mainly with administrative, technicians, doctoral students and researchers. Citing names will exceed the possibilities of this letter, aside the fact of unavoidable (and unpardonable) forgetfulness.

Now, what and how is IBAM after 10 years from its formal creation? As it is quite common in the Argentinean scientific environment, the institute has double dependence from both, the university (UNCuyo in this case) and CONICET. Roughly speaking, the first provides the building infrastructure and utilities, while the council supplies doctoral scholarships and salaries, while both contribute to the regular budget. This budget is complemented with extramural grants obtained from Scientific Agencies (both national and international) and,

in the case of IBAM, especially with contributions from the local agribusiness industry. In the latter case, such contributions are not provided as money but mainly supplied as productive infrastructure and consumables, as well as doctoral scholarships that have been and still are co-financed. In return, the IBAM members, apart their academic positions (in some cases) at UNCuyo, are strongly involved in teaching at both, undergraduate and postgraduate levels.

After 10 years of work the main scientific achievements may be resumed as follows:

Extensive research has been explored and results published regarding the plant's responses to environmental clues, which are light quality, water restriction and soil microflora. Most noticeably, the role of UV-B radiation in the grapes for red winemaking characteristics (especially of the cultivar Malbec, emblematic for Argentina's winemaking industry) has been apprised. Therefore, explaining the reason why "wines of altitude" (grapevines grown at more than 1300 m a.s.l.) show superior quality as compared to their low altitude counterparts. Such effects have been interacted with water restriction and plant hormones applications, so generating valuable tools for winegrowing industry. Analytical and tasting combined studies are currently under development in order to characterize different "terroir" for the cultivar Malbec. In addition, the influence of light spectra inside the grape canopy is also being analyzed, concerning the grapevine management system. Finally, several beneficial bacteria have been isolated and characterized their effects on grapevines (and the wine product) have been established. Soil characterization in vineyards of high value is in progress, both from the physic-chemical as well as the biological standpoints.

The importance of interspecific hybridization and polyploidy in the generation of genetic and epigenetic variability in Solanum species have been established. These mechanisms are key aspects for the improvement of potato breeding, since they allow the introduction of genes of interest from wild species to commercial cultivars. The investigations demonstrated that both mentioned mechanisms generated genetic and epigenetic changes, which may explain the yield increases of polyploidy hybrids (heterosis). As well, it has been found that, in wild populations, hybridization generates new epigenetic patterns that explain the adaptation to new environments. Recently, there has been advances in understanding how the epigenetic variability may explain phenotypic changes of plants in response to environmental clues, either in hybrids of wild and cultivated potato, and also in grapevines.

Another valuable contribution has been on the understanding of molecular mechanisms and horizontal transfer of genes in plants. Such observations are implied in the maintaining or potential expansion of genes introduced by genetic engineering (transgenic plants). Furthermore, it has been established that the molecular mechanisms for acquisition of foreign genes by mitochondria through genomic recombination, is related with quimera genes that cause cytoplasmic male sterility. A review article on this topic can be found in this issue.

Genomic studies involving selection and adaptation of the Malbec grape cultivar were also performed. Starting with the genomic sequencing of 4 grapevine clones (two of Malbec, two of Cot) the genotyping of more than 220 different Malbec clones has been accomplished. In addition, advances on the understanding of the genetic regulation of Resveratrol and Anthocyanins (important in grape and wine quality and as anti-oxidants) were achieved. Transcription factors elicitated by the plant hormone jasmonic (JA) acid were identified, and the biosynthesis of Resveratrol and Anthocyanin increased in the red varieties Malbec, Bonarda, Syrah, Cabernet Sauvignon and Pinot Noir cultured in different regions (cold and warm) through application of abscisic acid (ABA) and JA. It has been found that the detrimental effect of high temperatures on Anthocyanin content in berries may be counteracted by combining ABA and salicylic acid (SA) with water stress in the varieties Malbec and Bonarda.

With respect to plant pathogenesis, several *Phylloxera* genotypes present in argentinean grapevines and not found in other regions of the world were identified, in ecological associations with the plague in different Argentinean vineyards. Eventhough different levels of susceptibility of Vitis Vinifera cultivars and rootstocks were assessed for tolerance,

no correlation was found between the pathogen harmfulness and the plant´s watering management. Another important research lines include the biology of new plagues for grapevines, like Lobesia botrana.

Pioneering studies with Eutectic Natural Solvents have been endeavored, as solvents in Green Chemistry and their functional role in cells and organisms.

The analytical profile of different foods, noticeably grapes, wine, olives and byproducts of the winemaking and olive industries, have been evaluated by using physic-chemical. Methods to improve their scrutiny have also been developed. Likewise, the bioactivity of extracts from such foods and byproducts (including wastes) were tested in biological systems, seeking applications in human health and as nutraceutic supplements. Another pursued goal has been the use of extracts from autochthonous species and from agri-business byproducts in the control of different pathogenesis.

The reproduction of the native grass *Thricloris crinita* has been established as sexual, autogamous and tetraploid. Molecular markers have been developed for different cultivars of the species, and the stress tolerance, productivity and forage quality established for different genotypes. This will permit a better use of this Gramineae in order to restore degraded areas and/or as forage.

It is worth to mention that, along the process of generating knowledge that produced more than 200 scientific indexed articles, numerous human resources were trained in research and development (36 doctoral thesis defended to this day). Many of the former graduated students are now investigators at IBAM, other CONICET institutes, scholars at UNCuyo and other universities, or incorporated to Instituto Nacional de Tecnología Agro-pecuaria (INTA) and the agribusiness sector.

Considering, altogether, the scientific production, human resources formation and collaborative work with the productive sector, IBAM has given fresh impetus to the idea that investigations with international impact but strongly involved with the territory are possible. This has established the paradigm that academia and industry may invest asso-ciatively in research as a way to optimize resources.

Finally, even though the initial envision seems to be fulfilled, it is quite clear, for the IBAM's members, that the story just begins.

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Seasonal isothiocyanates variation and market availability of Brassicaceae species consumed in Mendoza

Recomendaciones de consumo para aprovechar los fitoquímicos bioactivos presentes en *Brassicaceae* a lo largo del año en Argentina

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Abstract

Along with the recommendation of a healthy diet, it is suggested to increase the consumption of fruits and vegetables. Among the latter, Brassicaceae species are preferred, because they show many phytochemicals mainly belonging to the isothiocyanates (ITCs) family compounds, with proven activities related to the prevention of chronic diseases and cancer. A survey about seasonal availability and phytochemical levels of Brassicaceae species in the total of vegetables marketed in the province of Mendoza (located in the centre west of Argentina) was done. Results throw that Brassicaceae vegetables are an important part of Mendoza vegetable market reaching up to 23% of the vegetables commercialized. Regarding ITCs content, watercress and rocket were the vegetables with the highest ITCs levels, being, therefore, the most promising vegetables studied herein by their potential functional activities. Finally, high levels of variation (up to 10 times) on ITCs content along the year in a single species were found. These facts should be considered when designing Brassicaceae species phytochemical characterization assays to achieve more reliable results. This work represents the first report of Brassicaceae availability and seasonal phytochemical variability in local conditions.

Keywords
Cruciferae • isothiocyanates • vegetable availability • phytochemical seasonal variability

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Resumen

El consumo de frutas y hortalizas es recomendado para mantener una dieta saludable. Dentro de las hortalizas, las especies de la familia Brassicaceae se destacan por poseer grandes contenidos de diversos fitoquímicos que han demostrado su acción en la prevención de enfermedades crónicas y cáncer. En el presente trabajo, se realizó un relevamiento sobre disponibilidad estacional y niveles de fitoquímicos de especies de Brassicaceae considerando el total de hortalizas comercializadas en la provincia de Mendoza (ubicada en centro oeste de Argentina). Los resultados demostraron que los vegetales de la familia Brassicaceae contribuyen de modo importante en el mercado representando hasta un 23% de las hortalizas comercializadas. Respecto del contenido de ITCs (isotiocianatos), los mayores niveles fueron encontrados en berro y rúcula y como consecuencia de ello representan los vegetales más prometedores por sus potenciales propiedades funcionales. Además, se pudo observar que la variabilidad en los niveles de ITCs a lo largo del año en una misma especie, puede ser muy amplia (hasta 10 veces). Por esta razón es que podemos recomendar tener en cuanta la fluctuación estacional al evaluar niveles de fitoquímicos en especies de esta familia. Cabe destacar, que este trabajo representa el primer reporte de la disponibilidad de hortalizas de la familia Brassicaceae y la variabilidad fitoquímica estacional en las condiciones locales.

Palabras clave
Crucíferas • isotiocianatos • disponibilidad • variación estacional de fitoquímicos

Introduction

Brassicaceae family contains more than 350 genera and 3,000 species that are distributed worldwide (5). Despite the great diversity among the Brassicaceae family members, only a few genera are consumed, mainly those belonging to the *Brassica* genus. These vegetables have been consumed for their distinctive flavor and for their health enhancing properties. Consumption of Brassicaceae vegetables has been associated with a reduced risk of cardiovascular disease and different kinds of cancer (4). These beneficial properties have been attributed to a group of sulphur compounds, the isothiocyanates (ITCs), which possesses a characteristic biting taste and pungent odour. ITCs are produced when plant tissue is damaged, allowing the hydrolysis of glucosinolates (GLS), catalysed by the action of myrosinase enzyme (thioglucoside glucohydrolase, EC 3.2.1.147). Although ITCs are the primary reaction products, depending on the medium conditions such as pH, availability of ferrous ions and activity level of specific co-factors as the epithiospecifier protein; other several breakdown products could be formed including nitriles, thiocyanates, epithionitriles and oxazolidine-2-thione (7).

Brassicaceae vegetables consumption can be highly variable across different cultures, ethnic groups and countries. Americans consume broccoli, cauliflower, and cabbage primarily; the residents of Japan prefers daikon, Chinese people consume mainly cabbage, wasabi, watercress, and oriental mustards; and those residing in the United Kingdom prefer cabbage, sauerkraut, and brussels sprouts (14). Although, these habits could be influenced by the Brassicaceae vegetables availability in each region; nowadays, the globalized market contributes getting access to species from the different geographic origin (11).

Intake data on phytonutrients from fruits and vegetables is limited (12). Therefore, knowing ITCs levels in different Brassicaceae species along the year is important for predicting health enhancing effects derived from these species, while achieving specific consumption recommendations. In this sense, Jiao *et al.* (1998) analysed total ITCs content in nine Brassicaceae species commonly consumed in Asia (including species scarcely consumed in America such as choi sum, kai choi and bok choi among others). On the other hand, Tang *et al.* (2013) also measure ITCs content in *Brassica* vegetables but in those consumed in the United States (including broccoli, cabbage, cauliflower, Brussel sprouts, kale and collar green among others). To our knowledge, this is the first report of ITCs concentration data of Brassicaceae vegetables commonly consumed and cultivated in Central and South America. Moreover, there is no local data on the seasonal variation of these compounds in the different species.

The work was focusing on evaluate the availability of Brassicaceae vegetables in Mendoza, considering seasonal variability; and determination of total isothiocyanates levels content of all species funded in each season. Finally, consumption recommendation considering the phytochemicals role in cancer chemoprevention for the species studied was proposed.

Materials and methods

Sampling and sample conditioning

A survey of all vegetables marketed in the Cooperative Market of Godoy Cruz, Mendoza, was made (considering Brassicaceae species and non-Brassicaceae species) in order to define the representativeness of this botanical family in that market. Besides, the availability of Brassicaceae species during each season of the year was registered.

A total of 160 samples of nine commonly consumed Brassicaceae vegetables, including broccoli, cabbage, Brussels sprouts, radish, mustard green, cauliflower, rocket, and watercress, were purchased from local stores located in that market. One Kilogram of each species was purchased in five different stores. Later, a single batch of one kilogram was formed and processed in the laboratory. This process was repeated in four different sampling dates (July, October of 2016 and January and April of 2017).

A subsample of each homogenous batch was analysed in triplicate. The edible part of such vegetables (table 1) was conditioned by properly cleaning. Phytochemicals extraction and moisture content determination were made on the same day of purchase. For moisture assays, the samples were processed, weighted (3 g of each vegetable) and dried in a convection oven (DALVO, Santa Fe, Argentina) at 100 ± 10°C until constant weight was reached.

Phytochemical extraction

The extraction was carried out following an optimized technique previously reported by our group (6). Ten g of fresh vegetable was placed in a blender with 50 mL of water and homogenized for 9 min (Blender, 600 W, 60 Hz, model HR2030/10, Phillips, Buenos Aires, Argentina), then, the obtained juice was sonicated in an ultrasound bath for 5 min in a 100 mL glass beaker (US-bath, 40 kHz and 600 W, model tb 04, Testlab, Buenos Aires, Argentina).

Total ITCs content

Hydrolysis of GLS to ITCs was carried out by stirring an aliquot of 5 mL homogenate in a glass vial for two hours in a water bath at 37°C (2). The cyclocondensation reaction was carried out according to Tang *et al.* (2013). An aliquot of 250 µL of the hydrolysed homogenate was mixed with 250 µL of 100 mmol L^{-1} potassium phosphate buffer (pH 8.5) and 500 µL of 10 mmol L^{-1} benzene-1,2-dithiol solution in 2-propanol in a 4 mL vial and kept at 65°C for 2 hours.

Table 1. Availability of Brassicaceae vegetables in Mendoza, Argentine and edible parts used for analysis.

Tabla 1. Disponibilidad de las especies de Brassicaceae en Mendoza, Argentina y descripción de las partes comestibles analizadas.

Scientific name	Trivial name	Edible parts	Seasons availability*
Brassica juncea var. crispifolia	Mustard green	Leaves	1, 2, 3, 4
Brassica oleracea var. botrytis	Cauliflower	Inflorescences	1, 2, 4
Brassica oleracea var. capitata	Red cabbage	Leaves	1, 2, 3, 4
Brassica oleracea var. capitata	White cabbage	Leaves	1, 2, 3, 4
Brassica oleracea var. gemmifera	Brussels sprouts	Leaves	1, 4
Brassica oleracea var. italica	Broccoli	Inflorescences	1, 2, 3, 4
Eruca sativa	Rocket	Leaves	1, 2, 3, 4
Nasturtium officinale	Watercress	Leaves and stalks	1, 2, 3, 4
Raphanus sativus	Radish	Roots	1, 2, 4

* 1= winter; 2= spring; 3= summer and 4= autumn

* 1 = invierno; 2 = primavera; 3 = verano y 4 = otoño

Then, the mixture was centrifuged at 14000 rpm (15339 *g*) for 5 min and filtered with a 0.22 µm filter membrane before injection into the HPLC system. A liquid chromatograph Konik KNK-500 series, coupled to a UV/Vis detector (Konik, Barcelona, Spain) was used with a Waters C_{18} HPLC column (150 x 4.6 mm, I.D. 5 µm particle size) (Milford, Massachusetts, USA). Data obtained was processed by EZ Chrom Chromatography Data System Version 6.8 software. HPLC-UV conditions were chosen according to a previous report (6) with slight modifications: isocratic elution using as mobile phase 80:20 (v/v) MeOH-water solution at 1 mL min^{-1} and the detection wavelength was fixed at 365 nm (13). Peak identification was carried out by comparing retention times of the synthesized standard compound (1,3-benzodithiole-2-thione) according to Kristensen *et al.* (2007). Levels of total ITCs were quantified by a standard calibration curve and expressed in µmol per 100 grams of dry matter (µmol % g DW). The assays were carried out in triplicate.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The data were analyzed by ANOVA using INFOSTAT software. Tukey's test compared the mean of each treatment group. p-values < 0.05 were considered significant.

Results and Discussion

Table 1 (page 405), shows the results of the market survey on the availability of the Brassicaceae family for each season. It was possible to find between six and nine different species in each season. Cauliflower and radishes were not available in the summertime, as well as Brussels sprouts, which were also not available in spring.

The survey considering more than 40 different vegetables offered in the Cooperative Market reveals an important commercial supply of Brassicaceae vegetables throughout the year, representing a 15 - 23 % of the total of marketed species considering number of different species.

Moisture contents of these vegetables revealed significant differences ($p< 0.05$) among the seasons ranging from 83 to 95 %. This analysis allows us to express the evaluated phytochemical levels in dry weight (DW) and avoid water content influence.

The study throughout the year implied 160 samples for the analysis of total ITCs. The results obtained were analysed by ANOVA, revealing significant differences ($p< 0.05$) for the same vegetable among seasons (table 2, page 407). Overall ITCs levels ranged from 21.5 to 1465.4 µmol % g DW in white cabbage in winter and rocket in autumn, respectively. The highest ITCs levels were found in rocket despite the season of the year (average value= 1233.9 µmol % g DW) and the lowest ITCs levels were found in cauliflower (average value= 33.4 µmol % g DW). It is also interesting to consider that ITCs fluctuations along the year could reach up to 10 times, which should be taken into account when characterizing these species in a single season or at a specific date of the year.

Winter and autumn were the seasons in which the greatest variety of Brassicaceae species could be found. Seven of the nine evaluated species evidenced significantly higher levels of total ITCs in autumn (table 2 and table 3, page 407). These findings are in concordance with the results reported by Rosa and Rodrigues (2010) for broccoli crops, which conclude that total or individual GLS (ITCs precursors) concentrations were higher from September to December (autumn in the North Hemisphere). The authors found lower biomass production in this season, but the level of organosulfur compounds was higher than for the other seasons.

Rockets and watercress were the vegetables that evidenced the highest ITCs levels throughout the year (table 2, page 407). These results are consistent with a previous report (8) which found that watercress evidenced the highest ITCs levels, up to 144.6 µmol % g, while regarding rockets, levels similar to those previously reported were measured (15).

Table 3 (page 407) shows the richest ITCs species along the year and allows inferring the ideal season for each species consumption: excluding rocket and watercress, in summertime the highest levels of ITCs were found in broccoli, red and white cabbage. In spring, the outstanding plants concerning ITCs were cauliflower, mustard green and radish; and finally, in autumn, the significant levels were found in broccoli and radish.

Table 2. Total ITCs concentration of Brassicaceae vegetables throughout the year.

Tabla 2. Concentración total de ITCs en hortalizas de la familia Brassicaceae en diferentes estaciones del año.

Brassicaceae VEGETABLES	[Total ITCs] µmol % g DW*				
	Winter	Spring	Summer	Autumn	Mean value (variation§)
Broccoli	65.0 ± 7.9^{a}	129.2 ± 14.0^{b}	177.4 ± 2.4^{c}	307.0 ± 11.3^{d}	169.6 (4.7)
Brussels Spouts	23.5 ± 1.7^{a}	--	--	63.9 ± 2.7^{b}	43.7 (2.7)
Cauliflower	21.7 ± 3.0^{a}	52.1 ± 1.2^{b}	--	26.4 ± 1.3^{a}	33.4 (2.4)
Mustard green	50.1 ± 4.0^{a}	71.4 ± 1.1^{b}	57.9 ± 4.2^{a}	77.0 ± 2.2^{c}	64.1 (1.5)
Radish	104.0 ± 11.1^{a}	134.0 ± 15.9^{b}	--	145.9 ± 0.4^{b}	127.9 (1.4)
Red Cabbage	25.5 ± 7.5^{a}	40.6 ± 4.6^{a}	257.1 ± 20.2^{b}	76.5 ± 0.2^{a}	99.9 (10)
Rocket	1044.2 ± 51.0^{a}	1294.3 ± 93.1^{b}	1132.0 ± 9.0^{a}	1465.4 ± 17.1^{c}	1233.9 (1.4)
Watercress	700.4 ± 28.1^{b}	803.0 ± 52.9^{c}	537.9 ± 2.1^{a}	906.3 ± 48.0^{d}	736.9 (1.7)
White Cabbage	21.5 ± 4.4^{a}	23.5 ± 3.5^{ab}	76.0 ± 7.0^{c}	32.5 ± 1.9^{b}	38.37 (3.6)

* Results expressed as mean ± SD; 95% confidence interval; µmol % g DW. Values followed by a different superscript lowercase letter are significantly different ($p<0.05$) on ITCs concentration (in ascending order), for each vegetable between seasons. (--) The vegetable was not available on that season. §Variation level calculated as: maximum value/minimum value

* Resultados expresados como media ± DE; Intervalo de confianza del 95%; µmol% g peso seco. Los valores seguidos por una letra minúscula de superíndice diferente son significativamente diferentes ($p <0,05$) en concentración de ITCs (en orden ascendente), para cada vegetal entre estaciones. (--) El vegetal no estaba disponible en esa temporada. §Valor de variación calculado como: valor máximo/valor mínimo

Table 3. Seasons of the year with the highest ITCs concentration for each Brassicaceae species.

Tabla 3. Estaciones del año con la concentración de ITCs más alta para cada especie de Brassicaceae.

Brassicaceae VEGETABLES	Season of the year			
	Winter	Summer	Autumn	Spring
Broccoli		**	*	
Brussels Spouts	*		**	
Cauliflower			*	**
Mustard green			*	**
Radish			**	*
Red Cabbage		**	*	
Rocket			**	*
Watercress			**	*
White Cabbage		**	*	

Where ** Shows the highest ITCs concentration and * The second largest ITCs concentration.

Donde ** Indica la concentración máxima de ITCs y * Indica la segunda concentración mayor.

Our current findings expand the previously reported (1, 3, 8, 13) because here the samples were taken from all the seasons and all the available Brassicaceae species were considered, including vegetables of global interest, usually less studied than *Brassica* genus. Consequently, taking into account that autumn was the season with both, highest species availability and ITCs levels, this season is recommended for consuming these vegetables, for the maximum phytochemical's achievement.

Conclusions

The present work demonstrates that Brassicaceae vegetables are an important part of Argentinean diet reaching up to 23% of the vegetables commercialized, considering total number of different species. In addition, it is interesting to take into account that due to the great diversity of species that are offered, there is availability of at least some of these species throughout the year. Regarding ITCs content, watercress and rocket were the vegetables with the highest ITCs levels, being the most promising vegetables of this family, given their potential functional activities. Finally, the variation level of ITCs content

along the year in a single species resulted to be remarkable, reaching up to ten times. This should be considered when designing Brassicaceae species characterization studies, in order to achieve more reliable comparisons among results. These findings will contribute to complete a comprehensive database that will be available for consumers and the scientific community.

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Historical overview and perspectives of the Instituto de Biología Agrícola de Mendoza (IBAM): since its birth in 2009 to 2019

Revisión histórica y perspectivas del Instituto de Biología Agrícola de Mendoza (IBAM): desde su creación en 2009 hasta el 2019

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Abstract

The Instituto de Biología Agrícola de Mendoza (IBAM) belongs to the Universidad Nacional de Cuyo and CONICET. It has the mission of generating knowledge in basic aspects and applied to irrigated agriculture. The purpose of this review is to analyze the articles published during 10 years, since its creation. All publications were collected from 2009 to the present and the outcoming data was evaluated according to funding, collaborations, disciplines, equipment, journal quality indicators and IBAM's h-index. Taking the Scimago ranking into account, it was concluded that 58% of the publications are in quartile 1 (Q1). When the disciplines were analyzed, those related to the crops of the Mendoza region, such as vine, potato, garlic and olive, were the most studied. Most of the projects that finance publications come from national institutions. Interinstitutional collaborations are mainly with national entities. IBAM has been growing over the past 10 years in terms of human resources and in the quality of its research.

Keywords
publication analysis • IBAM staff • CONICET • Universidad Nacional de Cuyo • Q-index • equipment • research funding • institutional collaborations • publications topic • H-index

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Resumen

El Instituto de Biología Agrícola de Mendoza (IBAM) tiene la misión de generar conocimientos en aspectos básicos y aplicados a la agricultura de regadío y pertenece a la Universidad Nacional de Cuyo y al CONICET. El objetivo de este trabajo es analizar los artículos publicados en los 10 años desde la creación del Instituto. Para ello se recopilaron todas las publicaciones desde el 2009 a la actualidad y se trabajó con los datos que se desprendieron de los mismos, como el financiamiento, las colaboraciones, las disciplinas, el equipamiento, los indicadores de calidad de las revistas y el índice h del IBAM. Teniendo en cuenta el ranking de Scimago, se concluyó que el 58% de las publicaciones se encuentran en el cuartil 1 (Q1). En cuanto a las disciplinas, donde hubo más producción fueron las relacionadas con los cultivos de la región de Mendoza, como por ejemplo la vid, papa, ajo y olivo entre otras. El 94% del financiamiento para las publicaciones proviene de instituciones nacionales. Las colaboraciones interinstitucionales son principalmente con entes nacionales. El IBAM ha ido creciendo a lo largo de los 10 años en cuanto a recursos humanos como en la calidad de sus investigaciones.

Palabras clave

análisis de publicaciones • recursos humanos de IBAM • CONICET • Universidad Nacional de Cuyo • índice Q • equipamiento • financiamiento para investigación • colaboraciones institucionales • temas de publicación • índice H

Introduction

The Instituto de Biología Agrícola de Mendoza (IBAM) was created in October of 2009, as a need of many researchers that worked in several research groups and Laboratories of the Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina. The Institute belongs to the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad Nacional de Cuyo (UNCuyo) and is located within the campus of the Facultad de Ciencias Agrarias (FCA, UNCuyo).

IBAM is constituted by ten research groups, working on different areas such as molecular biology and genetics, analytical and biological chemistry, plant physiology, and plant pathology. A total of 59 members were part of IBAM in 2018, including 26 researchers, 28 PhD students and postdoctoral fellows, one administrative secretary, and four research support staff (CPA).

The main purpose of IBAM is to generate knowledge in basic and applied aspects of irrigated agriculture, developing tools that improve production and reduce the negative factors on crops and the environment. IBAM´s researchers conduct different projects in the areas described above, and the results of their studies are shared with the scientific community through publications in high standard national and international journals. Also, the researches contribute to solving regional community problems through the generation of technical services like DNA and metabolomics analysis.

This work presents an overview of the scientific production of IBAM during its first 10 years by collecting data of the research topics, funding, relationships with other institutions, h-index, equipment used, among others.

To the best of our knowledge, there are no reports of publication analysis of other CONICET Institutes. However, there are articles that analyse the bibliometric data of FCA, UNCuyo projects (3), and argentinian Agriculture Multidisciplinary publications (4).

Materials and Methods

All publications from the period 2009-2018 involving a member of IBAM were evaluated (Supplementary Table). The data collection included year of publication, authors, total number of citations, equipment used, Journal Quality index, funding sources, number of PhD graduates, main research topics, collaborations between IBAM and other governmental or private institutes inside Argentina or international.

The authors of the publications were classified in terms of researchers, PhD students, postdoctoral fellows, or research support staff. The total number of citations was extracted from the database Scopus (6). For assessing the equipment used, only those belonging to IBAM were considered. The journal quality was assessed by the indicators developed by Scimago Journal Rank (SJR) (5), ranging from Q1 to Q4 (1) and according to the discipline of each research. The funding information supporting the published research was taken from the publications. The main research topics were established through the analysis of the key words. All collaborations with other institutions were identified from the affiliation data of the authors.

Results and Discussion

Publications along a 10-year period

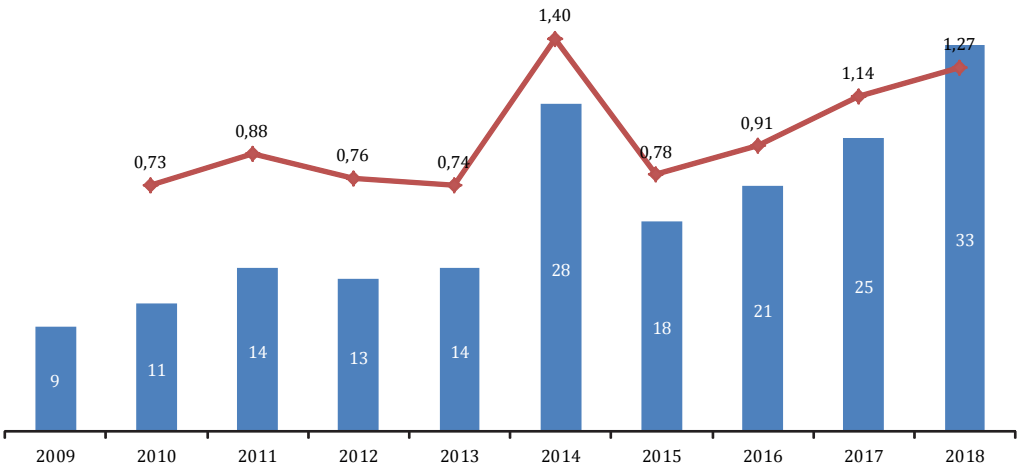
Considering the Scopus database, the number of publications during the 10 years since the creation of IBAM, reached a total of 186 scientific articles (figure 1). Interestingly, the number of annual publications increased since 2009, with a minimum of 9 publications and a maximum of 33 in 2018. The relationship between the number of articles/researcher/year varied from 0.73 in 2010 to 1.4 in 2014. This ratio in 2014 was due to an exceptional number of publications made, and not for a decrease of researchers. The number of publications per year reflects the continuous strengthening of IBAM's staff, including PhD students and researchers.

IBAM staff

During the first years of IBAM, the staff increased significantly, in particular from 2010 to 2013 (figure 2, page 412). In the following years, the number of members of the institute was constant, with a slowly but steady increase in the number of researchers. The main reason of that increase is that once a PhD student graduates, they can apply to be a researcher at IBAM following an intensive and competitive evaluation. An alternative, for the recent PhDs, is to apply for jobs in the private sector or public institutions like UNCuyo.

The research support staff (CPA, Carrera del Personal de Apoyo a la Investigación y Desarrollo) are professionals with different expertise and academic formation that collaborate with researchers and PhD students or postdoctoral fellows. In this context, they plan, carry out and execute technical support work, according to the requirements of the researchers. As shown in figure 2 (page 412), the CPA staff grew in the first years, but has remained constant since 2012 with four professional members.

A remarkable aspect to highlight about IBAM's researchers is that 81% of them are also professors at UNCuyo, giving them the opportunity to share their experiences and knowledge with students.



The red line shows the ratio of publications/year/researcher.

La línea roja muestra la relación entre las publicaciones anuales por investigador.

Figure 1. The number of publications per year are represented in blue bars.

Figura 1. Las publicaciones de cada año están representadas en barras azules.

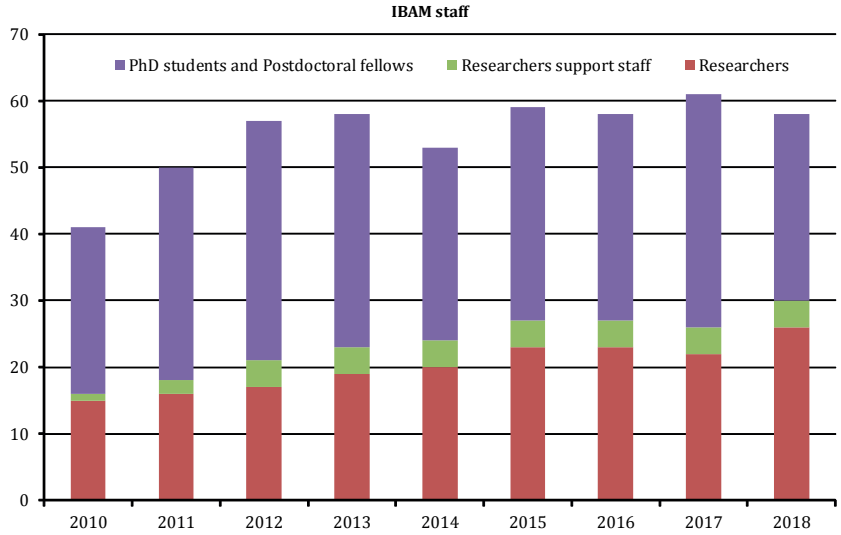


Figure 2. Total IBAM staff is represented in bars. Researchers are coloured in red, research support staff in green and PhD students in blue.

Figura 2. El personal del IBAM está representado en barras: los investigadores en rojo, los CPA en verde y los becarios en azul.

Journal quality indicators

With the aim to assess the journal quality, the Q index established by SJR was used. This index considers the importance of journals per research area, based principally on the number of citations. As can be seen in figure 3, most of the publications are placed in journals within the top category, Q1, showing that the majority of IBAM´s scientific articles are of the highest quality worldwide.

It is important to highlight that even though IBAM is one of the smallest Institutes in CONICET Mendoza and has a very short history, it could be recognized as one of the most outstanding centres of investigation in agricultural issues.

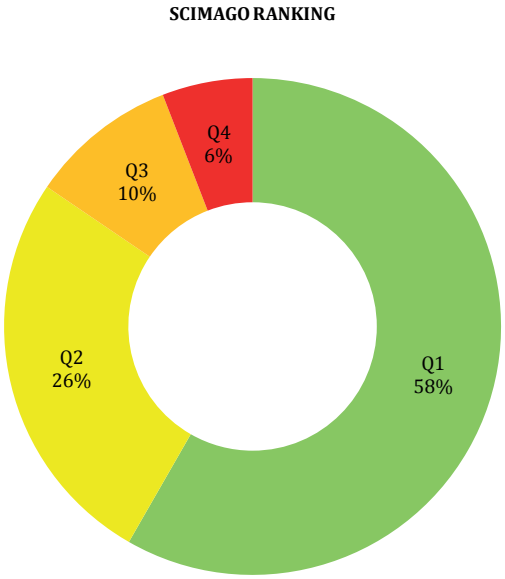


Figure 3. Total IBAM publications grouped according to SCIMAGO ranking.

Figura 3. El total de las publicaciones del IBAM se representan agrupadas de acuerdo con el ranking de SCIMAGO.

Research funding

Considering financial support, all the investigations that were published were supported by diverse funding agencies. Even though the funding source was included in the publications, the funding amounts were not detailed. Published research at IBAM received funding from national (94%) and international (6%) sources (figure 4A).

The national funding was provided by UNCuyo, Agencia Nacional de Promoción Científica y Tecnólogica, and CONICET (figure 4B). The double affiliation of IBAM is coherent with the fact that large part of the funding comes from UNCuyo and CONICET. Although in figure 4 the private sector financing (*Companies*) seems to be poor, it does not reflects the real situation: there is a continuous and substantial collaboration of this sector with IBAM, but it is not represented in the information given in the articles published. Actually several authors, mainly doctoral and postdoctoral students have been awarded fellowships in a cofounding format with the productive sector. Furthermore, much experimental work has been carried out in private locations.

Institutional collaborations

With the aim to identify the Institutional relationships between researchers from different organizations, author affiliations were considered.

As mentioned above, IBAM is organized in different research groups, focusing on several areas. However, as shown in figure 5 (page 414), there is a continuous interaction among research groups evidenced by joint publications (9.2%, shown as IBAM-IBAM). Interestingly, there is a considerable commitment between IBAM and FCA researchers (14.6%), working side by side and exploiting the potential of the agricultural area.

Moreover, IBAM researchers also collaborate with international research groups (21.8%) mainly from Spain, USA, and France. These interactions reflect the global recognition of IBAM.

Furthermore, IBAM researchers interact with other national institutions (52.9%), principally with INTA (Instituto Nacional de Tecnología Agropecuaria), and several other national universities such as Universidad Nacional de San Luis and Universidad de Buenos Aires. Finally, it is very important the collaborative work with other institutes from CONICET Mendoza, such as IMBECU and IANIGLA.

Taking into account the relationship of IBAM and private companies, only Catena Zapata Institute appears on publications, even though other agreements have been established between IBAM and private companies.

Also it is important to highlight that more than 20% of the collaborations are with international institutions.

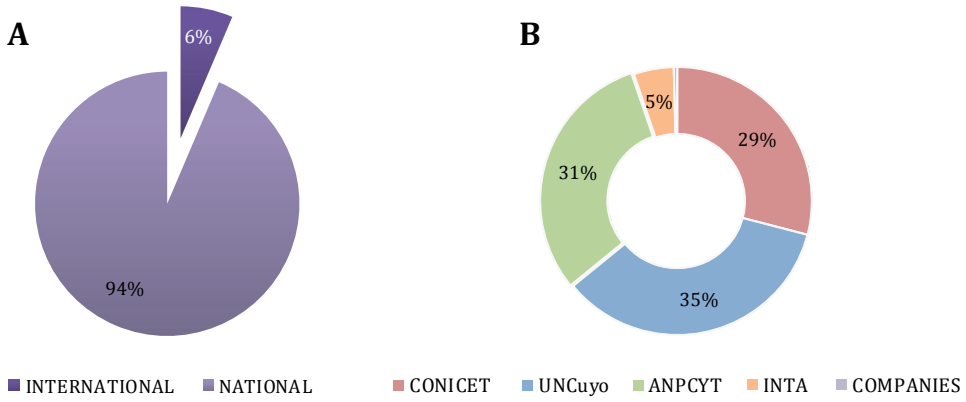


Figure 4. A, funding declared in publications according to national and international contribution based on the number of projects financed. B, national funding was split according to the Institution that financed the research.

Figura 4. A, Financiamiento declarado en las publicaciones de acuerdo con subsidios nacionales e internacionales, basado en el número de proyectos. B, el financiamiento nacional sectorizado en las distintas instituciones que contribuyeron a las publicaciones.

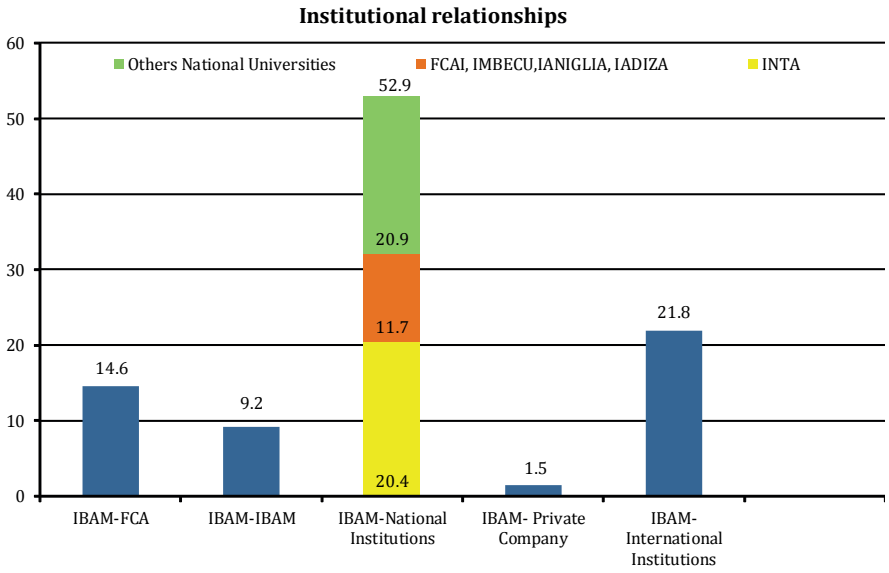


Figure 5. Institutional relationships between IBAM and several institutions.
Figura 5. Relaciones institucionales entre los integrantes del IBAM y otras instituciones.

Equipment used

Several complex equipment, located at IBAM, are shared among IBAM researchers and are available for investigations performed in FCA. They were acquired through different financing projects, as those mentioned above.

The equipment declared in publications is presented in figure 6. Among them, high performance liquid chromatography (HPLC) was the most used (26.7%), followed by gas chromatography (17.2%), thermocyclers (16.4%) and spectrophotometers (12.1%). These data is in agreement with the main research areas of IBAM, analytical chemistry and plant genetics. It important to highlight that one of the institutional priorities is the constant modernization of its equipment, as a way to be competitive worldwide.

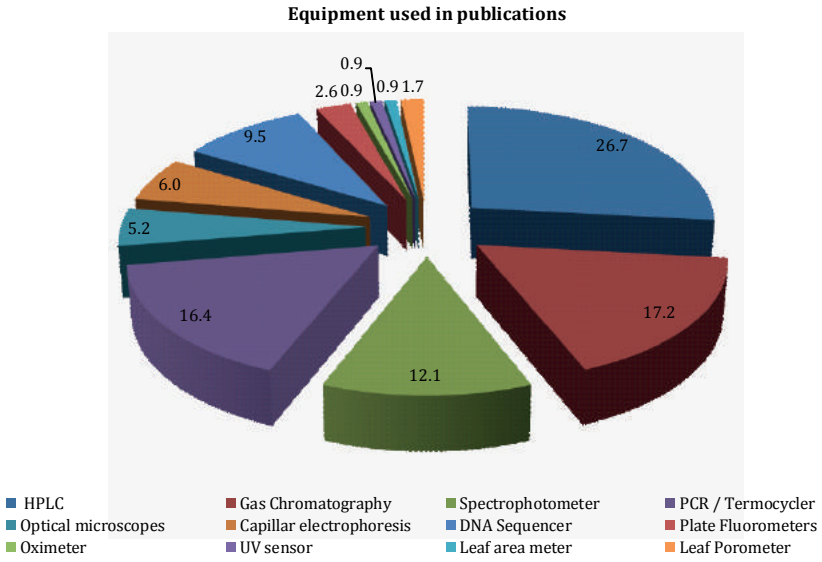


Figure 6. Equipment used in publications represented in percentage.
Figura 6. Equipamiento utilizado en las publicaciones.

Publication topics

IBAM is a multidisciplinary Institute that focuses on a variety of research areas. The key words from all the publications were classified in two areas: crop studies (77%) and other topics (23%) such as analytical chemistry, microbiology and evolution (figure 7A). Among the crop-related articles, grapevine studies were the most numerous, followed by potato, garlic, and olive (figure 7B). This distribution is in accordance with the productive interests of the Cuyo region, showing the commitment of IBAM to the local development. In addition, it is important to highlight the valuable studies conducted with non-cultivated plants. These species allow the study and discovery of basic biological mechanisms, vital for basic research.

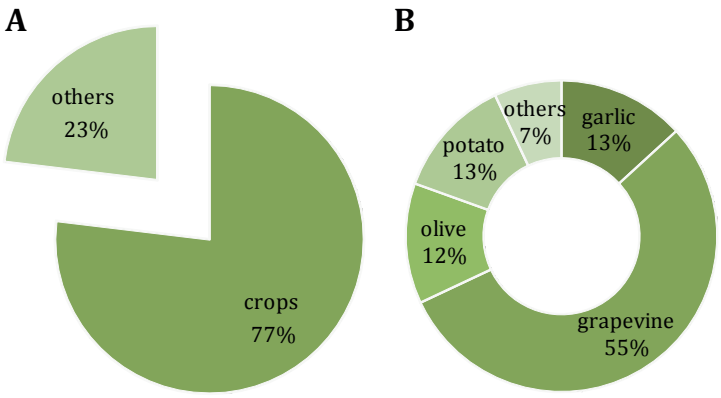


Figure 7. A, Publication topics classified as crop or others (analytical chemistry, microbiology and evolution). B, Crops publications divided into the different farming vegetables studied.

Figura 7. A, La temática de las publicaciones clasificadas de acuerdo con cultivos y otros (química analítica, microbiología y evolución). B, las publicaciones referidas a cultivos se dividieron en diferentes áreas estudiadas.

When analysing grapevine studies, the investigations include the determination of different metabolites, genetic and epigenetic studies, effects of different light quality and altitudes on plant physiology, among others. It is important to highlight that grapevine is object of interest from the vineyard, through the winemaking process and finishing with the alternative use of wine residues for biotechnology purpose. In the vineyard, IBAM conducts several studies of genetic and environmental effects on grape quality, as well as grape interaction with pathogens and plagues. The winemaking process is investigated and controlled by determining different secondary metabolites in wine, following different analytical methods. In addition, wine is tasted by specialists with the purpose of achieving a complete characterization. Ultimately, the vinification process generates great quantities of grape pomace. In this sense, IBAM studies are focused on its reutilization, as a sustainable source of bioactive compounds.

IBAM's h-index

The h-index is an indicator of the number of publications and citations of a single researcher or from an Institution (2). The IBAM h-index was 28 (figure 8, page 416), indicating the good quality of the scientific production during the 10 years of its existence.

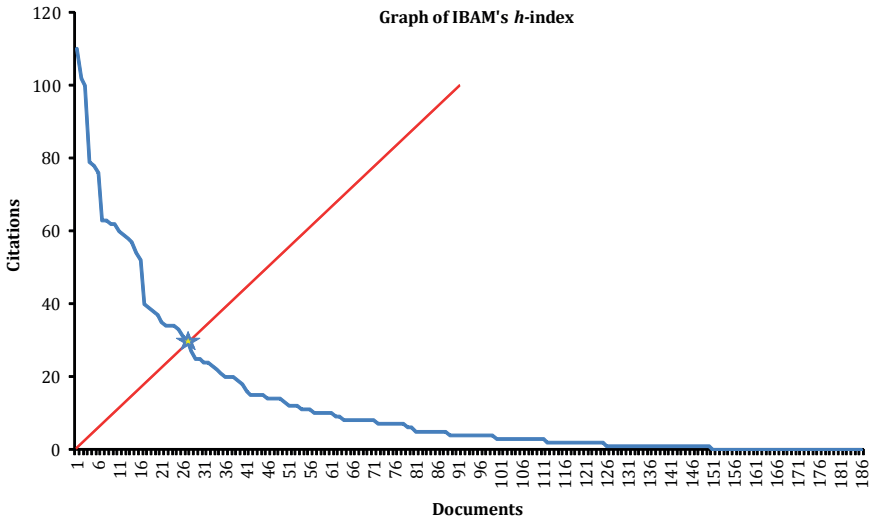


Figure 8. The intersection of the 45 degree line with the curve giving the number of citations *versus* the paper number gives h. The total number of citations is the area under the curve.

Figura 8. Índice h del IBAM: la intersección de la línea a 45 grados con la curva, resultante del número de citaciones, con respecto al número de publicaciones da como resultado el valor h. El número total de citas es el área debajo de la curva.

IBAM and the society

IBAM promotes an active policy of knowledge transfer to different sectors of society. Through services, agreements, and consultancies, the Institute makes all its research expertise and development experience available, responding to the concerns raised by public and private entities that seek to solve problems related to regional agricultural activity. Currently, 17 high-level technological services (STANs) are active. The equipment, infrastructure, and specialized human resources of the Centres, Institutes and Laboratories related to it are generally used for its provision.

More information in: https://www.mendoza.conicet.gov.ar/portal/ibam/paginas/index/servicios97

Supplementary materials

Supplementary materials with this article can be found, in the online version, at:

https://drive.google.com/file/d/13f8fzXaXpaeGDP-IefGyznWHUVuKvjDb/view?usp=sharing

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Assessment of grapevine stems as source of phenolics with antioxidant properties

Escobajos de la vid como fuente de compuestos fenólicos con propiedades antioxidantes

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Abstract

Winemaking industry generates considerable amounts of bunch stems that are usually wasted despite their potential value as source of bioactive compounds. Phenolic profiles and antioxidant capacity (AC) of bunch stem extracts from eight grape varieties of *Vitis vinifera* L. were determined. Sixteen phenolic compounds (PC) were quantified by high performance-liquid chromatography-diode array detection (HPLC-DAD). The maximum concentrations corresponded to the flavanols (+)-catechin (6462 µg g^{-1} DW) and procyanidin B1 (1987 µg g^{-1} DW), followed by the hydroxycinnamic acid caftaric acid (2967 µg g^{-1} DW). Naringin, myricetin and OH-tyrosol were identified for the first time in grape bunch stems. Total phenolic content (TPC) of extracts, assessed as gallic acid equivalents (GAE), ranged between 47 and 125 mg GAE g^{-1} DW. The AC of extracts was appraised by ORAC, ABTS and DPPH assays, with a good correlation between TPC and AC when measured by ABTS and DPPH ($r \geq 0.92$), while for ORAC the correlation was lower ($r \leq 0.41$). Samples of *cv.* Malbec, the most representative variety of Argentina, presented the highest contents in PC, particularly flavanols. The results showed that grape bunch stems may be an inexpensive, sustainable and high value source of bioactive compounds as functional ingredients.

Keywords
antioxidant capacity • bioactive compounds • grape bunch stems • industry by-products.

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Resumen

La industria vitivinícola genera cantidades considerables de escobajo que generalmente se desperdician a pesar de su valor potencial como fuente de compuestos bioactivos. En este trabajo se determinaron los perfiles fenólicos y capacidad antioxidantes (CA) de extractos de escobajo de ocho variedades diferentes de *Vitis vinífera* L. Se cuantificaron 16 compuestos fenólicos (PC) utilizando cromatografía líquida de alta resolución acoplada a detector de arreglo de diodos (HPLC-DAD). Las concentraciones más elevadas obtenidas correspondieron a los flavanoles (+)-catequina (6462 μg g^{-1} peso seco) y procianidina B1 (1987 μg g^{-1} peso seco), seguido del ácido caftárico (2967 μg g^{-1} peso seco). La naringenina, miricetina y OH-tirosol fueron identificados por primera vez en escobajos. El contenido total de compuestos fenólicos (TPC) de los extractos determinado con equivalentes de ácido gálico (GAE) presentó valores entre 47 y 125 mg GAE g^{-1} peso seco. La CA de los extractos fue determinada mediante las técnicas ORAC, ABTS y DPPH, evidenciando una buena correlación entre TPC y la CA medida mediante ABTS y DPPH ($r \geq 0,92$), mientras que para ORAC la correlación fue más baja ($r \leq 0,41$). La muestra de variedad más representativa de Argentina, cv. Malbec, presentó los mayores niveles de PC, particularmente flavanoles. Los resultados evidencian que los escobajos pueden ser una fuente económica, sostenible y de alto valor de compuestos bioactivos para su utilización como ingredientes funcionales.

Palabras clave

capacidad antioxidante • compuestos bioactivos • raquis de uva • subproductos industriales

Introduction

Viticulture is one of the world's most important agro economic activities with an annual production of more than 70 million t of berries coming from a cultivated area of 7.5 million ha (24). Currently, Argentina's vineyards represent around 3% of the grape cultivated area worldwide, indicating the economic importance of this activity in the region (17). About 97.8% of the grape produced in this area is used in the wine and must industry (16), which generates considerable amounts of woody wastes, especially bunch stems. Bunch stems consists of a main axis (rachis) and primary and secondary branches that ended in pedicels supporting the berries. This underused ligno-cellulosic residue, which accumulates during the process of eliminating the stems, constitutes 5% of the processed grape (12).

In the last years the potential valorization of this by-product has been increased, because of the information available regarding their content of purportedly health-promoting phytochemicals. Particularly, there are some reports informing about its richness in phenolic compounds (PC) belonging to the families of flavonoids, phenolic acids and stilbenes (1).

Furthermore, this raw material has also been showed as a rich source of dietary fiber (13). Normally, bunch stems are used for animal feeding and as organic fertilizer (compost) (1, 3), which limited their potential value as a source of bioactive compounds. Hence, the use of this raw material as source of PC would increase its economic value and reduce the environmental impact of winery activity (19).

Several studies have shown different biological properties for bunch stem extracts, mainly because their antioxidant capacity (AC) (31). The *in vitro* assays most commonly used to measure AC in food and biological samples have been oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,20-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The ORAC reaction mechanism is based on hydrogen atom transfer, while DPPH and ABTS methods are based on electron transfer (10). Thus, the information given by PC profiles and the material AC may help to understand which compounds, either acting individually or in interaction with others, are responsible for the antioxidant effects shown by the extracts, so providing an additional value of the extract.

For all the above, this raw material could be a rich potential source of PC with antioxidant biological properties that would provide added value and reduce the environmental impact of viticulture activity. This work presents the characterization of PC in bunch stems of different grapevine varieties cultivated in Argentina. The objective was to identify and quantify by HPLC-DAD, the individual PC of different families (flavanols, flavonols, stilbenes and phenolic acids) in bunch stem extracts of eight grape varieties cultivated in the region. Moreover, the *in vitro* AC (by ABTS, DPPH and ORAC) and TPC were measured to study the correlation amongst qualitative and quantitative profiles of individual PC.

Materials and methods

Chemicals

HPLC-grade standards of PCs (purity ≥ 95%), Trolox reagent (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), NaH2PO4.2H2O, Na2HPO4.12H2O, and fluorescein were from Sigma-Aldrich (Steinheim, Germany). HPLC-grade acetonitrile (MeCN), formic acid (FA) and methanol were from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Ethanol and Folin-Ciocalteu reagent were from Merck (São Paulo, Brazil). Other reagents used were of analytical grade. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA).

Sample preparation

This study was conducted with different bunch stem samples from vineyards of *V. vinifera* of the cultivars Malbec (MB), Cabernet Sauvignon (CS), Cabernet Franc (CF), Chardonnay (CH), Sauvignon Blanc (SB), Pinot Noir (PN), Petit Verdot (PV) and Syrah (SY). Samples were obtained after the separation of bunch stems from berries at the beginning of the winemaking process during season (2018) from vineyards of different locations of Mendoza's region, Argentina. Each sample was freeze-dried for 5 days. The dried bunch stems were powdered in an analytical mill (A 11 basic; IKA, Staufen, Germany) and stored protected from light at room temperature in plastic tubes until extraction.

Extract preparation

Extraction of PC from samples was performed by solid-liquid method according to previous reports (9), with slight modifications. Briefly, 1 g of powdered sample was extracted with 50 mL ethanol/water (50:50 v/v) in ultrasonic washer at 50 Hz and 60°C during 60 min. The mixture was centrifuged 10 min at 3000 rpm and filtered through filter paper. Then, the extract was stored in sealed dark-glass bottles at -20 °C prior to analysis. Extractions were performed in triplicate. Finally, an aliquot of each extract was filtered through a 0.2 µm PTFE vial filter and analyzed by HPLC-DAD.

Chromatography

Analysis of PC were done using a Dionex Ultimate 3000 HPLC-DAD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) and a reversed phase Kinetex C18 column (3.0 mm x 100 mm, 2.6 µm) (Phenomenex, Torrance, CA, USA). As mobile phases ultrapure water with 0.1% FA (A) and MeCN (B) were used. Analytes were separated using a previously reported method (11) with the following gradient: 0-1.7 min, 5% B; 1.7-11 min, 30% B; 11-14 min, 95% B; 14-15.5 min, 95% B; 15.5-17 min, 5% B; 17-20, 5% B. The mobile phase flow was 0.8 mL min^{-1}. The column temperature was 35°C, and the injection volume was 1 µL. The quantification wavelengths for different families of analytes were 254 nm, 280 nm, 320 nm, and 370 nm. Analytes present in the samples were quantified using an external calibration with pure standards also used to achieve identification. Results were expressed as µg g^{-1} PC of dry weight (DW) of bunch stem. The software used to control the HPLC-DAD system and to process data was Chromeleon™ 7.1.

Total phenolic content

The TPC was spectrophotometrically measured with an UV-vis spectrophotometer Cary-50 (Varian Inc., Mulgrave, Australia) from an aliquot of the extract. To quantify total PC Folin-Ciocalteu assay (FC) as reported by Antoniolli *et al.* (2015) at 765 nm and the direct reading of the absorbance at 280 nm of the sample diluted 1:100 v/v was used.

Results were expressed as mg GAE g^{-1} DW from calibration curves made with the standard solutions (three replicates) in the range between 20 and 200 mg L^{-1} (R2 = 0.998 and R2 = 0.999, respectively for each method).

Antioxidant capacity

The AC of bunch stem extracts were evaluated by ORAC, ABTS and DPPH assays (10). These methods were selected because they are based on different reaction mechanisms, as explained above. Trolox was employed as standard and results expressed as µmol of Trolox equivalents per gram of bunch stem dry weight (µmol TE g^{-1} DW) as mean ± standard deviation (SD).

The ORAC assay was performed as previously reported (2), with some modifications. Stem extract solutions were diluted ranging from 1: 750 to 1: 1500 v/v in 75 mmol L^{-1} NaH2PO4-Na2HPO4 (pH 7.0), due to their differences in PC content. Later, 50 µL aliquots of diluted samples and Trolox standards (0 - 50 µmol L^{-1}) were added to a 96-well plate. Then, 100 µL of fluorescein solution were added and the mixture incubated 7 min at 37°C before addition of 50 µL of 140 mmol L^{-1}peroxyl radical generator AAPH. Fluorescence was monitored at 485 nm excitation and 538 nm emission with 1 min intervals for 90 min using a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc, Wilmington, DE). The area below the curve of the fluorescence decay during 90 min was calculated for each sample by integrating the relative fluorescence curve.

For ABTS assay the method described by Ferreyra *et al.* (2019) was employed. ABTS radical cation (ABTS•+) was produced by mixing 2.5 mL of 7 mM ABTS stock solution and 44 µL of 140 mM K2S2O8, both diluted with ultrapure water. This mixture was stored 12-16 h in darkness and then diluted with 80% methanol solution to an absorbance of 0.70 ± 0.02 at 734 nm. After addition of 10 µL of Trolox (0 - 2000 µmol L^{-1}) or the diluted sample to 2.5 mL of diluted ABTS•+ solution, absorbance readings were taken after 7 min of the initial mixing with an UV-vis spectrophotometer Cary-50.

The DPPH assay was based on Ferreyra *et al.* 2019. A stock solution of 61 µM DPPH• was prepared in methanol and then stored at room temperature in darkness before use. The stock solution was diluted with methanol to an absorbance of 1.0 ± 0.1 at 515 nm. An aliquot of each diluted extract sample (10 µL) was added to 2.5 mL of fresh DPPH• solution, shaken and incubated in darkness. Decrease in mixture absorbance was recorded after 30 min with an UV - vis spectrophotometer Cary-50.

Statistical analysis

Values of PC, TPC and AC were analyzed by Pearson Correlation test with Statgraphics® Centurion XVI v.16.0.7 (Statpoint Technologies Inc., Warrenton, VA, USA) statistical software. Pearson value (r) and p-values were computed.

Results and discussion

Identification and quantification of bioactive compounds

Table 1 (page 421) shows individual PC from bunch stem extracts of the different varieties. In terms of total low molecular weight PC (LMW-PCs) concentration, expressed as the sum of quantified compounds, samples MB and PN exhibited the highest amounts.

For all the analyzed samples, the flavanol family presented the highest levels, with concentrations ranging between 1442 and 10605 µg g^{-1} DW (data not shown). The cultivars that presented highest amounts were Malbec (MB), followed by Pinot Noir (PN) and Chardonnay (CH). The major flavanol compound was (+)-catechin with the highest level in samples MB and PN, representing about 25% of total assessed PC. It was found at levels between 1249 (PV) and 6462 µg g^{-1} (MB), in accordance with previously published data (21).

Table 1. PC of bunch stem extracts from different *V. vinifera* L. varieties. Average contents (μg g^{-1} DW), n = 3 replicates. Samples identification: MB= Malbec; CH= Chardonnay; PN= Pinot Noir; SB= Sauvignon Blanc; PV= Petit Verdot; CS= Cabernet Sauvignon; CF= Cabernet Franc; SY= Syrah.

Tabla 1. PC de extractos de escobajo de diferentes variedades de *V. vinífera* L. Contenidos promedios (μg g^{-1} peso seco), n = 3 réplicas. Identificación de muestras: MB= Malbec; CH= Chardonnay; PN= Pinot Noir; SB= Sauvignon Blanc; PV= Petit Verdot; CS= Cabernet Sauvignon; CF= Cabernet.

	MB	CH	PN	SB	PV
Hydroxybenzoic acids					
Gallic acid	75 ± 2	50 ± 1	58 ± 1	90 ± 1	145 ± 2
Syringic acid	90 ± 1	90 ± 3	89 ± 1	32 ± 1	44 ± 1
Hydroxycinnamic acids					
Caftaric acid	2520 ± 9	2967 ± 52	2196 ± 18	891 ± 38	814 ± 6
Stilbenes					
ε-viniferin	107 ± 1	84 ± 7	612 ± 52	1373 ± 7	326 ± 20
Flavanols					
Procyanidin B1	1987 ± 58	1317 ± 60	1230 ± 23	n.d	n.d.
(+)-catechin	6462 ± 229	4471 ± 46	6204 ± 13	2209 ± 170	1249 ± 15
Procyanidin B2	232 ± 2	217 ± 4	186 ± 1	69 ± 1	57 ± 1
(-)-epicatechin	354 ± 27	100 ± 6	116 ± 3	107 ± 5	23 ± 1
(-)-epigallocatechin gallate	749 ± 28	707 ± 2	701 ± 13	45 ± 2	112 ± 1
(-)-epicatechin gallate	804 ± 40	369 ± 7	735 ± 42	58 ± 3	n.d.
Naringin	18 ± 0.6	10 ± 0.6	19 ± 0.5	19 ± 0.2	n.d.
Flavonols					
Quercetin-3-galactoside	n.d.	1347 ± 20	n.d.	n.d.	16 ± 0.8
Quercetin-3-glucoside	n.d.	57 ± 4	n.d.	19 ± 0.5	10 ± 0.3
Myricetin	71 ± 1	72 ± 1	74 ± 1	n.d	75 ± 1
Astilbin	1347 ± 20	734 ± 4	937 ± 27	n.d.	63 ± 2
Other compounds					
OH-tyrosol	162 ± 5	140 ± 1	102 ± 4	91 ± 2	145 ± 3
Total LMW-PCs	14978	11531	13257	5002	3080

n.d.: not detected.
n.d.: no detectado.

However, our results for (+)-catechin were higher than in other reports, with values ranging from 120 to 1858 μg g^{-1} (1, 14). It is interesting to point out that the (+)-catechin concentrations in grape stem are higher than those described in skins and seeds (33). This compound is very labile under oxidative conditions, which generate protective effect over macromolecules in the presence of free radicals (15).

Recent studies have shown that catechin inhibit the growth of foodborne pathogens (*E. coli* and *Salmonella*) and its potential use as antibiotic substitute (20). Its isomer, (-)-epicatechin, was also found with the maximum level of 354 μg g^{-1} DW, which is in agreement with other studies (14, 21). Nevertheless, our results for (-)-epicatechin were lower than those published by Püssa *et al.* (2006), with values ranging from 600 to 1300 μg g^{-1} DW. The differences amongst the data obtained with those available in the literature confirms the influence of viticultural management and environmental factors in the final composition of by-products. The highest concentration of procyanidin B1 and B2 corresponded again to sample MB (1987 and 232 μg g^{-1} DW, respectively). Similar values of these dimmers were found by other authors (14, 29), which have shown these compounds can significantly contribute to the anti-inflammatory capacity of extracts (15). In this work, traces of naringin were identified in this plant material for the first time. Naringin have protective effects against metabolic diseases, therefore it may be used for the prevention and management of these types of diseases (7).

For the hydroxycinnamic acids family, caftaric acid was the most abundant, with levels ranging between 355 and 2967 μg g^{-1} DW, and the CH sample showed the maximum content. This compound has shown able to inhibit the oxidative damage of free radicals at a biological level, so the exploration of this byproduct as potential source of the compound is important

for future biotechnological applications in food systems (18). Howbeit, Dominguez-Perles *et al.* (2016) reported superior values ranging from 2180 to 19460 μg g^{-1} DW. In all the analyzed samples, gallic and syringic acids were found at quantifiable levels. The first compound was the most abundant of this family, with maximum concentration in sample SY (146 μg g^{-1} DW), followed by syringic acid, in agreement with what was informed by Teixeira *et al.* (2014).

Concerning flavonols content, MB showed as the best variety, with level of 1418 μg g-1 DW (data not shown). Astilbin resulted as the major flavonol, with concentrations between 63 and 1347 μg g^{-1} DW, which is in agreement with reported by Gouvinhas *et al.* (2019). This compound have a many bioactivities, such as antibacterial and AC, being this last similar with commercial antioxidants used in the food industry (33). Myricetin was characterized for the first time in bunch stems, with a maximum level of 82 μg g-1 DW, although traces of their glycosylated derivatives have been previously informed (15). Myricetin have beneficial effects against several human diseases such as cardiovascular pathologies and diabetes mellitus, being also a potent anticarcinogen (5).

The analysis of stilbenes content in bunch stems showed the presence of ε-viniferin, being SB and PN the best sources of this compound. Previous studies of bunch stems showed values ranging from 47 to 5820 μg g^{-1} DW for different cultivars (6, 26), and the data reported in this paper is within this range. The *trans*-resveratrol dimer ε-viniferin has been reported as having beneficial health properties as cancer chemo-preventive (25), showing anti-obesity and anti-inflammatory effects *in vitro* and *in vivo* (22). This fact highlights the relevance of the high concentrations found in this work for some samples and the potentiality for future applications.

OH-tyrosol was found in all the analyzed samples at levels higher than 78 μg g^{-1} DW. Its high antioxidant power and preventive capacity in several pathologies (8), demonstrate its importance as phenolics source with health beneficial effects.

Taking into account the grape varieties from where the bunch stems were obtained, the sample from Malbec stand out from the other cultivars in terms of its high content of most PC, especially of the flavanol family. Malbec is the main cultivar of Argentina (37% of the red grape area), making it source of most by-products with biological and technological interesting applications.

Antioxidant capacity and TPC

Table 2 (page 423), presents the results for the bunch stem extracts. Sample MB showed the maximum levels of TPC both by FC and 280 nm lecture (125 and 85 mg GAE g^{-1} DW, respectively), while SB (62 and 47 mg GAE g^{-1} DW) had the lowest. Melo *et al*. (2015), obtained similar values of TPC (between 51 and 125 mg GAE g^{-1} DW) while Teixeira *et al.* (2014), reported lower values (between 27 and 36 mg GAE g^{-1} DW), although from different cultivars. In our work, the highest levels of PC were found in bunch stem extracts of MB and SY.

ORAC, ABTS and DPPH assays were chosen to determine AC in extracts, since they are based in different reaction mechanisms. ORAC employs hydrogen atom transfer reaction, which resembles actual reactions *in situ*, while ABTS and DPPH assays are based on electron transfer (30). As it is shown in table 2 (page 423), the range of AC by ORAC was between 292 (CS) and 1506 (CH) μmol TE g^{-1} DW. Previous studies reported values of AC ranged from 29 to 1508 μmol TE g^{-1}, but they were obtained from dry stem extracts (4, 28). The samples CS and SB showed the lowest values, while CH and PN had the highest. The AC determined by ABTS and DPPH varied from 504 to 1003 μmol TE g^{-1} DW and from 434 to 1097 μmol TE g^{-1} DW, respectively. Melo *et al.* (2015) found slightly superior values ranging from 805 to 2419 μmol TE g^{-1} DW by ABTS, while DPPH values were in the range of 473 and 1086 μmol TE g^{-1} DW, which are similar to ours.

Because of the potent AC of PC, the correlation coefficient (r) between the TPC and AC of bunch stem extracts were studied. A positive correlation between TPC assays measured by FC and 280 ($p \leq 0.05$, $r \geq 0.89$) was observed. The correlation between the TPC measured by both methods (FC and 280 nm) with ORAC values was lower ($r \leq 0.41$). The results are summarized in table 3 (page 423). This poor correlation may be attributed to specific PC present in extracts with a given AC, the action of other phytochemical compounds, and/or the interaction between them (either synergism or antagonism) (3).

Table 2. Levels of TPC and AC evaluated by ORAC, ABTS and DPPH assays. Results are expressed as mean ± SD, n = 3 replicates. Samples identification as table 1 (page 421).

Tabla 2. Niveles de TPC y AC evaluados por los ensayos ORAC, ABTS y DPPH. Los resultados se expresan como media ± SD, n = 3 réplicas. Identificación de muestras como en tabla 1 (pág. 421).

	MB (mg GAE g^{-1} DW)	CH (mg GAE g^{-1} DW)	PN (μmol TE g^{-1} DW)	SB (μmol TE g^{-1} DW)	PV (μmol TE g^{-1} DW)
MB	125 ± 2	85 ± 0.4	784 ± 24	1003 ± 43	1047 ± 18
CH	96 ± 0.2	62 ± 0.4	1506 ± 69	770 ± 14	822 ± 13
PN	103 ± 1	71 ± 0.5	894 ± 83	858 ± 16	867 ± 30
SB	62 ± 1	47 ± 1	340 ± 15	504 ± 24	434 ± 17
PV	73 ± 0.2	64 ± 0.3	344 ± 10	677 ± 14	617 ± 25
CS	68 ± 1	57 ± 0.3	292 ± 15	556 ± 33	562 ± 6
CF	101 ± 3	82 ± 3	625 ± 17	837 ± 18	888 ± 60
SY	111 ± 6	85 ± 3	605 ± 8	918 ± 19	1097 ± 77

Table 3. Pearson's correlation coefficients between the TPC and the AC in bunch stem extracts (n = 9).

Tabla 3. Coeficientes de correlación de Pearson's entre el TPC y la AC en los extractos de escobajo (n=9).

	TPC FC	TPC 280	ORAC	ABTS	DPPH
TPC FC	1	0.891*	0.409	0.985*	0.964*
TPC 280		1	0.066	0.921*	0.924*
ORAC			1	0.380	0.380
ABTS				1	0.970*
DPPH					1

*, correlation is significant at p ≤ 0.05.

*, la correlación es significativa a p ≤ 0,05.

Nevertheless, the correlation between both TPC and the other AC assays (ABTS and DPPH) were high (r ≥ 0.92, table 3, page 423). Our study also showed that values found for ABTS and DPPH (r ≥ 0.97) methods are well correlated, but there is no good correlation with the results of ORAC (r ≥ 0.38).

Villaño *et al.* (2005) observed a similar behavior in their report. This study intends to determine the main individual PC responsible of the AC (table 4, page 424). The antioxidant effectiveness of these compounds is proportional to the number of -OH groups present in the aromatic ring(s). At the same time, the mechanism of reaction and availability of antioxidant groups related to the medium-solvent, as well as pH could affect the AC (23). The results showed that caftaric acid had a high correlation with AC measured by ORAC, following procyanidin B2 and (-)-epigallocatechin gallate (r=0.86, r=0.84 and r=0.83). The high AC of caftaric acid could be explained by the presence of catechol (1,2-dihidroxybenzene) group and side chain conjugated double bonds that allow for delocalisation of the electrons improving stabilize the phenoxy radical formed. Therefore, cinnamic acids are better antioxidants than benzoic acids due to the presence of the -CH=CH-COOH group which enhances the AC more than the -COOH group. In the case of (-)-epigallocatechin gallate respect to procyanidin B2, its lowest correlation could be explained by glycosylation of 3-OH group that reduce the antioxidant properties of compounds (23). In contrast, the results showed that astilbin and syringic acid had strong correlation (r = 0.84 and r = 0.78, respectively) with AC measured by ABTS and DPPH. In both methods, there are evidence of formation of covalent adducts between the phenolic compounds and cation radical (ABTS or DPPH). Thus, the new compounds exhibit antioxidant properties, which not reflect the antioxidant activity of compound itself, but the sum of antioxidant activities, which have a parent compound and a product of its reaction with the cation radical. This makes prediction of actual antioxidant properties according to chemical structure were complicated (32). However, the antioxidant efficiency of astilbin could be given by catechol group, -OH groups and glycosylation of 3-OH group, making it more effective than its aglicons.

Table 4. Correlation coefficient (r) values of PC concentration *versus* ORAC, ABTS and DPPH values of bunch stem extracts.

Tabla 4. Valores del coeficiente de correlación (r) de la concentración de PC frente a los valores de ORAC, ABTS y DPPH de los extractos de escobajo.

	ORAC	ABTS	DPPH
Gallic acid	-0.668 *	-0.023	0.035
Syringic acid	0.820 *	0.820 *	0.785 *
Caftaric acid	0.865 *	0.535	0.460
ε-viniferin	-0.402	-0.384	-0.408
Procyanidin B1	0.708 *	0.732 *	0.656
(+)-catechin	0.588	0.793 *	0.775 *
Procyanidin B2	0.839 *	0.664	0.604
(-)-epicatechin	0.124	0.737 *	0.732 *
(-)-epigallocatechin gallate	0.826 *	0.792 *	0.758 *
(-)-epicatechin gallate	0.333	0.542	0.543
Astilbin	0.552	0.840 *	0.742 *
Naringin	0.211	0.231	0.112
Quercetin-3-galactoside	0.493	0.235	0.312
Quercetin-3-glucoside	0.730 *	-0.066	0.031
Myricetin	0.281	0.588	0.623
OH-tyrosol	0.469	0.541	0.416
Total LMW-PCs	0.685 *	0.768 *	0.718 *

*, correlation is significant at p ≤ 0.05

*, la correlación es significativa a p ≤ 0,05

On the other hand, the AC of syringic acid was favored by two methoxy substitutions at the *ortho* position relative to the hydroxyl (23). Apostolou *et al.* (2013) reported that polyphenols (-)-epicatechin and *trans*-caftaric acid were significantly correlated with IC50 values of dried stem extract measured by ABTS.

However, it is not easy to compare the AC data of bunch stem extracts with those reported in other studies, especially because of the differences in the methods and standard units used, type of cultivar and climate conditions, among others (1, 32).

The results reported in the present work indicate that bunch stem extracts constitute a rich source of bioactive PC together with other different biological compounds previously reported, like cellulose, xyloglucans and lignins (15). In addition, the extracts showed great AC, which make them potential alternatives for economic exploitation and utilization in the cosmetic, food and pharmaceutical industries as antioxidant source for several technological purposes (15).

Conclusions

Contents of PC in bunch stem extracts obtained from different grape varieties cultivated in Argentina and their correlation with AC are presented. The results showed that bunch stem extracts from *V. vinifera* species constitutes a useful source of bioactive flavanol and hydroxycinnamic acid derivatives, with high AC. One important aspect to consider from this work, is the report, for the first time, of naringin, myricetin and OH-tyrosol. Regarding grape varieties, Malbec was highlighted from the other cultivars for its high PC content. In turn, the information generated here allow for explain the antioxidant properties of extracts and to establish possible synergic effects between compounds. In this sense, bunch stems can be considered an inexpensive and sustainable potential source of bioactive compounds of high value for future applications as functional ingredients.

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ECo-friendly postharvest protection: *Larrea cuneifolia*-nades extract against *Botrytis cinerea*

Control sustentable poscosecha: extractos de *Larrea cuneifolia* mediados por nades frente a *Botrytis cinerea*

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Abstract

Botrytis cinerea is a ubiquitous fungus causing gray mold, the main postharvest disease in fruit, which implies important economic losses in agriculture. With growing concern over health and environmental effects of pesticides, the search for eco-friendly alternatives is a clear priority. Plant extracts represent a rich source of biocompounds with attractive antimicrobial properties. In the last decade, Natural Deep Eutectic Solvents (NADES) has emerged as an auspicious green extraction media to achieve bioextract for a sustainable postharvest control. In the present study, a novel *L. cuneifolia* NADES-based bioextract was evaluated against *B. cinerea*. To this purpose, a NADES composed by lactic acid, glucose and water (LGH) was used as extracting agent and compared with traditional solvents in terms of antioxidant capacity and total phenolic content. Furthermore, the bioextract antifungal activity was tested *in vitro* and also *in vivo* on artificially inoculated grapes, in order to obtain preliminary data about the efficacy on gray mold development. The antimicrobial activity of the bioextract was assessed using agar diffusion method against *B. cinerea*, inhibition of 92% was achieved with the bioextract at 2%. Notably, *L. cuneifolia* bioextract showed an excellent performance for gray mold control on grapes, supporting their potential as alternative green fungicide.

Keywords
natural deep eutectic solvents • biocompounds • antimicrobial activity • medicinal plant • postharvest control

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Resumen

Botrytis cinerea es un hongo ubicuo que ocasiona la podredumbre gris, una de las principales enfermedades de fruta en poscosecha, siendo responsable de pérdidas económicas. Debido a la creciente preocupación por los efectos adversos del uso de pesticidas, la búsqueda de alternativas se presenta como una meta prioritaria. En este contexto, los extractos de plantas representan una rica fuente de biocompuestos con atractivas propiedades antimicrobianas. Recientemente, los solventes eutécticos naturales (NADES) se han propuesto como agentes extractantes sustentables de compuestos bioactivos a partir de plantas. En el presente estudio, se evaluó un bioextracto de *L. cuneifolia* basado en NADES hacia *B. cinerea*. Para este propósito, se usó un NADES compuesto por ácido láctico, glucosa y agua (LGH) como agente de extracción y se comparó con solventes tradicionales en términos de capacidad antioxidante y contenido fenólico total. Además, la actividad antimicrobiana del bioextracto se evaluó *in vitro* e *in vivo* en uvas inoculadas artificialmente. A una concentración del 2% el bioextracto fue capaz de inhibir el crecimiento miceliar de *B. cinerea* en un 92%. Interesantemente, *L. cuneifolia* mostró un excelente rendimiento para el control de la podredumbre gris en uvas, demostrando su potencial como alternativa sustentable a los fungicidas sintéticos.

Palabras claves

solventes eutécticos naturales • biocompuestos • actividad antimicrobiana • plantas medicinales • control poscosecha

Introduction

Botrytis cinerea (Pers. ex. Fr) is a ubiquitous fungus with a wide host range including vegetables, ornamental plants and fruits. This pathogen causes gray mold, the main postharvest disease in fruit, leading to important economic losses in agriculture (1, 2, 24). The chemical control of *B. cinerea* has been encumbered by the emergence of resistant strains. Moreover, the synthetic pesticides present high toxicity and low biodegradability. One of the greatest challenges that agriculture faces is the need of safer approaches for sustainable crop protection. In this sense, plant extracts with fungistatic or fungicidal activities have shown potential as effective alternatives for the control of several postharvest crop diseases (14, 16, 20, 38).

Ethnobotanical studies support the use of several Argentinean autochthonous plants for antimicrobial purposes. Among these, the genus *Larrea* (Zygophyllaceae) is one of the most notable (3), being *L. ameghinoi, L. cuneifolia*, *L. divaricata,* and *L. nitida* the four species found in this country (32). *L. cuneifolia* extracts have been used as anti-inflammatory, antirheumatic, dysphoretic, amenagogic, antimicrobial and antioxidant agents (36). These properties have been attributed to the presence of bioactive compounds, being the phenolic compounds one of the most relevant group (21). Interestingly, certain classes of phenolics, such as hydroxybenzoic and hydroxycinnamic acid derivatives, flavonoids, and tannins have been explored for a long time as postharvest alternative control (22).

Extraction of plant phenolic compounds is traditionally performed with solvents such as methanol, ethanol, hexane, chloroform and diethyl ether and water (5, 11, 34). Even though these extracts are obtained from natural sources, their preparation using toxic organic solvents has many disadvantages for human health and for the environment. In this sense, the development of eco-friendly solvents is identified as a clear priority to achieve a sustainable extraction processes (28).

In the last decade, a new generation of solvents, called Natural Deep Eutectic Solvents (NADES), has been proposed as promising green extraction media (8, 17). NADES are mixtures consisting of natural metabolites that are naturally present in all types of cells and organisms such as sugars (glucose, sucrose, fructose, etc.); organic acids (lactic, malic, citric acids, etc.); urea and choline chloride (6, 9). NADES offer outstanding advantages including biodegradability, low toxicity, solute stabilization, sustainability and low cost (12, 27). It has to be pointed out that NADES are considered food grade solvents.

In the present study, a novel *L. cuneifolia* NADES-based bioextract was evaluated against *B. cinerea*. To this purpose, a NADES composed by lactic acid, glucose and water (LGH) was used as extracting agent and compared with traditional solvents in terms of antioxidant capacities and total phenolic contents. Furthermore, the bioextract antifungal activity was tested *in vitro* and also *in vivo* on artificially inoculated grapes, in order to obtain preliminary data about the efficacy on gray mold development.

Materials and methods

Chemicals and equipments

Compounds for LGH preparation including glucose anhydrous (≥ 99%), L (+) lactic acid (85-90%) were purchased from Biopack. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA) and Methanol (MeOH) was purchased from Baker (USA). 2,2'-azinobis(3-ethylbenzothiaziline-6- sulfonic acid (ABTS), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent and gallic acid 99% (Gal) were obtained from Sigma Aldrich (St. Louis, MO,USA). Sodium carbonate anhydrous (Na^2CO^3) and Potato Dextrose Agar media (PDA) were obtained from Biopack. Sulfur dioxide generating pads as a commercial fungicide postharvest were purchased from PROPEL (Mendoza, Argentina).

Ultrasound Cleanson, Argentina, 200 W output power, 20 kHz frequency; Centrifuge Presvac DCS-16-RV and a Spectrophotometer Spectrum SP 2000 UV were used for extraction and determinations.

NADES preparation

LGH was prepared using a method previously described by Dai *et al.* (2013). The two-component mixture (lactic acid and dextrose; 5:1) with 15% of H_2O (v/v) was placed in a 20 mL amber glass vial. After, the mixture was heated in a magnetic stirrer with temperature control (Fisatom model 752A, Brasil) at 40°C for 60 min.

Plant material and extract preparation

L. cuneifolia plants were cultivated at a greenhouse and were identified by means of morphological, anatomical, and histochemical analyses. Leaves were harvested during flowering period and immediately frozen in liquid nitrogen, then lyophilized in darkness. Before the extraction, lyophilized material was grounded up to a fine powder with liquid nitrogen.

Extraction was performed according to Espino *et al.* (2018). Lyophilized plant material and extraction solvent (LGH, methanol or water) were placed in a 15 mL centrifuge tube (ratio plant- solvent of 75 mg mL^{-1}), homogenized by a vortex during 15 s and processed by ultrasound during 42 min at 40°C (±2°C). Then, the system was centrifuged for 30 min and the supernatant was filtered (0.45 µm). The extraction was performed in triplicate.

Total phenolic content

Total polyphenols were determined using Folin-Ciocalteu (FC) method described by Singleton and Rossi (1965) with modifications. For this determination, dilutions of the extracts were assessed at 5 % with LGH, MeOH or water. In a test tube, 50 µL of each extract dilution previously obtained, were mixed with the Folin-Ciocalteu reagent (200 µL) and, after 5 min, with an aqueous solution of Na_2CO_3 (1250 µL, 5 % w/v). Then, ultra-pure water was added to a final volume of 5000 µL. The mixture was incubated for 60 min in the dark, at room temperature, and the total phenol content was determined absortiometrically at 750 nm. Gallic acid calibration curve was prepared in the concentration range of 0-1000 µg mL^{-1} (R^2 = 0.9904) and results were expressed in µg of gallic acid per mL of extract. Each determination was performed in triplicate.

Antioxidant activity

DPPH (2,2´-diphenyl-1-picrylhydrazyl) assay*

The radical scavenging activity was measured in the extracts following the methodology described by Nuutila *et al.* (2003). The discoloration of the stable radical, 2,2'-diphenyl-1-picrylhydrazyl was tested. For this determination, dilutions of the extracts were

assessed at 2.5 % with LGH, MeOH or water. Then, 3.5 mL of DPPH* methanolic solution (0.045 mg mL^{-1}) was rapidly mixed with 250 µL of each extract dilution. After 5 min, the absorbance was measured at 515 nm (A_E). The decline in DPPH* concentration indicated the radical scavenging activity of the plant extracts. The initial absorbance of DPPH* solution was 1.375 (A_{DPPH}). The experiment was carried out in triplicate. Gallic acid solution (1000 µg mL^{-1}) was used as a reference (A_{REF}) and radical scavenging activity of the extracts were calculated as inhibition percentage (I %) as follows (Eq. 1) :

$$\%I_{DPPH*} = \frac{A_{DPPH*} - A_E}{A_{DPPH*} - A_{REF}} \quad (1)$$

ABTS (2,2´-azinobis(3-ethylbenzothiaziline-6- sulfonic acid) assay

Antioxidant activity was also measured following the method proposed by Re *et al.* (1999), using 2,2'-azinobis(3-ethylbenzothia-zoline-6-sulfonic acid) diammonium salt (ABTS). An ABTS ethanolic solution (2 mM) was added with potassium persulphate solution (2.45 mM) in order to produce radical cations (ABTS*). After 16 hours in dark, ABTS* solution was diluted with ethanol, to an absorbance of 0.70 (±0.02) at 734 nm (AABTS*). For this determination, dilutions of the extracts were assessed at 2.5% with LGH, MeOH or water. Each extract dilution (80 µL) was mixed with ABTS* ethanolic solution (3920 µL) and after 7 min the absorbance was measured (A_E). All the determinations were carried out three times and the absorbance sample was considered. A gallic acid solution (1000 µg mL^{-1}) was used as reference (A_{REF}) and results were calculated according to the following formula (Eq. 2):

$$\%I_{ABTS*} = \frac{A_{ABTS*} - A_E}{A_{ABTS*} - A_{REF}} \quad (2)$$

Antimicrobial activity

Microorganisms

The isolates of *B. cinerea* were obtained from the microorganism's collection of the Cátedra de Fitopatología (Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina).

Antimicrobial activity of L. cuneifolia bioextract by solid agar assay

L. cuneifolia bioextract was filtered (0.2 µm) and added to sterile Potato Dextrose Agar (PDA) at different concentrations (0.05, 0.1, 0.25, 0.5, 1, 1.5, 2 % (v/v)) in Petri dishes (5.2 cm in diameter). A pathogen agar disk (diameter 4 mm), removed from an actively growing culture, was placed in the centre of each plate. A solvent control for the seven extract dilutions was included to confirm that LGH did not present antifungal effect. Three replicate plates for each concentration as well as control were prepared. The Petri plates were kept at 25 ± 2 °C for 4 days. After the incubation period the test was considered concluded. In order to evaluate the mycelial growth inhibition; the mean colony area was determined. These mean growth values were calculated as the inhibition percentage of mycelial growth related to the control treatment according to the following equation (Eq. 3):

$$\% \text{ mycelial growth inhibition} = ((c-t)100)/c \quad (3)$$

where:

c = control mean colony area

t = is treated mean colony area

Antimicrobial activity of L. cuneifolia bioextract in commercial grapes

Experiments were conducted with commercial grapes *cv* Red globe following the procedure proposed by Boiteux *et al.* (2015).

Fruits free from injuries and infections were selected. Grape bunch with similar shape and size, containing each of them between 10-13 grapes were selected. *B. cinerea* was cultured on PDA petri plates for three weeks at 25°C. Then, the spore suspension was prepared in sterile water at a concentration of 1×10^6 conidia mL^{-1}. In order to study the protective and curative activity of bioextract at 2 and 10% (v/v), grapes skin were sprayed with 3 mL of the bioextract 1 day before or after the pathogen inoculation. Two controls were performed, one using sterile water and the other with a postharvest commercial fungicide (SO_2 generating pads). Grapes were put in closed plastic boxes to maintain a relative humidity of approximately 90 % and incubated for 7 days at 22°C. The experiment was performed in triplicate. The efficacy of the bioextract was calculated according to the following formula (Eq. 4):

$$\% \text{ effectiveness} = ((C-T)/C) * 100 \quad (4)$$

where:

C= (number grapes affected with gray mould/number total grapes inoculated for control with commercial fungicide)*100

T= (number grapes affected with grey mould/number total grapes inoculated for each treatment) *100

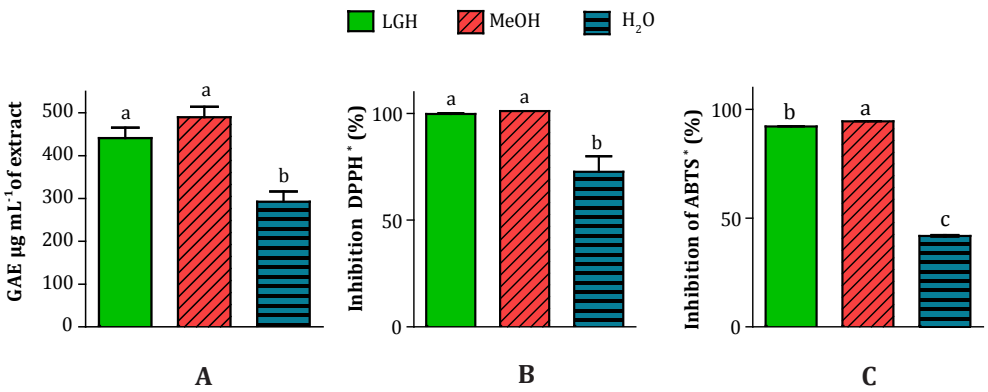
Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA), and means were compared using Tukey test. All the analyses were done in triplicate. The results were significant at $p < 0.05$ unless specified otherwise. Statistical analyses were carried out using Statgraphics Centurion XVI.II and GraphPad Prism 5.0 Software.

Results and discussion

Total phenolic content and antioxidant capacity of extracts

Folin-Ciocalteau assay is widely applied to estimate the total phenol content (TPC) in plant extracts. Thus, this technique was used to compare the TPC in the methanolic, aqueous and LGH extracts of *L. cuneifolia*. As can be seen in figure 1, water extract showed the lowest TPC for all the solvents under study. Interestingly, LGH bioextract presented a satisfactory performance when compared with methanolic extract.



Different letters represent significant differences.

Letras distintas indican diferencias significativas.

Figure 1. Total phenolic content (**A**) and antioxidant capacity determined by DDPH* (**B**) and ABTS* (**C**) methods of *L. cuneifolia* extracts obtained with different solvents (MeOH, H_2O and LGH).

Figura 1. Contenido de polifenoles totales (**A**) y capacidad antioxidante determinada mediante los métodos de DDPH* (**B**) y ABTS* (**C**) de los extractos de *L. cuneifolia* obtenidos con diferentes solventes (MeOH, H_2O and LGH).

Many studies have shown that plants rich in phenolic compounds also exhibit potent antioxidant activity. In general, the methods for determining the antioxidant capacity of plant extracts can deactivate radicals by two major mechanisms: assays based on the single electron transfer (SET) reaction and assays based on a hydrogen atom transfer (HAT). The DPPH test is SET-based method and ABTS used both HAT and SET mechanisms (18). Thus, in this work both assays were used to evaluate antioxidant activity of *L. cuneifolia* extracts (figure 1, page 431). The results demonstrated that for the two methods studied, LGH bioextract showed similar antioxidant activity than methanolic extract, whereas water extract presented the lowest activities.

According to our results, LGH reveals a great potential as green extraction media to obtain bioextracts rich in bioactive compounds in comparison with traditional solvents. Previous studies demonstrated that this green solvent has outstanding extractability for both polar and weak polar phenolic compounds compared to conventional solvents (15). We have developed in our lab a HPLC-DAD methodology for the determination of phenolic compounds in *Larrea* (13). The results of sample analysis validated the TPC values reported in the present study.

Recently NADES have been introduced as environmentally benign solvents for the bioextract preparation with antimicrobial properties (30). Therefore, LGH-bioextract was selected for evaluating the biological activity against *Botrytis cinerea.*

Antifungal activity of *L. cuneifolia* bioextract by solid agar bioassay

In order to evaluate the antifungal activity of *L. cuneifolia* bioextract, different concentrations (0.05, 0.1, 0.25, 0.5, 1, 1.5 and 2% (v/v)) on the mycelial growth of *B. cinerea* were tested (photo 1).

All the bioextract concentrations were able to inhibit the growth of *B. cinerea* in different percentages (figure 2, page 433). As can be seen, pathogen inhibition was observed even at low concentrations. The IC_{50} (concentration of the extract that inhibited 50 % the pathogen growth) was 0.67 % (0.5 g L^{-1}). At the maximum concentration tested (1.5 g L^{-1}), a 92 % of *B. cinerea* inhibition was achieved.

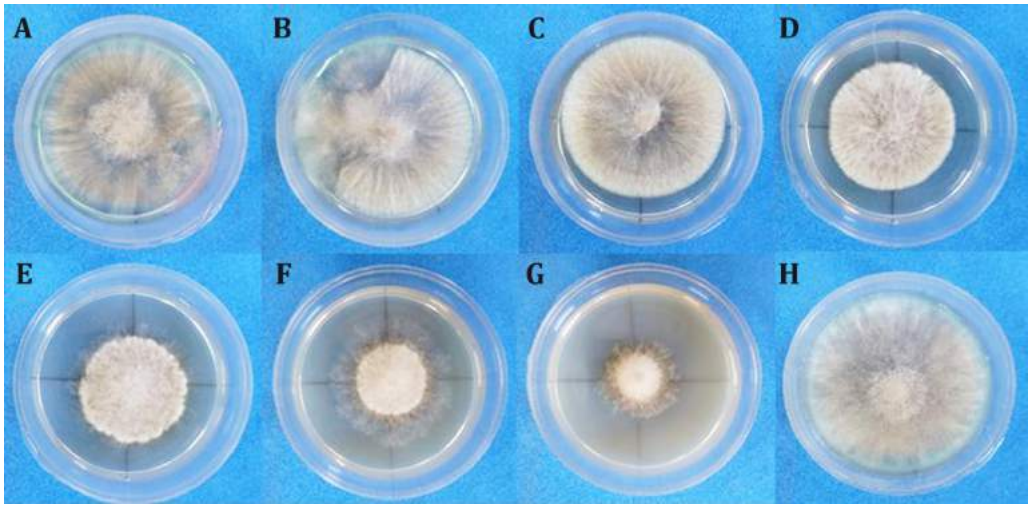


Photo 1. Mycelial growth of *B. cinerea* at different concentration of *L. cuneifolia* bioextract **A:** 0.05%; **B:** 0.1%; **C:** 0.25%; **D:** 0.5%; **E:** 1 %; **F:** 1.5%; **G:** 2% and **H:** control.

Foto 1. Crecimiento miceliar de *B. cinerea* a diferentes concentraciones del bioextracto de *L. cuneifolia* **A:** 0,05%; **B:** 0,1%; **C:** 0,25%; **D:** 0,5%; **E:** 1%; **F:** 1,5%; **G:** 2% y **H:** control.

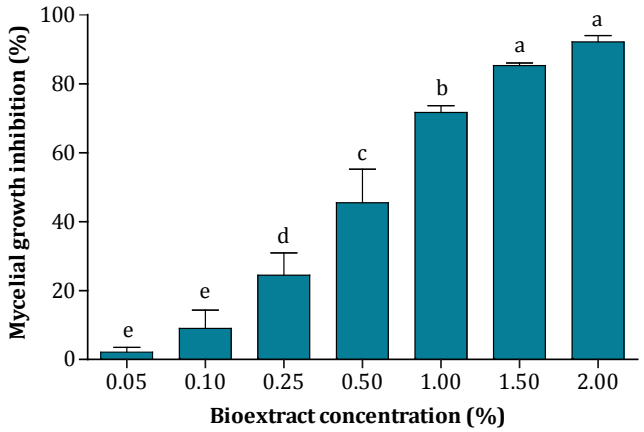


Figure 2. Percentage of *B. cinerea* mycelial growth inhibition at different concentrations of *L. cuneifolia* bioextract.

Figura 2. Porcentaje de inhibición del crecimiento miceliar de *B. cinerea* a diferentes concentraciones del bioextracto de *L. cuneifolia.*

Our results highlight that NADES-*Larrea cuneifolia* extracts shows outstanding activity against *B. cinerea*. Vast scientific knowledge supports the applications of the genus *Larrea* in antimicrobial assays. Alcoholic extracts of *L. divaricata* and *L. cuneifolia* showed considerably activity against filamentous fungi (*Lenzites elegans*, *Schizophyllum commune*, *Pycnoporus sanguineus*, *Ganoderma applanatum*, *Fusarium oxysporum*, *Penicillium notatum, Aspergillus niger* and *Trichoderma spp*) (29). Zampini *et al*. (2007) demonstrated the activity of *Larrea* ethanolic extracts against antibiotic-resistant bacteria.

Antimicrobial activity of our bioextract against *B. cinerea* mycelial growth was compared with plant extracts previously reported (table 1). NADES extract exhibits a much better pathogen inhibition efficiency than organic solvents extracts (7, 35). With regard to aqueous extracts, higher concentrations were required to achieve a similar *B. cinerea* inhibition to that obtained with the LGH extract (23). This could be explained by the great capacity of NADES to solubilize and stabilize bioactive compounds (10, 25, 37).

Table 1. Antimicrobial activity of plant extracts with different solvents against *B. cinerea*.

Tabla 1. Actividad antimicrobiana de extractos de plantas obtenidos con diferentes solventes hacia *B. cinerea*.

Plant material	Solvent	Concentration	Mycelial growth inhibition	References
Larrea cuneifolia	LGH	1.5 g L^{-1} (2 % v/v)	92%	**Present study**
Fluorensia cernua	water	4 g L^{-1}	66%	(11)
Zanthoxylum rhoifolium	chloroform/ methanol	1 g L^{-1}	70%	(5)
Lippia origanoides	ethanol	0.5 g L^{-1}	44%	(34)
Thymus vulgaris	ethanol	0.5 g L^{-1}	37%	(34)
Calendula officinalis	ethanol cold water hot water	2.26 % (v/v) 3.23 % (v/v) 10 % (v/v)	≈40% 100% ≈75%	(23)
Dolichos kilimandscharicus	methanol	1 g L^{-1}	60-80%	(35)
Phytolacca dodecandra	methanol	1 g L^{-1}	40-50%	(35)
Maerua subcordata	methanol	1 g L^{-1}	30-40%	(35)
Ottonia martiana	ethanol	1 g L^{-1}	69%	(7)

NADES are recently introduced as environmentally benign solvents for the bioextract preparation with antimicrobial properties. Rajan *et al.* (2015) studied the antibacterial activity of the extract of ginger rhizome (*Zingiber officinale* Roscoe), prepared with different Natural Deep Eutectic Solvents. NADES extracts exhibited prominent antimicrobial activity against *Staphylococcus aureus*, *Streptococcus viridans, Salmonella typhi, Bacillus cereus, Klebseilla pneumonia, Vibreo cholera* and *Escherichia coli* using paper disc diffusion methods.

In order to assess the efficacy of *L. cuneifolia* bioextract on gray mold development, its antimicrobial activity was evaluated in commercial grapes.

Antimicrobial activity of *L. cuneifolia* bioextract in commercial grapes

The protective and curative activity of different concentrations of *L. cuneifolia* bioextract against *B. cinerea* were tested in grapes *cv* Red Globe (photo 2). Regarding that the extract at 2 % achieved the highest inhibition of the pathogen mycelial growth for in vitro assays, this concentration and 10% were chosen for in vivo tests.

Analyzing the obtained results (figure 3, page 435), a similar trend was observed on curative and protective application. Even though data showed no significant differences between the *L. cuneifolia* extracts at 10% and 2%. When comparing the two treatments, the protective assay presented the greatest effect against *B. cinerea*, showing an efficacy between 70 and 80 % in relation with chemical control. It has to be pointed out that the 2% (1.5 g L^{-1}) bioextract not only showed a high *B. cinerea* inhibition *in vitro*, but also an important effect for disease control in grapes.

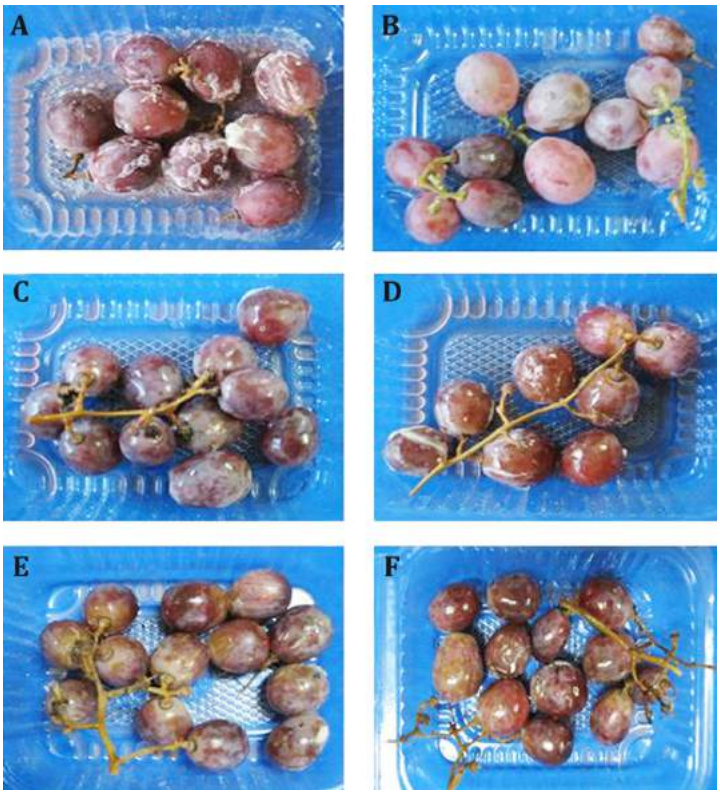
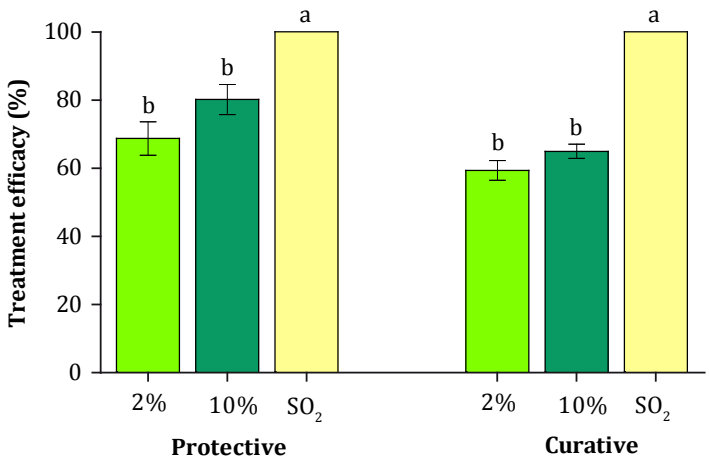


Photo 2. *In vivo* antimicrobial assay in *B. cinerea*-inoculated grapes: (**A**) sterile water, (**B**) postharvest commercial fungicide (SO_2 generator), (**C**) curative 2%, (**D**) curative 10%, (**E**) protective 2%, (**F**) protective 10%.

Foto 2. Racimos *cv* Red Globe inoculadas artificialmente con *B. cinerea* sometidos a diferentes tratamientos: (**A**) agua estéril, (**B**) fungicida comercial (generador de SO_2), (**C**) tratamiento curativo 2%, (**D**) tratamiento curativo 10%, (**E**) tratamiento preventivo 2%, (**F**) tratamiento preventivo 10%.



Different letters indicate significant differences.

Letras distintas indican diferencias significativas.

Figure 3. *In vivo* antimicrobial efficacy (%) of *L. cuneifolia* bioextract (2 and 10%) in protective and curative test over *B. cinerea*-inoculated grapes after 7 days at room conditions.

Figura3. Porcentaje de eficiencia del bioextracto de *L. cuneifolia* (2 and 10%) para el control de *B. cinerea* en racimos de uva inoculados artificialmente luego de 7 días de incubación.

Previous reports carried out on table grapes had demonstrated that plant extracts have remarkable potential as biopesticides; Kanetis *et al.* (2017) demonstrated that the acetonic extract of *Salvia fruticosa* was effective for the control of *B. cinerea* on this fruit. Also, the extracts of *Borago officinalis, Orobanche crenata, Plantago coronopus, P. lanceolata, Sanguisorba minor, Silene vulgaris, Sonchus asper, Sonchus oleraceus,* and *Taraxacum officinale* induced a significant reduction of grey mould disease (22).

Conclusions

This work highlights the ability of NADES as solubilisation vehicles for plant derived postharvest protection agents. LGH reveals a great potential as green extraction media to obtain bioextracts rich in total phenolic content and similar antioxidant activity when compared with traditional solvents. The bioextract obtained presented an effective antimicrobial activity against *Botrytis cinerea*. Notably, *L. cuneifolia* bioextract on grapes showed an excellent performance for gray mold control in protective assay, supporting their potential as alternative green fungicide. Further researches are needed for the applicability of this bioextract in commercial processes.

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Effect of irrigation and soil texture on grape phylloxera (*Daktulosphaira vitifoliae* Fitch) population and grapevine damage

Efecto del riego y textura de suelo sobre la población de la filoxera de la vid (*Daktulosphaira vitifoliae* Fitch) y su daño en plantas

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Abstract

It is believed that phylloxera grows better in clay soils and/or under drip irrigation than in sandy soils and/or flooding. To test these hypotheses, phylloxera damage and population growth were evaluated in potted *V. vinifera* cv. Malbec under two irrigation methods and soil textures in growth chambers at 16 h of photoperiod and 28°C ± 3°C. In a first experiment, phylloxera damage and population were analyzed in infested (*P*) and uninfested (*C*) plants, drip (*D*) or flood (*F*) irrigated. A second experiment consisted in infested (*P*) and uninfested (*C*) plants in clay (*CL*) or sandy (*S*) soil. *D* x *P* reduced leaf number, while *P* x *C* increased photosynthesis rate. In the irrigation experiment, *P* reduced leaf area, shoot length and root dry weight and increased stomatal conductance. Irrigation methods did not influence variables related to root damage or phylloxera population. In the texture experiment *CL* x *C* showed a greater leaf area. *P* also reduced shoot length and root dry weight while *CL* had a higher number of leaves and less root dry weight. Despite *CL* developed more phylloxera root symptoms, texture did not affect the number of insects found on roots. Possibly, neither irrigation methods nor soil texture *per se* are limiting factors for phylloxera performance, but their influence on the vigor of the plants could affect the plant-insect interactions. This is one of the first reports about the influence of soil conditions on phylloxera in a controlled environment and provides a foundation for further studies.

Keywords
phylloxera • *Daktulosphaira vitifoliae* • irrigation • soil texture • *Vitis vinifera*

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Resumen

Durante años, la afirmación sobre que la filoxera crece mejor en suelos arcillosos y/o bajo riego por goteo, que en suelos arenosos y/o riego superficial, se ha considerado cierta. En este experimento se evaluó el daño y la población de filoxera en *V. vinifera* cv. Malbec, en macetas bajo dos métodos de riego y texturas del suelo, en cámaras de crecimiento con 16 h de luz a 28°C ± 3°C. En un primer experimento, los tratamientos consistieron en plantas infestadas (*P*) y no infestadas (*C*), regadas por goteo (*D*) o riego superficial (*F*). Otro experimento consistió en plantas infestadas (*P*) y no infestadas (*C*) en suelo arcilloso (*CL*) o arenoso (*S*). *D* x *P* disminuyó el número de hojas, mientras que *F* x *C* mostró una mayor tasa de fotosíntesis. *P* redujo el área foliar, longitud del brote y peso seco de la raíz y aumentó la conductancia estomática. Los métodos de riego no influyeron en las variables relacionadas con el daño de la raíz o la población de filoxera. En el segundo experimento *CL* x *C* mostró una mayor área foliar. *P* también redujo la longitud del brote y el peso seco de la raíz mientras que *CL* mostró más hojas y menos peso seco de la raíz. A pesar de que *CL* desarrolló más síntomas de filoxera en la raíz, la textura no afectó la cantidad de insectos encontrados en las mismas. Posiblemente, ni el método de riego ni la textura del suelo *per se* sean factores limitantes para la filoxera, pero su influencia sobre el vigor de las plantas podría afectar las interacciones planta-insecto. Este es uno de los pocos estudios en macetas y condiciones controladas que estudia la influencia de los factores de campo sobre la filoxera proporcionando las bases para futuros estudios.

Palabras clave
filoxera • *Daktulosphaira vitifoliae* • riego • textura de suelo • *Vitis vinifera*

Introduction

Grape phylloxera (*Daktulosphaira vitifoliae* Fitch; Hemiptera; Sternorrhyncha; Phylloxeridae) is an obligated biotrophic monophagus insect which feeds on *Vitis* spp. producing leaf galls (mainly in native American *Vitis* spp.) and root nodosities and tuberosities (mainly in non-American *Vitis* spp.). Native to North America, the pest coevolved with American *Vitis* spp. and the severe damage apparently only occurs under cultivated situations (17). Grape phylloxera (from now on: phylloxera), as other root herbivores, lives in constant physical contact with the surrounding soil; therefore variables such as climate, vegetation and cultural practices that affect the spatial-temporal dynamics of the 3 phases that make up the soil (solids, water and gas) (34), also influence phylloxera's behavior. Soils are a particular heterogeneous environment and the range of abiotic conditions that influence phylloxera could be both diverse and complex (4). Some of these factors could be manipulated to control phylloxera; however, information regarding these interactions is scarce and poorly understood.

Soil texture and structure affect root herbivore insects' physiology by modeling other abiotic attributes (4). For instance, water retention of fine-textured soils (6) makes these soils less prone to desiccation, increasing survivorship of several insect species (4) while coarse-textured soils normally are associated to negative impacts on root herbivore physiology. One example of the latter is the survivorship decrease of *Diabrotica undecimpunctata* (Coleoptera) larvae in sandy soils, which could be caused by the abrasiveness of the sand particles on their cuticle (33). In the case of phylloxera, soil texture could affect survivorship and dispersion; however, this phenomenon has not been widely studied. Chitkowski and Fisher (2005) suggested that the information that has led to believe that some type of soils, especially sandy soils and soilless agricultural substrates, do not admit *D. vitifoliae* populations is anecdotic. Probably the most cited literature regarding this topic is based on researches by Nougaret and Lapham (1928) and Davidson and Nougaret (1921). In these articles authors speculated that sandy soils would constrain phylloxera movement through the soil due to the lack of cracks, resulting in the immunity of vines growing in sandy soils. Also, de Klerk (1974) found a relationship between high levels of phylloxera infestation in heavy-textured soils and low levels of infestation in lighter textured soils in South African vineyards. Furthermore, in Australian vineyards Powell *et al*. (2003) also

found a relationship between soil type and levels of infestation, although they could not attribute the observed differences to texture. In contrast, Buchanan (1987) could not find this association in other Australian vineyards, and Chitkowski y Fisher (2005) found that the insect was able to develop in 6 different types of soils and a soilless mix. Similarly, a link between soil texture and phylloxera infestation levels has not been established in Argentinean vineyards (3).

Water is another crucial factor influencing soil dynamics and, in this context, winter flooding has been used as a first attempt to eradicate phylloxera due to its hypothetical drowning and dragging effect. However, laboratory essays have shown that phylloxera can survive up to 21 days submerged under water (29) and a 24 h stream of water, applied directly upon phylloxera has been ineffective to remove it from the roots (10). Inundation could cause an indirect benefit by promoting an increase in the vine's vigor, but it is only possible to use it in non-permeable soils, it incurs in high economical costs, and it requires access to great amounts of water and additional fertilization to replace the soluble nutrients lixiviated out of the soil profiles (28). Moreover, the efficacy of such practice could depend on the phylloxera strain virulence and other biotic and abiotic factors.

In Argentina, phylloxera is present in most viticultural regions (13). For years, it has been commonly stated that since local vineyards are mainly flood irrigated and soils are mostly sandy or loam-sandy, phylloxera do not affect the vines to the point of big economical losses, even though about 90% of vineyards are own-rooted *V. vinifera* (23). Again, we have not found associations between phylloxera infestation levels and irrigation methods (drip or flood) in the Argentinean viticultural areas (3). Interestingly, most of the infested vineyards did not present visual aerial symptoms and the pest was unnoticed (Arancibia, 2013-2019 personal observations). However, current changes in viticultural practices, especially the conversion from flood irrigation to drip irrigation could impact phylloxera behavior and change this scenario (2). In view of this background, climate change and its potential consequences in water availability, especially in irrigated areas (20), will force the preference of more efficient watering systems such as drip, therefore it is essential to have scientific information, about the interaction between soil pests and irrigation, and other abiotic factors such as soil textures.

In this work, we examined the effect of irrigation (drip and flood) and soil texture (clay and sandy) on phylloxera population growth and vine damage, in two different experiments, under controlled conditions, using *V. vinifera* cv. Malbec, the emblematic grape of Argentina. A better understanding of these interactions is essential for decision-making regarding water, soil and pest management at vineyard planting and in established vineyards and it will have long term implications for the economy of the industry.

Materials and methods

Plant material

Wood cuttings of *V. vinifera* cv. Malbec (clon 12 INTA) from the experimental field of Cátedra de Fisiología Vegetal-Facultad de Ciencias Agrarias (Mendoza, Argentina) were forced to root by submerging the base in 1000 ppm IBA (indol-butiric acid) 40% hydro-alcoholic solution for 10 seconds followed by culture in perlite, in a rooting chamber. Cutting bases were kept at 20 ± 2°C to stimulate callus formation and rootlet emission, while the upper parts were kept at 10 ± 2°C to prevent bud break. The photoperiod regime was 16 h light and 8 h of darkness. When roots were approximately 4 cm long, vines were transferred to 4.4 L pots with corresponding soil texture. Plants were grown in a growth chamber with 16 h light and 8 h of darkness and an average temperature of 28°C ± 3°C and light intensity of 40 µEinstein m^{-2} s^{-1}. Vines were trained to a single shoot and selected for inoculation 3 months after transplant.

Inoculation

Phylloxera eggs were obtained from Argentinean genotype B grape phylloxera (3) grown on Malbec excised roots in Petri dishes at 24°C± 2°C, in the darkness. Fifty phylloxera eggs were transferred to a 1.5 µL Eppendorf® tube, which was subsequently laid down approximately 4 cm under the soil surface, in the central part of the pot, close to the roots. Inoculation

was repeated 3 weeks later to insure effective infestation (9). In order to prevent insects from escaping and/or infesting control plants, all pots were wrapped and closed to the base of the plant with frost cloth. Plants were pruned at 30 cm together with the first inoculation.

Irrigation experiment

Plants were potted in sterile loam soil. Twenty-four vines were infested with phylloxera eggs and other 24 plants were left non-infested as control plants. Twelve infested and 12 uninfested plants were drip irrigated, while other 12 infested and 12 uninfested plants were surface watered. Irrigation treatments started together with first inoculation. Drip irrigation method consisted of 350 mL of water applied by plastic drip lines, with drippers (2.1 L h^{-1}). Flood irrigation consisted of 350 mL of water applied by a dripping plastic hose, with bubblers (10.5 L h^{-1}). In this case, the water flow was higher than soil basic infiltration, so the volume between the pot rim and the soil surface was full of water during irrigation; in drip irrigated pots, due to lower water flow of the drippers, water penetrated the soil immediately. Plants were watered twice per week. Differences in humidity profiles between irrigation methods were measured with Decagon DS5 (Decagon devices Inc., Washington, USA) humidity sensors (figure 1) showing a differential water distribution in the pots of the different irrigation methods. Six pots were placed under 8 irrigation lines each, alternating drip and flood methods. In each irrigation line infested and control plants were placed randomly. Treatments were designated *infestation* (*phylloxera* (*P*) and *control* (*C*)) and *irrigation method* (*flood* (*F*) and *drip* (*D*)). Analysis included only data from plants in which all variables were measured. For this reason, the final number of replicates was: 7 *F* x *P*, 5 *F* x *C*, 6 *D* x *P* and 7 *D* x *C*.

Soil texture experiment

Plants were potted in sterile agricultural sandy or clay soil (86 mL and 102 mL sediment volume, respectively) three months before inoculation. Fourteen plants in clay and 16 in sandy soil were infested with phylloxera and 14 plants in clay and 16 in sandy soil were left uninfested as control plants. Plants in sandy soil were watered with 200 mL and plants in clay soil with 350 mL to reach field capacity, about twice per week depending on the atmospheric demand.

Pots were randomly arranged in the growth chamber. Treatments were designated *infestation* (*phylloxera* (*P*) and *control* (*C*)) and *soil texture* (*sandy* (*S*) and *clay* (*CL*)). Analysis included only data from plants in which all variables were measured. For this reason, the final number of replicates was: 6 *CL* x *P*, 8 *CL* x *C*, 6 *S* x *P* and 5 *S* x *C*.

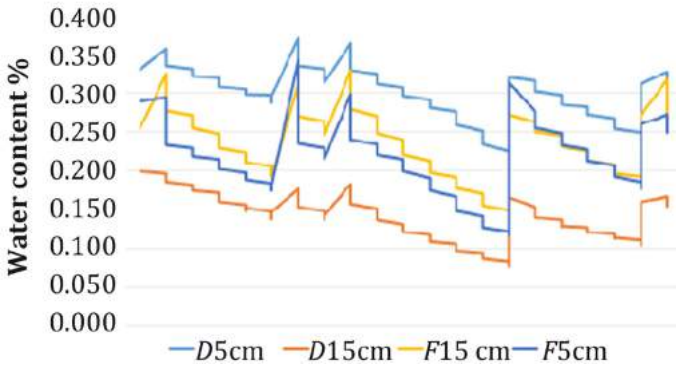

Figure 1. Volumetric water percentage measured every 24 hours in pots under drip (*D*) and flood (*F*) irrigation.

Figura 1. Porcentaje de volumen de agua medido cada 24 horas en macetas bajo riego por goteo (*D*) y riego superficial (*F*).

Measured variables

In both experiments, main shoot length, number of leaves and relative chlorophyll content (RCC) were measured 60 and 90 days after inoculation (dai). RCC was measured in leaves from position 4 to 8, counting from the apex, using a SPAD-502 Chlorophyll Meter (Minolta Corp., Ramsey, NJ) and data was averaged to yield a single value per vine. Stomatal conductance was measured at the same time than RCC, using a Leaf Porometer SC^{-1} (Decagon devices Inc., Washington, USA), in leaves from position 3 to 5, counting from the apex, and then averaged to yield a single value per vine. Photosynthetic rate was assessed using a CIRAS-2 Portable Photosynthesis System (PP Systems, Amesbury, USA), 120 dai. Plants were removed from the pots 120 dai to count nodosities, tuberosities, phylloxera eggs, nymphs, and adults on the roots under a Carl Zeiss stereo microscope 35X. Total individuals were calculated as the sum of eggs, nymphs and adults. Root dry weight (RDW) was assessed by placing roots at 55°C until constant weight. Number of eggs, nymphs, adults and total individuals were related to RDW. Total leaf area was measured with a LI-COR 3000ª (LI-COR, Inc., Lincoln, USA) and then dried at 55°C until constant weight to calculate leaf dry weight (LDW) and LDW/total leaf area ratio. A similar procedure was followed to determine shoot dry weight (SDW).

Statistical analysis

Principal coordinate analyses (PCoA) were performed to examine variables in reduced dimensions in both experiments. Photosynthetic rate (mmol CO_2 m^{-2} s^{-1}), number of leaves, stomatal conductance (mmol H_2O m^{-2} s^{-1}), leaf area (cm^2), LDW/total leaf area ratio (g/cm^2), RCC (SPAD unities), SDW (g), main shoot length (cm) and RDW (g) were included in the PCoA to evaluate the effects of phylloxera on the aerial part of the plants. In this analysis, for those variables measured more than once, only last measurement was included. Data was standardized, Euclidean distance was used and the distance function applied was Mij=-0.5*Dij*Dij. Similarly, PCoA was applied to number of eggs, nymphs, adults, total individuals, tuberosities and nodosities to analyze phylloxera population density and damage in roots. Data were analyzed by generalized linear mixed models (GLMM) and Akaike and Bayesian Information Criteria were applied to select the best models. Factors *infestation*, *irrigation method* and *soil texture* were computed as fixed effects. In the irrigation experiment, drip line (1 to 6) and pot locations in the growth chamber (border or interior) were computed as random effects. When variables were measured more than once, *time* was considered a fixed effect. An independent error correlation structure was applied and, when necessary, heteroscedasticity was corrected using the VarIdent function. Mean comparison was estimated by DGC test (14). All described statistical analyses were carried out using InfoStat v. 2017 software (15). In the soil texture experiment, inoculation of roots was not 100% successful and Fisher's exact test (1) was applied to examine the statistical association between soil texture and infestation success using www.langsrud.com/fisher.htm.

Results and discussion

For many years, it has been widely accepted that phylloxera prefers heavy clay soils and that flood irrigation can help to lower the population levels. Although these claims are supported by several reports (10, 12, 25, 31), many others have refuted them (8, 9, 27); so, more studies are needed to clarify these interactions. In this project we present the results obtained from two experiments involving the interaction between irrigation and soil texture with phylloxera population and grapevine damage.

Table 1 (page 443) shows, GLMM results of the statically significant variables found in the irrigation experiment; results of non-significant analyses ($p > 0.05$) are not shown. As expected, total leaf area, RDW and shoot length were affected by *infestation*, showing that phylloxera damage was reflected in the aerial part of the plants.

Several significant interactions were also found. A reduction in number of leaves of *D* x *P* was possibly caused by water stress in parts of the root system, given the humidity gradient in the drip irrigated pots which, in addition to the interaction with the insect, could have affected leaf production or abscission.

Table 1. Means, P values and standard errors for irrigation experiment significant variables.

Tabla 1. Medias, valores P y errores estándar para las variables significativas del experimento de riego.

VARIABLE	TREATMENT	p VALUE	MEAN ± SE
LEAF AREA	***infestation***	0.0055	
	C		173.84 ± 18.94 A
	P		83.88 ± 17.99 B
NUMBER OF LEAVES	***irrigation method* x *infestation***	0.0001	
	D x *P*		13.57 ± 2.8 B
	D x *C*		21.6 ± 2.73 A
	F x *P*		21.34 ± 2.57 A
	F x *C*		18.55 ± 2.84 A
ROOT DRY WEIGHT	***infestation***	0.006	
	C		8.32 ± 0.54 A
	P		6.01 ± 0.42 B
SHOOT LENGHT	***infestation***	0.0119	
	C		47.19 ± 2.39 A
	P		39.78 ± 2.33 B
STOMATAL CONDUCTANCE	***infestation***	0.0154	
	C		25.42 ± 1.97 B
	P		31.51 ± 1.93 A
PHOTOSYNTHETIC RATE	***irrigation method* x *infestation***	0.0016	
	D x *P*		2.55 ± 0.27 B
	D x *C*		1.77 ± 0.25 B
	F x *P*		1.99 ± 0.25 B
	F x *C*		3.44 ± 0.3 A
RCC	***time***	0.0001	
	60		36.52 ± 1.12 A
	90		31.17 ± 1.12 B

SE: standard error. Same letters do not represent a statistical difference. DGC test ($p < 0.05$).

MEAN: media; SE: error estándar. Letras iguales no presentan diferencias significativas. DGC test ($p < 0,05$).

The increase in photosynthetic rate in *F* x *C*, could be due to homogeneous water availability in the pots and to the fact that, even in small populations, phloem sucker insects can decrease the photosynthesis rate of their hosts (22) by repressing genes required by Rubisco and photosystem II activity (19). Furthermore, the healthy root system of control plants may have permitted an adequate water and nutrient intake that stimulated photosynthesis process. Also, in the field, leaf chlorosis is often the initial visual indication of phylloxera infestation; however, in this experiment phylloxera did not reduce RCC, in contrast to other studies (5). Only time reduced RCC as result of a natural ontogenic process. Phylloxerated plants showed reduction of 52% in leaf area. Similar results were obtained by with Eitle *et al.* (2017) in grafted and ungrafted rootstocks, however LDW/total leaf area ratio remained unaffected. Phylloxera was responsible for 18% shoot length reduction and since the number of leaves remained unaltered, internodes were shortened (a common symptom in phylloxerated vineyards). A suboptimal cell elongation originated by a restriction of water intake due to a limited root system could cause this phenomenon. SDW was not affected by any treatment. Phylloxerated plants showed 28% less root mass than *C* plants, contrary to the increase found by Eitle *et al.* (2017), and our results can be attributed to a secondary pathogen attack (non-tested), a deviation of photoassimilates to biosynthetic pathways implicated in the plant's defense mechanism or to a different grapevine genetic background used in this experiment. In general, the reduction of vines biomass in *P* can be explained by the metabolic cost incurred due to infestation (35); either as an infestation induced response (oxidative enzymes activity, hormones and phenolic compounds accumulation) (28), or as part of a phylloxera manipulation towards plant's metabolism genes to assure a source of nutrients for it (18). The increase of stomatal conductivity in *P* could be due to a larger stomatal cell opening or a higher density of stomata (not measured). As Nabity *et al.* (2013) observed, leaf phylloxera can manipulate grapevine

primary metabolism and induce stomata formation on adaxial leaf surface, therefore it is possible that chemical signals related to phylloxera infestation move from the roots through the vascular system and induce stomata formation or regulate its opening. The multidimensional scaling approach by the PCoA revealed the grouping of *D* x *P* plants on the negative side of axis 1, while the rest of the combined treatments were almost exclusively on the positive side of the axis at random positions (figure 2). In general, the combined treatments were not able to predict the response of these variables (in concordance with the observed through GLMM in the individual analyses of the variables); the grouping of *D* x *P* plants must have responded to the stronger influence of number of leaves variable.

The number of insects at different life stages (eggs, nymphs, adults and total individuals) and the number of tuberosities and nodosities were not affected by the *irrigation method* and these results agree with previous observations in Argentinean vineyards, that did not find a statistical association between levels of infestation and type of irrigation (3). The biplot obtained from the multidimensional scaling approach of these variables (figure 3, page 445) when *irrigation method* was used as a classification criterion, did not show any obvious groupings, reveling again the lack of ability of *F* or *D* to predict the behavior of such variables under our experimental conditions. Many studies have revealed that the strength of plant-herbivorous relationship may be based on plant health (9). The *Plant Vigor Hypothesis* proposes that plants or plant organs that grow vigorously and are relatively large are favorable to herbivorous attack; in such interaction it is suggested that galling insects normally attack the most vigorous plants or plant organs (30). On the other hand, *The Plant Stress Hypothesis* proposes that plants undergoing some type of physiological stress are more susceptible to herbivory, given a reduction on protein synthesis and an increment of amino acids in tissues, thus generating an improved nutritious source of food for nitrogen-limited organisms (30).

In this study, although only photosynthetic rate mean was significantly higher in *F* x *C*, 6 other variables were also higher (non-significant) in *F*, including dry weights. These results suggest that *The Plant Vigor Hypothesis* could explain the tendency of *F* to harbor more insects, as found by Kimberling *et al.* (1990) in phylloxera leaf galling attacks.

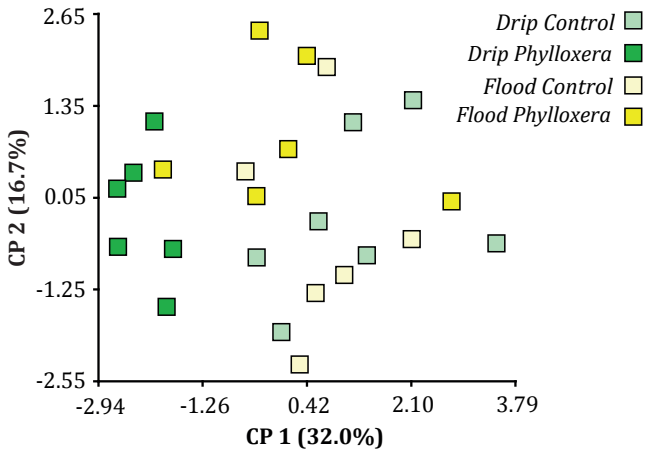

Figure 2. ACoP Biplot of photosynthetic rate; chlorophyll relative content; stomatal conductance; main shoot length; number of leaves; leaf area; main shoot length dry weight; leaf dry weight/leaf area ratio and root dry weight, using *combined treatments* as classification criterion.

Figura 2. Biplot del ACoP para las variables: tasa fotosintética, contenido relativo de clorofila, conductancia estomática, longitud de brote, número de hojas, área foliar, peso seco de brote, peso seco de raíces y área foliar/peso seco, tomando los *tratamientos combinados* como criterio de clasificación.

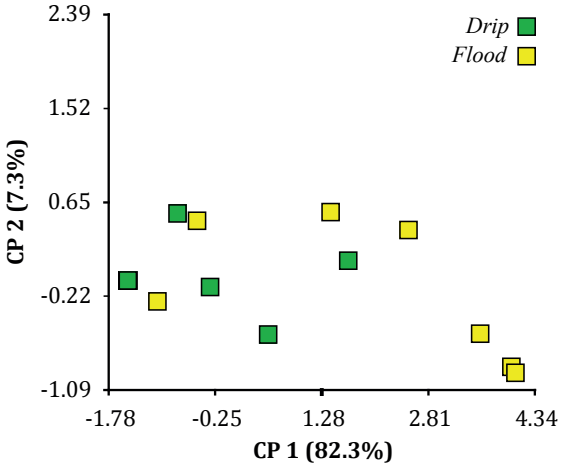

Figure 3. ACoP Biplot for variables measured on phylloxerated plants: tuberosities and nodosities, adults, nymphs, eggs and total individuals expressed per root dry weight, using *irrigation method* as classification criterion.

Figura 3. Biplot del ACoP para las variables de las plantas del tratamiento filoxera: tuberosidades y nudosidades, adultos, ninfas, huevos, individuos totales, todos expresados por peso seco de raíz en gramos tomando el factor método de riego como criterio de clasificación.

To date, the predominant irrigation system in Argentina has been flooding. Water availability is subjected to climate and government regulations, among other factors. Nevertheless, even in regions where water availability, soil conditions and economic resources allow flooding, the killing of phylloxera under these circumstances is debatable. Most likely soil moisture is important at both ends of the spectrum, especially because of its influence on plant growth. That is, the direct effect of flooding on the insect would not be as important as the effect on vine physiology, whether the plant-insect interaction responds to the hypothesis of vigor or the hypothesis of the stress. Also, both irrigation methods present differences in cultural practices associated to soil management that can also affect phylloxera indirectly through variations in soil micro-flora and fauna.

GLMM results of the statically significant variables ($p < 0.05$) in the soil texture experiment are shown in table 2 (page 446) (non-significant analyses are not shown). *Clay Control* plants showed a larger leaf area than the rest of the *combined treatments*. This could be the effect of the greater number of leaves observed in *CL* plus a healthy root system growing in a soil with higher water retention capacity than a sandy soil (32). The steady water supply would provide the necessary turgor for cell enlargement and maximum leaf expansion. Grapevines under *CL* generated around 33% more leaves than those in *S*. It is possible that the lower water retention and field capacity in *S* could have caused temporal water stress events, and consequently, induced abscisic acid synthesis followed by stomata closure and a decrease of CO_2 uptake. Decreased photosynthesis would have, in turn, inhibited leaf production, as a strategy to prevent water loss through the canopy (11).

As observed in the irrigation experiment, *P* also decreased root dry weight by 30%. Similar results were reported by Omer *et al.* (1995) in a pot trial with *V. vinifera* cv. Chardonnay. Furthermore, this variable was 40% lower in *CL*, supporting the prediction of smaller root systems in fine-textured soils (32). It is possible that the higher water content available for plants growing in *CL* favored carbohydrate partitioning to the shoot over the root.

As in the irrigation experiment, shoot length was also reduced around 28% by phylloxera. The analysis of all these variables through a multifactorial approach, showed a biplot where *CL* x *P* and *CL* x *C* appeared mainly on the positive side of principal coordinate 1 and *S* x *P* and *S* x *C* on the negative side (figure 4, page 446) , which suggests that only texture was able to predict the response of such variables. This was supported by the fact that *soil texture* statically influenced 6 variables, while *infestation* affected 3, and *soil texture* x i*nfestation* only one.

Table 2. Mean, P value and standard error for soil texture experiment significant variables.

Tabla 2. Medias, valores P y errores estándar para las variables significativas del experimento de texturas de suelo.

VARIABLE	TREATMENT	pVALUE	MEAN ± SE
LEAF AREA	***infestation***	0.0219	
	C		151.63 ± 12.17 A
	P		106.64 ± 13.5 B
	soil texture	0.0002	
	S		87.27 ± 7.51 A
	CL		170.99 ± 16.55 B
	soil texture* x *infestation	0.027	
	CL x *C*		224.4 ± 21.67 A
	CL x *P*		117.58 ± 25.02 B
	S x *C*		78.86 ± 10.13 B
	S x *P*		95.69 ± 11.1 B
NUMBER OF LEAVES	***soil texture***	<0.0001	
	S		13.15 ± 0.78 B
	CL		19.62 ± 0.85 A
ROOT DRY WEIGHT	***infestation***	0.0094	
	C		3.93 ± 0.29 A
	P		2.75 ± 0.29 B
	soil texture	<0.0001	
	S		4.76 ± 0.31 A
	CL		1.92 ± 0.27 B
SHOOT LENGHT	***infestation***	0.0206	
	C		35.48 ± 3.27 A
	P		25.42 ± 2.11 B
NODOSITIES AND TUBEROSITIES (ROOT DRY WEIGHT)$^{-1}$	***soil texture***	0.0089	
	S		10.92 ± 2.95 B
	CL		38.13 ± 7.87 A

SE: standard error. Same letters do not represent a statistical difference. DGC test ($p < 0.05$).

MEAN: media; SE: error estándar. Letras iguales no presentan diferencias significativas. DGC test ($p < 0,05$).

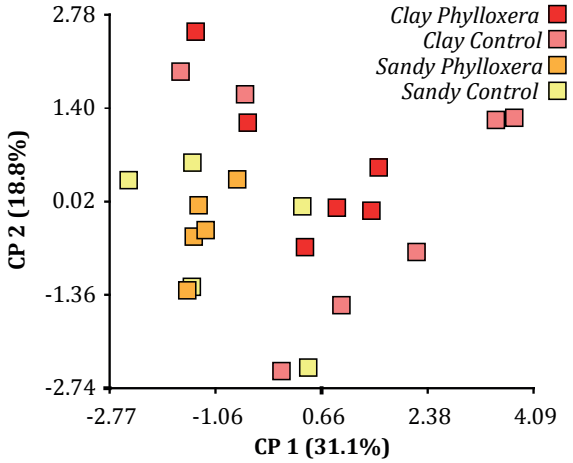

Figure 4. ACoP Biplot of photosynthetic rate; chlorophyll relative content; stomatal conductance; main shoot length; number of leaves; leaf area; main hoot length dry weight; leaf dry weight/leaf area ratio and root dry weight, using *combined treatments* as classification criterion.

Figura 4. Biplot del ACoP para las variables: tasa fotosintética, contenido relativo de clorofila, conductancia estomática, longitud de brote, número de hojas, área foliar, peso seco de brote, peso seco de raíces y área foliar/peso seco, usando los tratamientos combinados como criterio de clasificación.

In this trial, *soil texture* did not affect egg survival, hatching and survival of the nymphs, as revealed by Fisher's exact test; there was no association between soil texture and phylloxera infestation success (p = 1) (table 3). Phylloxera life stage means did not differ between *soil texture* treatments (p > 0.05). These results are supported by Chitkowski and Fisher (2005) who testing 8 different types of soils from Oregon and Washington found a normal phylloxera growth population in all of them. Unexpectedly, *CL* plants had more nodosities and tuberosities than those in *S*, however this response did not appear to affect plant growth. Again, even under infestation it is possible that better water availability had promoted vine growth and improved tolerance to the pest.

The fact that more root symptoms in *CL* were not accompanied by a larger phylloxera population could be explained by the proposed physical resistance of sandy soil to phylloxera movement. It is possible that in *CL*, phylloxera could have moved easier to fresh roots when root deterioration progressed. On the contrary, *S* would have limited such mobility. Finally, the biplot did not expose clear groupings between levels, when variables related to phylloxera population and root damage were analyzed by PCoA, using *soil texture* as classification criterion (figure 5). The results of this experiment contradict the findings of Nougaret and Lapham (1928), who concluded that sandy soils prevent or suppress phylloxera establishment (9). Other reports have also suggested that sandy soil do not inhibit phylloxera growth (3, 8, 9, 27).

Table 3. Number of successful and non-successful infested plants in clay and sandy soil.
Tabla 3. Número plantas con infestaciones exitosas y no exitosas en macetas de suelo arcilloso y arenoso.

	Non successful infestation	Successful infestation	Total
CLAY	8	6	14
SANDY	10	6	16
Total	18	12	30

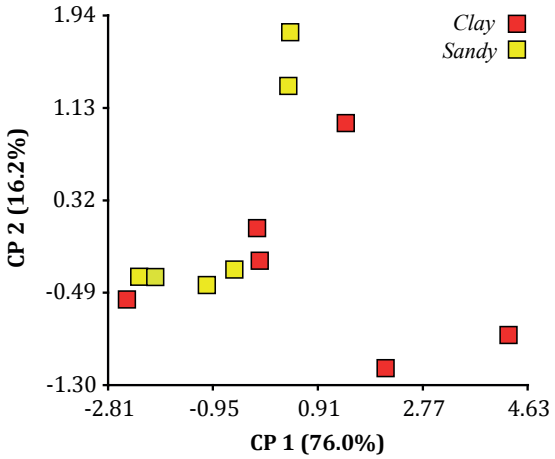

Figure 5. ACoP Biplot for variables measured on phylloxerated plants: tuberosities and nodosities, adults, nymphs, eggs and total individuals expressed per root dry weight, using *soil texture* as classification criterion.

Figura 5. Biplot del ACoP, Análisis para las variables de las plantas del tratamiento filoxera: tuberosidades y nudosidades, adultos, ninfas, huevos, individuos totales, todos expresados por peso seco de raíz en gramos tomando el tratamiento *textura de suelo* como criterio de clasificación.

The results of this study suggest that the irrigation method and soil texture have little incidence on phylloxera development, at least under these experimental conditions. This is in agreement with field observations in the Argentinean viticultural area, which revealed that phylloxera exists in a broad type of texture and compaction degree spectrum and did not find associations between infestation level and soil texture or irrigation method (3).

Nougaret and Laphman (1928) found that in deep, friable and recent-alluvial soils with porous subsoils, destruction caused by phylloxera infestations is slow. This could be the case of Argentinean vineyards, mostly located in Entisols y Aridisols (of different suborders), generally unstructured, with depths up to 2 meters, which allow good infiltration and an optimal development of the root system.

Non-specific stress symptoms are difficult to separate from other conditions ranging from water or nutrient stress to phylloxera or disease burden (7). Moreover, the dynamics of vineyard soils can be as diverse as the factors influencing them and their numerous combinations. This complexity makes hard to establish a direct relation between a specific factor and its influence on phylloxera's population. It is possible that texture and irrigation of the soil might play an important role in the dispersion, rather than in phylloxera survival. Possibly, these influences are indirect, and interact with other factors involved in the plant-soil-insect system rather than as a *per se* factor. For instance, is likely that other ecological actors such as soil bacteria, mycorrhiza and other soil fungus play an important role as beneficial or harmful agents under different soil textures and humidity conditions, affecting vine and phylloxera physiology. Under *The Plant Vigor Hypothesis*, synergy between factors such as low incidence of biotic diseases, high energy supply, optimum climate, good cultural management, deep soils and adequate irrigation, create a perfect combination for the development of a good plant defense system able to cope with the plague's attack and may be the key for the coexisting situation of phylloxera and *Vitis* spp., at levels that do not generate big economical losses. In view of these results and the accumulated experimentation of other researchers, field experimentation based on an adequate and precise methodology is essential to formulate accurate conclusions of these phenomena. Since water resources will be challenged by the effects of climate change (20), its management will be a critical factor in the years to come, at regional and global scale. Understanding the plant-soil-water-insect interactions under field conditions is crucial for an effective and efficient water management and the sustainability of the grape industry.

Conclusion

The effects of two irrigation methods and soil textures were studied on *V. vinifera* cv. Malbec in pots under controlled conditions. Phylloxerated plants under drip irrigation decreased the number of leaves while uninfested plants under flood had a higher photosynthetic rate. Phylloxera itself reduced leaf area, shoot length and root dry weight and increased stomatal conductance. Irrigation methods did not affect variables related to root damage or phylloxera population. On the other hand, uninfested plants on clay soil had a greater leaf area and more leaves. Phylloxera also reduced shoot length, root dry weight while plants on clay soil showed less root dry weight. Finally, more phylloxera root symptoms were found in plants in clay soil; however, texture did not affect the number of insects. Moreover, after inoculation soil texture had no influence on the infestation success of the plants.

It is possible that neither irrigation methods nor soil texture *per se* are a limiting factor for phylloxera performance, however their influence on the vigor of the plants will affect plant interactions with the pest. It is possible that the growing conditions present in Argentinean vineyards promote a balance in which the health of the vineyards is not dangerously compromised. This is the first study that analyzes the effects of irrigation and soil texture using Argentinian phylloxera strains and can constitute the base for field trials necessary to bring integrative results to develop phylloxera control management strategies in Argentina.

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Abscisic acid and methyl jasmonic acid module anthocyanins and *trans*-resveratrol accumulation in berry skin of five red *Vitis vinifera* cvs. in two contrasting viticultural regions of Mendoza-Argentina

El ácido abscísico y metil jasmonato modulan la acumulación de antocianinas y *trans*-resveratrol en hollejos de bayas de cinco cultivares tintos de *Vitis vinifera* en dos regiones vitícolas contrastantes de Mendoza, Argentina

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Abstract

Berry skins from red grape cultivars contain significant amounts of polyphenols that contribute to wine quality and provide health benefits. These compounds can be elicited by plant hormones. The aim of this work was to increase the content of anthocyanins (ANT) and *trans*-resveratrol (*T*-RES) by application of abscisic acid (ABA) and methyl jasmonate (MeJA) in five red *V. vinifera* cvs. (Bonarda, Malbec, Syrah, Cabernet Sauvignon, and Pinot Noir), in two Argentinean contrasting growing regions (Santa Rosa and Valle de Uco). Results showed positive and differential effects of ABA and MeJA on the total ANT content for the diverse cultivars with changes in the proportions of blue and red ANT. ABA increased total ANT in both viticultural region, while MeJA had a positive effect only in Santa Rosa. Also, ABA and MeJA induced an accumulation of *T*-RES in different cultivars, regardless of the region; *T*-RES accumulation elicited by ABA was not previously described. This work brings out the possibility to use these hormones as practical tools to produce high-quality red wines in two contrasting viticultural regions.

Keywords
elicitors • phenolic compounds • grapevine • plant hormones

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Resumen

Los hollejos de las uvas tintas contienen cantidades significativas de polifenoles que contribuyen a la calidad del vino y proporcionan beneficios para la salud. Estos compuestos pueden ser elicitados por hormonas vegetales. El objetivo de este trabajo fue aumentar el contenido de antocianinos (ANT) y *trans*-resveratrol (*T*-RES) mediante la aplicación de ácido abscísico (ABA) y jasmonato de metilo (MeJA) en 5 *V. vinifera* cvs. (Bonarda, Malbec, Syrah, Cabernet Sauvignon y Pinot Noir), en dos contrastantes regiones vitícolas argentinas (Santa Rosa y Valle de Uco). Los resultados mostraron un efecto positivo y diferencial de ABA y MeJA en el contenido total de ANT para los diversos cultivares, con cambios en las proporciones de ANT azul y rojo. ABA aumentó los ANT totales en ambas regiones vitícolas, mientras que MeJA tuvo un efecto positivo solo en Santa Rosa. Además, ABA y MeJA indujeron una acumulación de *T*-RES en diferentes cultivares, independientemente de la región; la acumulación de *T*-RES provocada por ABA ha sido previamente reportada. Este trabajo pone de manifiesto la posibilidad de utilizar estas hormonas como herramientas prácticas para producir vinos tintos de alta calidad en dos regiones vitícolas contrastantes.

Palabras clave
elicitores • compuestos fenólicos • vid • hormonas vegetales

Introduction

Wine is a traditional beverage that has been associated with both healthy and harmful effects. Scientific evidence has demonstrated a tight correlation between a mild but regular red wine consumption and a healthy cardiovascular system in populations where wine accompanies everyday meals as a habit (28); benefits are mostly conferred by the presence of certain phenolic compounds that possess antioxidant activity (14). Phenolic compounds (mainly located in berry skin and seeds) have also an important role in determining the final oenological quality of red wines (29), being red wine known to contain 10-fold more phenolic compounds than white wine.

The biological potential of the wide range of chemical of polyphenols compounds in wine has been examined in extensive reviews (6, 24). In general, anthocyanins (ANT) are excellent antioxidants since they are easily oxidized under stress circumstances, having a protective effect on human health, regarding degenerative and chronic diseases (6). On the other hand, *trans*-resveratrol (*T*-RES) (a stilbene), is the most examined phenolic compound over the past decade due to its nutritional and medicinal value. *T*-RES exerts a plethora of biological functions, especially as a cardiovascular protective, antiplatelet, antioxidant, anti-inflammatory, blood glucose-lowering, anticancer, antiaging, neuroprotective, and anti-obesity compound (24).

Polyphenol content in grapes is very variable and it depends on several genetic, environmental and management factors (8). Among the environmental factor, the vineyard location has a very important role (3). For example, high altitude vineyards have a wider temperature range and grapes are richer in polyphenolic compounds than grapes from vineyards located in lower altitude and warmer regions (19). Due to the important properties of these compounds, there is an increasing interest in producing grapes or wines with higher contents, which have more nutraceutical value. Plant hormones, such as abscisic acid (ABA), play an important role in plant physiological and biochemical processes. Exogenous applications of ABA induce the accumulation of ANT in grape berries by enhancing the transcription of anthocyanins synthesis related genes, thus improving the color of the fruit (17). On the other hand, jasmonates like methyl jasmonic acid (MeJA) stimulate *T*-RES biosynthesis (3, 18). Several local studies regarding content and biosynthesis stimulation of certain phenolic compounds have been conducted in red grapevine cultivars grown in Mendoza (1, 3, 5) and, in this context, ABA has shown positive effects on grapes growing under high temperature conditions, countering the decrease of ANT caused by this environmental factor (22, 23). In addition, MeJA has significantly increased *T*-RES accumulation, by exacerbating the expression of genes whose products are involved in its synthesis, such as

several members of the Stilbene Synthase (*Vv*STS) multigenic family, transcription factors *Vv*MYB14 and *Vv*MYB15.2, and Phenylalanine ammonia-lyase (*Vv*PAL) gene in Malbec (10). Currently, there are no previous works assessing the different phenolic compounds induced by hormones such as ABA and MeJA simultaneously in more than one variety and located in contrasting viticultural regions with dissimilar climate conditions, trellis systems, irrigation methods and soil depth, among other factors, and although the impact of plant hormones and high temperatures alone has been widely studied, their combined effect with other vineyard factors on plants remains poorly understood.

In Argentina, the vineyards involved in the production of red wine, account for almost 50% of the total country vineyards, distributed across irrigated arid areas. Mendoza, is one of the most favorable viticultural locations in Argentina that produces premium red wines and its vast viticultural area comprises great differences that could play a role in the modulation of ABA and MeJA exogenous application on berries. The aim of this work was to evaluate the accumulation of ANT and *T*-RES in the berry skin of five red *V. vinifera* cvs., after direct spraying with ABA and MeJA, in two different Argentinean viticultural regions.

Materials and Methods

The study was conducted during 2016 season, in commercial vineyards of two contrasting viticultural regions of Mendoza-Argentina. One of the vineyards was located in Santa Rosa (68°03'28" W and 33°15'56" S; 590 m a. s. l.), in East Mendoza. These vineyards consisted in a spur-pruned overhead trellis system (vine spacing: 4 m x 4.5 m), flood irrigated. All East Mendoza viticultural region show high average daily temperatures and warm nights (4), deep soils, and overhead is the predominant trellis system. The other vineyard was located in Gualtallary (69°15'37" W and 33°23'51" S; 1450 m a. s. l.), Valle de Uco, a region located in South West Mendoza. These vineyards were trained in a spur-pruned vertical shoot position system (vine spacing: 2.2 m x 1.4 m), drip irrigated. In comparison to Santa Rosa, this region shows lower average temperatures and very cold nights (4), shallow soils, and vertical shoot position system predominates.

Climatic differences were detected between the two growing regions by the analysis of daily data from nearby meteorological stations provided by Dirección de Contingencias Climáticas de Mendoza. Temperatures from January to March (2016) were significantly higher in Santa Rosa than in Valle de Uco region while relative humidity was lower in Santa Rosa than in Valle de Uco (table 1, page 454). According to these data, Santa Rosa was considered to be warmer and drier than Valle de Uco during the period when the experiment was conducted.

Vineyards from both locations were 10-12 years old, not covered by anti-hail net and managed according to the standard viticultural practices for each region and cultivar. In both locations Bonarda, Cabernet Sauvignon, Malbec, Pinot Noir and Syrah own-rooted *Vitis vinifera* cvs. were selected. Within a plot, 20 plants of each variety, were chosen randomly from a set of plants comprising only those with trunk perimeter of the media of the block ± 1 standard deviation. A randomized complete block experimental design with five replicates was used, where the experimental unit was a plant and each block was composed of a row. Two independent hormone treatments were performed. An aqueous solution (distilled water) of 1 mM ABA plus Tween 20 0.1% and was applied directly on the berries with a handheld sprayer until runoff, at three opportunities (10 days apart), starting at veraison, according to Malovini (2017). According to Durán (2016), MeJA treatment consisted of 10 mM MeJA in 40% ethanol-water solution with Tween 20 0.1%, and was sprayed on the berries together with the last ABA application. ABA control solution consisted of distilled water and Tween 20 0.1%, while MeJA control solution consisted of 40% ethanol-water solution with Tween 20 0.1%. Bunches from all cultivars were harvested 4 days after the last ABA and the MeJA application. In the laboratory, grapes from the modal size of each variety were randomly chosen and kept at - 80°C. For phenolics extraction, samples consisted of 1 g of fresh berry skins (after being manually separated from the pulp), macerated in methanol-HCl 0.1% mixture at 4°C for 48 h in the dark and then filtered through a 0.45 µm cellulose acetate membrane.

Table 1. Temperature and relative humidity differences between the two studied regions. Values are means of growing season (January-March). Meteorological stations were located in Las Catitas (33°15'56" S; 68°03'28" W, Santa Rosa) and El Peral (33°20'48.2" S; 69°9'27.7" W, Valle de Uco).

Tabla 1. Diferencias de temperatura y humedad relativa entre las dos regiones estudiadas. Los valores son los medios para la temporada de cultivo (enero-marzo). Las estaciones meteorológicas se ubicaron en Las Catitas (33°15'56" S; 68°03'28" O, Santa Rosa) y El Peral (33°20'48,2" S; 69°9'27,7" O, Valle de Uco).

Season	Meteorological Station	Temperature				Relative humidity
		n	Maximum (°C)	Mean (°C)	Minimum (°C)	Mean (%)
2016	Santa Rosa	111	31.23 ± 0,45 a	22.08 ± 0.41 a	15.63 ± 0.42 a	66.78 ± 1.16 b
	Gualtallary	105	29.22± 0,45 b	20.96 ± 0.41 b	14.03 ± 0.40 a	72.41 ± 1.16 a

Different letters indicate significant differences between regions by DGC test ($p < 0.05$).

Letras diferentes indican diferencias significativas entre regiones determinadas por la prueba DGC ($p < 0,05$).

ANT and *T*-RES contents were assessed by HPLC-DAD (SPD-M10AVP, Shimadzu, and Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) according to the official OIV methodology proposed by Otteneder (2004). Total ANT content was computed as the sum of individual ANT (delphinidin-3-glucoside, malvidin-3-glucoside, petunidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, malvidin3-O-(6"-acetyl)glucoside, peonidin3-O-(6"-acetyl)glucoside, malvidin-3-O-(6"-*p*-coumaroyl) glucoside and peonidin-3-O-(6"-*p*-coumaroyl)glucoside) and it was expressed as a mg of malvidin-3O-glucoside equivalent per gram of fresh berry skin weight. Also, ANT chemical profile as red (cyanidin-3-glucoside and peonidin-3-glucoside) and blue (delphinidin-3-glucoside, malvidin-3-glucoside, and petunidin-3-glucoside) content was analyzed. *T*-RES content was expressed as μg g^{-1} of fresh berry skin weight. At harvest, soluble solids (° Brix) were measured with a portable automatic refractometer (Atago®), and also pH and berry size were assessed. Data were analyzed using mixed linear models (MLM) with factorial structure, several alternative correlation structures were evaluated, as well as different structures of residual variance. Since natural pH and ° Brix variations between the different cultivars and regions, at harvest, could influence ANT and *T*-RES content, these variables were considered as co-variables in the MLM. The best models were selected using the Akaike (AIC) and Schwarz (BIC) information criteria (InfoStat v.2016 software, Grupo Infostat, FCA-UNC, Argentina). When significant differences were found between treatments, means were compared using DGC method involving a comparison based on multiple hierarchical conglomerates, ($p < 0.05$) (7).

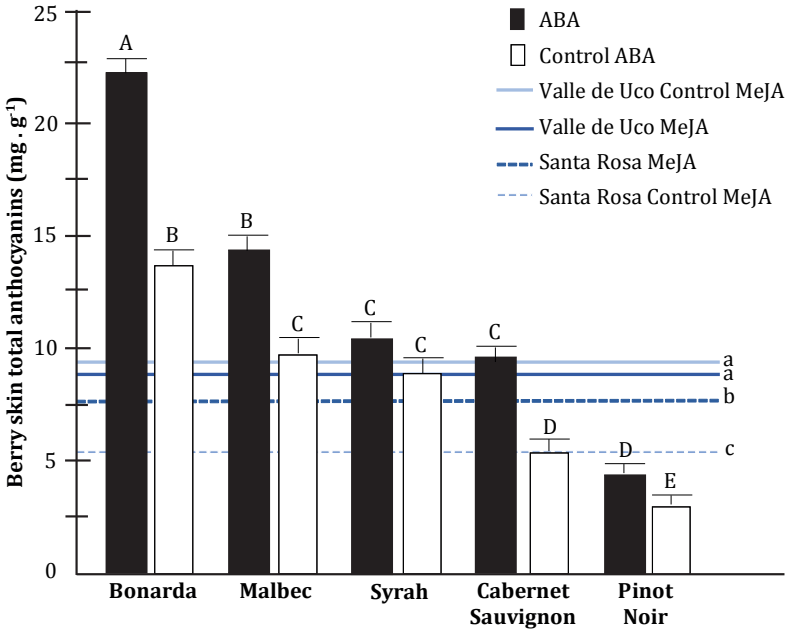
Results and Discussion

Anthocyanins and resveratrol are important factors that determine berry color and nutraceutical properties, relevant aspects for producing good quality red wines. Numerous reports have shown that exogenous applications of ABA and MeJA can improve these parameters in grape berry skin (9, 12, 26, 30, 31). In this trial, we examined the effect of these plant hormones in five different *V. vinifera* red cultivars in two contrasting viticultural regions.

ABA treatment increased total ANT concentration in all cultivars, except in Syrah (ABA treatment x Cultivar $p < 0.0001$) (figure 1, page 455). Such increments ranged from 35% in Malbec and 46% in Pinot Noir, to 73 and 70% in Bonarda and Cabernet Sauvignon. Since Syrah is known to have an anisohydric behavior (16), this could be due to deficient ABA receptors, and it could also explain the lack of response to ANT accumulation after ABA sprayed. On the other hand, the analysis revealed an interaction between cultivars and the viticultural region ($p < 0.0001$). Regardless of the many differences that characterize both regions, it could be expected that, given the lower temperatures in Valle de Uco, all cultivars would accumulate more total ANT, when compared to Santa Rosa. However, not all cultivars behaved in such a way. In Valle de Uco, Malbec showed a two-fold accumulation, Pinot Noir 53% and Syrah 40%, compared to Santa Rosa. Bonarda and Cabernet Sauvignon seemed to be more plastic cultivars; there were no differences in this variable between the two regions.

It seems most likely that the ANT accumulation is due to an upregulation of CHI, F3'H, DFR, and UFGT genes and the *Vv*MYBA1 and *Vv*MYBA2 transcription factors, as Koyama *et al.* (2018) demonstrated in *Vitis vinifera* x *Vitis labrusca* hybrid after elicitation with ABA. In this experiment, since no triple interaction between hormonal treatment, cultivar and regions was found, the two significant interactions mentioned above have additive effects. Overall, ABA effect on total ANT was not affected by the viticultural region ($p > 0.05$). This seems to indicate that ABA could turn into a good agronomic tool to be used in both regions. On the other hand, the application of MeJA was effective to induce the accumulation of total ANT only in Santa Rosa (p MeJA treatment x Region = 0.0289) (figure 1). One explanation for this phenomenon could be an effect of environmental factors, as previous studies have shown that the season had a significant effect on ANT accumulation in response to exogenous application of MeJA (10, 26). In Santa Rosa, when the hormone was applied, the difference with total ANT content observed in the control plants of Valle de Uco, was notoriously smaller (figure 1). This could be helpful to achieve wines of higher ANT content in a region where it is normally lower, like Santa Rosa.

Regarding ANT chemical profile, ABA increased red ANT in all cultivars, while blue ANT were increased in all, except on Syrah (ABA treatment x Cultivar for blue ANT $p < 0.0001$ and for red ANT $p = 0.0001$) (figure 2, page 456). This data suggested that the observed increment in total ANT with ABA application did not occur under an equal distribution of the two types of ANT. Bonarda, Malbec and Cabernet Sauvignon experienced a higher accumulation of the red than the blue type. The proportional increment of red over blue in Bonarda was 45%, 38% in Malbec and 19% in Cabernet Sauvignon. Interestingly, in Pinot Noir, the increment on total ANT responded to a higher relative increment of blue over red (43%), which has not been previously described and could be a desirable enological trait for blending. Finally, Syrah only had a significant increment of 44% in red ANT after ABA application.

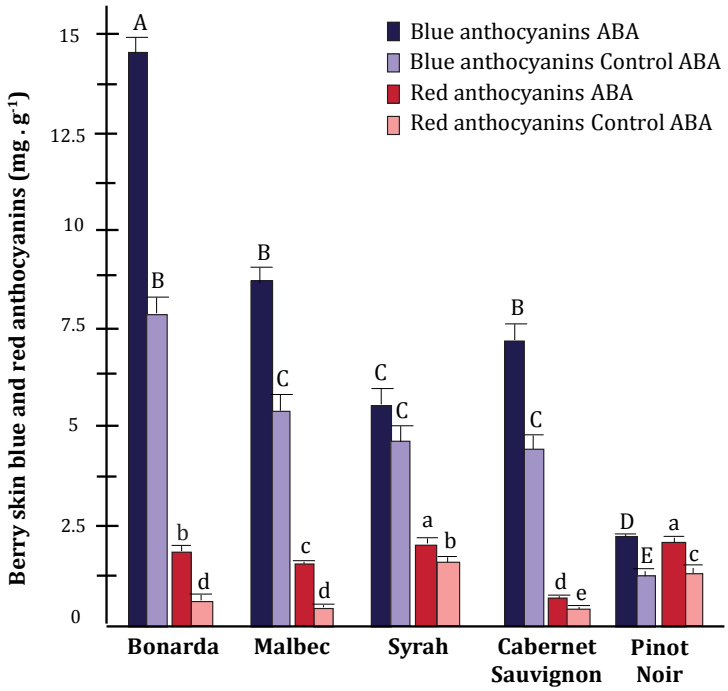


Different capital letters indicate significant differences between ABA and Control ABA treatments by DGC test ($p < 0.05$). Dashed lines represent means of the interaction of MeJA treatments and the viticultural regions; different lowercase letters next to the lines indicate significant differences by DGC test ($p < 0.05$).

Letras mayúsculas diferentes indican diferencias significativas entre el tratamiento ABA y el Control ABA, determinadas por la prueba DGC ($p < 0,05$). Las líneas discontinuas representan las medias de las interacciones de los tratamientos MeJA y las zonas vitícolas; letras minúsculas diferentes junto a las líneas indican diferencias significativas, determinadas por la prueba DGC ($p < 0,05$).

Figure 1. Bar graph for the interaction of hormonal treatments (ABA) and cultivars, and the interaction of hormonal treatments (MeJA) and the viticultural region, on total anthocyanins content in berry skin.

Figura 1. Gráfico de barras para la interacción entre el tratamiento hormonal (ABA) y los cultivares de vid, y la interacción del tratamiento hormonal (MeJA) con la región vitícola, sobre el contenido del total de antocianos en hollejo.



Different capital letters indicate significant differences between treatments, for blue anthocyanin type determined by DGC test ($p < 0.05$) and different lowercase letters indicate significant differences between treatments, for red anthocyanin type determined by DGC test ($p < 0.05$).

Letras mayúsculas diferentes indican diferencias significativas entre los tratamientos para las antocianos azules, determinadas por la prueba DGC ($p < 0,05$) y las letras minúsculas diferentes indican diferencias significativas entre los tratamientos para las antocianos rojos, determinadas por la prueba DGC ($p < 0,05$).

Figure 2. Bar graph for the interaction of ABA treatments and cultivars on blue and red anthocyanin contents in berry skins.

Figura 2. Gráfico de barras para la interacción de los tratamientos de ABA con los cultivares sobre el contenido en hollejo de antocianos azules y rojos.

On the other hand, MeJA did not show any influence on the blue ANT accumulation in berry skin, neither as a simple effect, nor in interaction with the other tested factors ($p > 0.05$). Regarding red ANT in grape skin, the accumulation of these compounds was affected by the triple interaction of the assessed factors (MeJA treatment x Region x Cultivar $p = 0.009$) (table 2, page 457). In Syrah and Pinot Noir, MeJA did not influence red ANT levels in plants of Valle de Uco, and when it was applied in Santa Rosa, these polyphenols reached comparable levels to those found in Valle de Uco. Cabernet Sauvignon showed no differences in red ANT levels between treatments, nor between regions. Finally, these compounds were not affected by MeJA applications on Bonarda and Malbec. They only showed a difference attributable to the region where they grew, an effect previously seen by other researchers (32).

Regarding *T*-RES content in berry skin, the effectiveness of both hormonal treatments was not influenced by the viticultural region ($p > 0.05$). ABA and MeJA showed an interaction with cultivars (MeJA treatment x Cultivar $p = 0.0043$ and ABA treatment x Cultivar $p = 0.0007$) (figure 3, page 457). The effect on *T*-RES accumulation triggered by the exogenous application of ABA, contradicts Wang *et al.* (2016) study. In both control treatments, Malbec, Syrah and Pinot Noir had the highest values, while Cabernet Sauvignon showed medium values, and Bonarda the lowest. This observation in Bonarda is coincident with the low *T*-RES observed in Bonarda wines from Mendoza-Argentina (11). ABA only increased the accumulation of this polyphenol in Malbec by 93% and in Syrah by 48%. Methyl jasmonate, on the other hand, only induced a significantly higher accumulation in Bonarda (150%) and Cabernet Sauvignon (53%).

On the other hand, Pinot Noir did not respond to hormonal applications. In this work, induction values described are comparable to those previously found in similar studies on Malbec (9), Syrah (12), Tempranillo, Graciano and Monastrel (13, 26). Finally, on the whole, grapes accumulated more *T*-RES in Valle de Uco than in Santa Rosa (p ABA treatment x Cultivar < 0.0001 and p MeJA treatment x Cultivar < 0.0001) (data not shown). This has an additive effect to the one on *T*-RES accumulation given by the interactions detected between ABA and MeJA treatments x Cultivar.

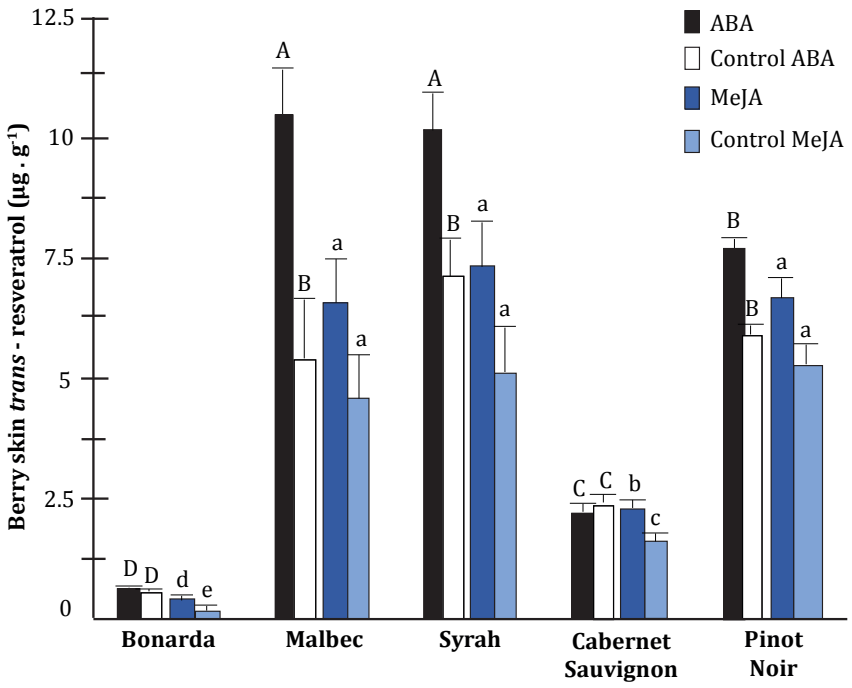
Table 2. Table of the interaction among cultivars, viticultural region and MeJA treatments on red anthocyanins (mg. g $^{-1}$) in berry skin.

Tabla 2. Tabla de la interacción entre cultivares, zona vitícola y tratamiento de MeJA sobre los antocianos rojizos (mg. g $^{-1}$) en hollejo.

Grape cultivar	Viticultural region	Treatment	Means ± E.E.
Bonarda	Valle de Uco	MeJA	1.25 ± 0.29 B
Bonarda	Valle de Uco	Control Me JA	1.25 ± 0.29 B
Bonarda	Santa Rosa	MeJA	0.33 ± 0.07 C
Bonarda	Santa Rosa	Control MeJA	0.4 ± 0.07 C
Malbec	Valle de Uco	Control MeJA	1.06 ± 0.16 B
Malbec	Valle de Uco	MeJA	0.96 ± 0.16 B
Malbec	Santa Rosa	Control MeJA	0.5 ± 0.05 C
Malbec	Santa Rosa	MeJA	0.48 ± 0.05 C
Syrah	Valle de Uco	MeJA	1.27 ± 0.19 B
Syrah	Valle de Uco	Control MeJA	1.46 ± 0.19 B
Syrah	Santa Rosa	MeJA	2.29 ± 0.26 A
Syrah	Santa Rosa	Control MeJA	1.26 ± 0.26 B
Cabernet Sauvignon	Valle de Uco	MeJA	0.36 ± 0.06 C
Cabernet Sauvignon	Valle de Uco	Control MeJA	0.49 ± 0.06 C
Cabernet Sauvignon	Santa Rosa	MeJA	0.39 ± 0.04 C
Cabernet Sauvignon	Santa Rosa	Control MeJA	0.31 ± 0.04 C
Pinot Noir	Valle de Uco	MeJA	1.99 ± 0.29 A
Pinot Noir	Valle de Uco	Control MeJA	1.84 ± 0.29 A
Pinot Noir	Santa Rosa	MeJA	2.81 ± 0.32 A
Pinot Noir	Santa Rosa	Control MeJA	0.88 ± 0.32 B

Different letters represent significant differences by DGC test ($p < 0.05$).

Letras diferentes indican diferencias significativas entre regiones determinadas por la prueba DGC ($p < 0,05$).



Different capital letters indicate significant differences between ABA and Control ABA treatments and cultivars by DGC test ($p < 0.05$). Different lowercase letters indicate significant differences between MeJA and Control MeJA treatments and grape cultivars by DGC test ($p < 0.05$).

Letras mayúsculas diferentes indican diferencias significativas entre los tratamientos ABA y Control ABA y los cultivares de vid, determinadas por la prueba DGC ($p < 0,05$). Letras minúsculas diferentes indican diferencias significativas entre los tratamientos MeJA y Control MeJA y los cultivares de vid, determinadas por la prueba DGC ($p < 0,05$).

Figure 3. Bar graph for the interaction of hormonal treatments and cultivars on *T*-RES content in berry skin.

Figura 3. Gráfico de barras para la interacción de los tratamientos hormonales y los cultivares sobre el contenido de *T*-RES.

The higher levels of erythemal weighted UV-B irradiance received in Valle de Uco, due to its altitude, could also be the cause of a higher *T*-RES content as it has been found to enhance PAL activity (1, 2). However, differences in trellis or irrigation systems that could potentially play a role in the ANT accumulation cannot be discarded. For example, it is possible that the higher vigor of vines in Santa Rosa conferred by their architecture, the irrigation method and the climate of the region lead vines to a different utilization of photosynthates compared to vines of Valle de Uco; this intricate interaction of factors is hard to tell apart. Even so, under the given experimental vineyards' conditions, the influence of the different thermal parameters between Santa Rosa and Valle de Uco are conspicuous and they might be playing the major role in the accumulation of *T*-RES and polyphenols in general.

In this study, pH and °Brix were evaluated to determine its influence on the analyzed polyphenols. They were used as co-variables in the GLMs and the analyses showed no correlation with total ANT, ANT chemical profile or *T*-RES. Regarding these parameters, Hiratsuka *et al*. (2001) suggested that the reason for ABA promoting the coloration of grapes might be due to the increase of soluble sugar content caused by this hormone. This would provide more substrate for the final production of ANT and promote the activation of ANT synthase or the expression of related genes. In this experiment, none of the hormonal treatments modified this parameter ($p > 0.05$). Similar results were previously described in Syrah berries (27). Regarding pH, berries sprayed with MeJA had a higher pH (3.92 ± 0.03) than those sprayed with control solution (3.87 ± 0.02) ($p = 0.0001$). Portu *et al.* (2018) found that MeJA applications on Graciano cultivar resulted in more acidic berries, while Fernández-Marín (2014) found that this hormone did not affect the acidity of Syrah grapes. Apparently, MeJA has an interaction with *Vitis* ssp. genotypes and, possibly, with the season (26). ABA did not modify pH values ($p > 0.05$), in accordance with the results showed by Sun *et al*. (2019) on *V. vinifera* cv. Merlot berries. Finally, none of the hormonal treatments influenced berry size ($p > 0.05$).

The results of this experiment show that ABA is a valuable agronomic tool for increasing ANT in grapes, in two contrasting Argentinean viticultural regions, Valle de Uco and Santa Rosa. Meanwhile, the application of MeJA only increased these compounds in Santa Rosa. These increases responded, at least in part, to changes in the blue and red ANT ratios induced by the hormones. In addition, the use of MeJA and ABA constitutes a promising innovation for both regions. It could increase wine's nutraceutical value by augmenting the contents of *T*-RES. In addition, this study has led to another experiment, where red wines with enhanced ANT and *T*-RES contents obtained with these hormones, are being studied for their psychotropic effects on animal models. This contributes to understanding the influence of wine consumption on human health. Also, current studies in similar contrasting regions are being carried out, including transcriptomic analysis of ANT and *T*-RES regulation mediated by ABA and MeJA.

Conclusion

The phenolic composition of the main red grape cultivars treated with ABA and MeJA in two contrasting viticultural regions (Santa Rosa and Valle de Uco, Mendoza-Argentina) was reported for the first time. A positive effect of ABA and MeJA on the total ANT content was observed, at different magnitudes, for the diverse cultivars. The increment of these compounds responded to changes in their chemical profile. ABA increased total ANT regardless of the viticultural region, while MeJA had a positive effect only in Santa Rosa. On the other hand, ABA and MeJA induced an accumulation of *T*-RES in different cultivars, while the region had no effect on these treatments. *T*-RES accumulation elicited by ABA was previously non-reported.

The use of these practical tools would allow the industry to produce high-quality red wines with enhanced organoleptic and nutraceutical value, in the mentioned contrasting areas.

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Wild potato Genetic Reserves in Protected Areas: prospection notes from Los Cardones National Park, Salta, Argentina

Reservas Genéticas de especies silvestres de papa en Áreas Protegidas: notas de prospección del Parque Nacional Los Cardones, Salta, Argentina

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Abstract

Wild potato species (WPS) are vital genetic resources to improve the productivity and sustainability of the third most important food crop worldwide. Although *in situ* conservation of this germplasm has been considered the most appropriate strategy, establishment of Genetic Reserves is still incipient. Northwest Argentina is among the priority regions for establishing WPS Genetic Reserves, whose designation within Protected Areas is accepted as the most efficient approach. In this work, we present results of the prospection and collection of WPS in Los Cardones National Park, a Protected Area with high environmental heterogeneity and diversity of plant communities. Four wild and one cultivated potato species were identified in different physiognomic vegetation units: *Solanum acaule*, *S. brevicaule*, *S. boliviense*, *S. vernei* and *S. tuberosum* group A*ndigenum*. In the four WPS, characters of interest for plant breeding have been described. Through the development of environmental education workshops and the monitoring over two consecutive years within a worldwide priority site, we have established a baseline on which *in situ* conservation will be projected to preserve an essential component of the natural and cultural America's patrimony.

Keywords
crop wild relatives • *in situ* conservation • plant genetic resources for food and agriculture • Protected Areas • *Solanum acaule* • *Solanum boliviense* • *Solanum brevicaule* • *Solanum vernei*

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Resumen

Las especies silvestres de papa (ESP) son recursos genéticos vitales para mejorar la productividad y sustentabilidad del tercer cultivo alimenticio en importancia mundial. Aunque su conservación *in situ* se ha considerado la estrategia más adecuada, el establecimiento de Reservas Genéticas es aún incipiente. El Noroeste Argentino figura entre las regiones prioritarias para establecer Reservas Genéticas de ESP, cuya designación dentro de Áreas Protegidas es aceptada como el enfoque más eficiente. En este trabajo presentamos resultados de la prospección y colecta de ESP en el Parque Nacional Los Cardones, un Área Protegida con una alta heterogeneidad ambiental y diversidad de comunidades vegetales. Cuatro especies silvestres y una cultivada de papa fueron identificadas en distintas unidades fisonómicas de vegetación: *Solanum acaule*, *S. brevicaule*, *S. boliviense*, *S. vernei* y *S. tuberosum* grupo *Andigenum*. En las cuatro ESP se han descripto caracteres de interés para el fitomejoramiento. A través del desarrollo de talleres de educación ambiental y del monitoreo durante dos años consecutivos dentro de un sitio prioritario a nivel mundial, establecimos una línea de base sobre la que se proyectará la conservación *in situ* para preservar un componente indispensable del patrimonio natural y cultural de América.

Palabras clave

Áreas Protegidas • conservación *in situ* • parientes silvestres de los cultivos • recursos fitogenéticos para la alimentación y la agricultura • *Solanum acaule* • *Solanum boliviense* • *Solanum brevicaule* • *Solanum vernei*

Introduction

Warranting global food security now and for the future is the greatest challenge. In order to face it, increases in productivity, resilience and sustainability of current agricultural systems are necessary. Crop wild relatives (CWR), which include crop ancestors and genetic related species, have useful genetic diversity to produce crop varieties with drought, heat and cold tolerance, high nutritional quality and pest and disease resistance. For these characteristics, CWR are essential components of the genetic resources for food and agriculture, contributing to address the productivity and sustainability of farming systems.

The potatoes represent an essential example addressing the importance of CWR germplasm in global food security needs (29). The potato (*Solanum tuberosum* L.) is the third more important food crop worldwide (17) and was probably one of the first cultivated species in which genetic improvement was carried out for resistance to diseases (23). The late blight disease in Ireland during the years 1845 and 1846 produced the total loss of potato production due to the low genetic diversity in cultivated germplasm. Towards the end of the 19th century and the beginning of the 20th century, potato improvement programs focused on obtaining *Phytophthora* resistance using a single wild potato species: *Solanum demissum* (24). This strategy changed completely in the 1920s, when efforts were made to collect wild germplasm in the Andean countries of South America and Mexico. Explorations were realized from 1925 to 1967, and at least, 33 prospecting and collection voyages of wild germplasm and cultivated potato were carrying out (22). This *ex situ* conservation strategy initially leaded by the Vavilov's team, continued with a broad development during the second half of the 20th century (27, 29), until some authors suggested that for some wild potato species no further collecting are required (9).

Alternatively, *in situ* conservation of potato wild relatives has an incipient development. Around 50 years ago, it was already recognized that wild species are most effectively preserved in their natural state (18). Later on, the Convention on Biological Diversity (10) in its Article 9 state that the *ex situ* conservation could be implemented predominantly for the purpose of complementing *in situ* measures. However, *ex situ* strategies still prevail in potato conservation and in contrast to the CBD recommendations *in situ* programs have rarely been assessed.

The great long-term challenge of *in situ* conservation of CWR is the creation of Genetic Reserves, which implies the location, designation, management and monitoring of wild populations in their natural habitat to maintain their genetic diversity. In economic and politic terms, the most effective method to implement Genetic Reserves is to establish them in existing Protected Areas (35). Also, it is necessary to count with information related to species distribution, phenology, demography, genetics, ecology, politic and socioeconomic studies (16).

Protected Areas are geographically defined zones, designated, regulated and administered in order to reach specific conservation objectives and are the central axis of the national and international strategies of *in situ* conservation of the CWRs. In Argentina, the National System of Protected Areas is under the application authority of the National Parks Administration (APN in Spanish). In addition, there are provincial systems of Protected Areas. Taken together, all Protected Areas in Argentina created and administered by national, provincial or municipal organizations, or by NGOs or private entities, are integrated in the Federal System of Protected Areas (SIFAP in Spanish).

In Argentina, since 2006 the Potato Active Genebank of the Balcarce Agricultural Experimental Station (BAL) of the National Institute of Agricultural Technology (INTA in Spanish) started with *in situ* conservation initiatives, prospecting and collecting potato wild relatives in national Protected Areas. As a result of these activities, 12 wild potato species were identified inside these areas (13). In the same vein, since 2011, the Institute of Agricultural Biology of Mendoza (IBAM) is developing a pioneering *in situ* conservation program of potato wild relatives within Protected Areas. This challenge was assumed in an integral way, contemplating the generation of knowledge, the interaction with governmental and private entities linked to the management of natural resources, the training of Protected Areas professionals and the spreading of this initiative in academia and among the general public by means of environmental education workshops. Working with *Solanum kurtzianum* Bitter & Wittm., the wild potato of Argentina best adapted to dry environments, a baseline was generated with distribution data, biotic and abiotic interactions, population dynamics, phenotypic and genetic variability of natural populations of this species (28, 33, 34). Based on this information, a management plan for *in situ* conservation of potato wild relatives was presented and a working protocol was generated to be implemented at the national and regional level (34). At the same time, in the Paititi Natural Reserve (Buenos Aires) started an *in situ* conservation initiative of *Solanum commersonii* Dunal (19), a wild potato species that is important for genetic improvement because it has genes for resistance/tolerance to biotic and abiotic stresses that affect the crop (14). In addition, it is cited by Sajama as the highest priority to carry out conservation actions since it is one of the wild potato species that is losing the most geographical range (43).

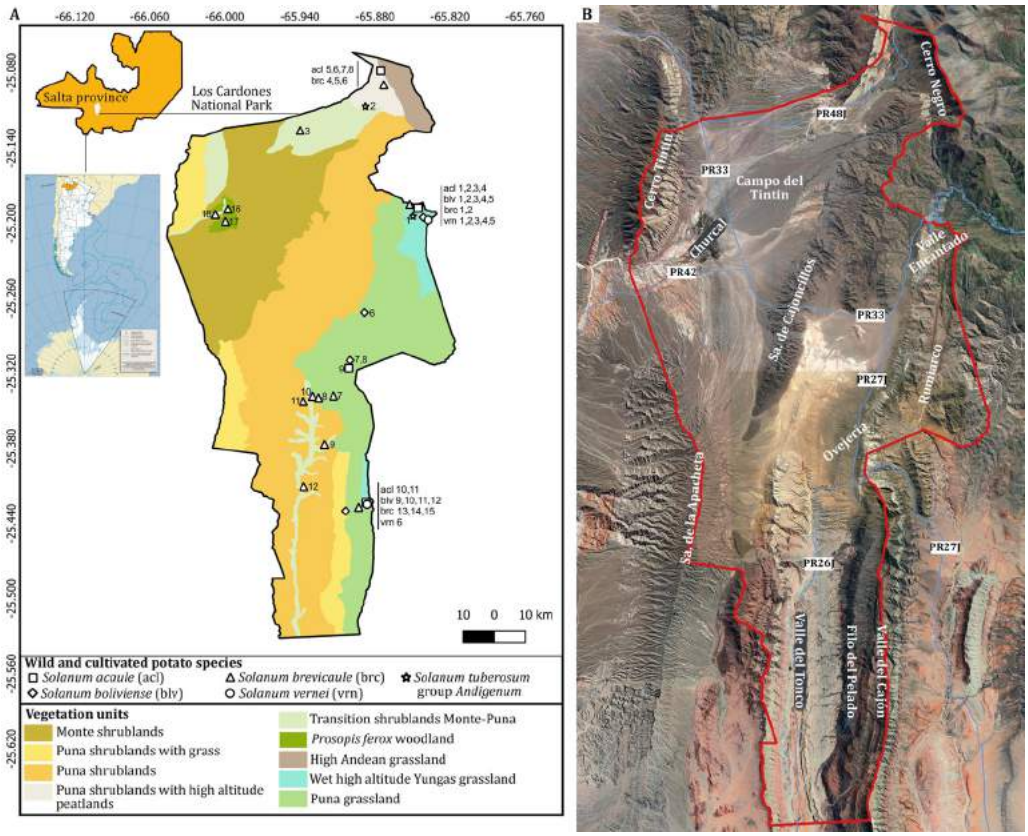
Based on the overlap of geographic coordinates of potato genebank accessions with those of the Argentine Protected Areas and on prospecting and collecting expeditions, Los Cardones National Park (LCNP) was identified as one of the priority sites for establishing a Genetic Reserve by its wild potato species richness (13, 34). LCNP was established in 1996. It is located in the western of Salta province in the localities of Cachi and San Carlos and covers an area of 64,117 hectares. Mountain ranges running north to south with a wide altitudinal range from 2600 to 5226 m a. s. l. (1), interspersed by narrow valleys that generates remarkable diversity of environments, based upon differences especially in precipitation and temperature. This environmental heterogeneity determines a high diversity of plant communities within the park. In fact, these characteristics are the basis of the creation of the LCNP. Five biogeographic provinces are represented in LCNP, namely Yungas, Monte, Puna, Prepuna and High Andean realms (6). According to Sánchez (2009), Puna and Monte biogeographical provinces occupy 90% approximately of the total area of LCNP and harbour the greatest number of vegetation units mainly shrublands but also grasslands. Yungas, Prepuna and the High Andes occupy a much smaller area, with unique vegetation units such as wet high-altitude Yungas grasslands, *Prosopis ferox* woodland, and High Andean grasslands respectively. We hypothesize that WPS from LCNP are associated with different vegetation units.

In the present work, we present results of two prospection and collection expeditions in Yungas, Monte, Puna and High Andean biogeographic provinces within LCNP and awareness activities through communication, education, and participative activities that were performed to establish a Genetic Reserve of wild relatives of one of the most important crop worldwide which is part of the natural and cultural heritage of the Americas.

Materials and methods

Two prospection and collection expeditions took place in LCNP, the first was achieved from March 6-11, 2017 and the second one was realized from March 5-9, 2018.

Five biogeographic provinces were recognized in LCNP according to Cabrera (1994): Yungas, Monte, Puna, Prepuna and High Andean. Within each biogeographic province different vegetation units were identified (45), where wild potato species were located: i) wet high-altitude Yungas grasslands (Site: Valle Encantado and Valle del Cajón), ii) Puna shrublands with high altitude peatland and transition shrublands Monte-Puna (Site: Cerro Negro, and road to Cerro Negro), iii) Puna grasslands (Site: Ovejería and Filo del Pelado), iv) transition shrublands Monte-Puna (site: Valle del Tonco) and v) *Prosopis ferox* woodland (Site: El Churcal) (figure 1 and table 1, page 465-466). We selected collection sites based on a previous expedition report carried out by INTA in 2010. Populations were defined *a priori* based on spatial distribution of plants and geographic characteristics (presence of physical barriers between populations). In general, plants spaced more than fifty meters were considered belonging to different populations. Population size was determined by counting all plants in an area of about 25x25 meters. Hybridization and gene flow are frequent in populations of wild potato species; therefore, we need to analyse the genetic structure of all sampled populations to corroborate the category of population assigned.



A. Distribution of populations of the wild potatoes species *Solanum acaule, Solanum boliviense*, *Solanum brevicaule* and *Solanum vernei*; and the cultivated species *Solanum tuberosum* group *Andigenum* in different vegetation units. **B.** Relief map of Los Cardones National Park and location of collection sites.

A. Distribución de poblaciones de las especies silvestres de papas *Solanum acaule*, *Solanum boliviense*, *Solanum brevicaule* y *Solanum vernei*; y la especie cultivada *Solanum tuberosum* grupo *Andigenum* en diferentes unidades de vegetación. **B.** Mapa de relieve del Parque Nacional Los Cardones y ubicación de los sitios de muestreo.

Figure 1. Potato species within Los Cardones National Park, Salta province, Argentina.

Figura 1. Especies de papa dentro del Parque Nacional Los Cardones, provincia de Salta, Argentina.

Table 1. Collected and monitored populations of wild and cultivated potato species in Los Cardones National Park during two years (2017-2018).

Tabla 1. Poblaciones de especies silvestres y cultivadas de papa recolectadas y monitoreadas en el Parque Nacional Los Cardones durante dos años (2017-2018).

Species	Population code	Collector number/ Germplasm collection[a]	Localization			Population size[b]		Phenological stage[c]	
			Site	Latitude (S) Longitude (W)	Altitude (m a. s. l.)	2017	2018	2017	2018
Solanum acaule	acl1	GaSAMaIb2/S, P, H	Valle Encantado	25°11.798' 65°50.454'	3083	>100	>200	Fr	Fr
	acl2	GaSAMaIb10/S, H	Valle Encantado	25°11.512' 65°50.386'	3110	~40	NM[b]	Fr	NM
	acl3	GaSAMaIb12/S, H	Valle Encantado	25°11.267' 65°50.726'	3174	~60	NM	Fr	NM
	acl4	DiLoMaKIb 6/S, H	Valle Encantado	25°11.691' 65°51.010'	3186	NM	~100	NM	Fl/Fr
	acl5	GaSAMaIb15/S, P, H	Cerro Negro	25°04.686' 65°52.527'	3952	>100	>500	Fl/Fr/Wi	Fl/Fr
	acl6	GaSAMaIb17/S, H	Cerro Negro	25°05.121' 65°52.469'	3878	>100	>500	Fr	Fr
	acl7	GaSAMaIb18/S, H	Cerro Negro	25°05.558' 65°52.186'	3867	>300	NM	Fl	NM
	acl8	DiLoMaKIb30/S, H	Cerro Negro (limit LCNP)	25°04.006' 65°52.546'	4024	NM	>200	NM	Fl/Fr
	acl9	GaSAMaIb20/S, P, T, H	Ovejería	25°18.948' 65°54.053'	3228	>100	>300	Fl/Fr	Fl/Fr
	acl10	DiLoMaKIb43/S, H	Valle del Cajón	25°25.446' 65°53.273'	3089	NM	>50	NM	Fl/Fr
	acl11	DiLoMaKIb44/S	Valle del Cajón	25°25.388' 65°53.225'	3095	NM	~20	NM	Fl/Fr
Solanum boliviense	blv1	GaSAMaIb3/S, P, H	Valle Encantado	25°11.784' 65°50.449'	3083	>100	>200	Fr	Fl/Fr
	blv2	GaSAMaIb6/S, P, H	Valle Encantado	25°11.128' 65°50.559'	3095	~40	NM	Fr	NM
	blv3	GaSAMaIb7/S, H	Valle Encantado	25°11.719' 65°50.484	3106	~100	NM	Fr/Wi	NM
	blv4	GaSAMaIb9/S, H	Valle Encantado	25°11.512' 65°50.386'	3110	>300	NM	Fr/Wi	NM
	blv5	DiLoMaKIb3/S, H	Valle Encantado	25°11.838' 65°50.244'	3168	NM	~60	NM	Fl/Fr
	blv6	DiLoMaKIb11/S, H	Ovejería	25°16.288' 65°53.298'	3154	NM	>100	NM	Fl/Fr
	blv7	GaSAMaIb19/P, H	Ovejería	25°18.568' 65°53.991'	3219	>1000	>1000	V	Fl/Fr
	blv8	DiLoMaKIb15/S, P, H	Ovejería	25°19.008' 65°54.056'	3229	NM	>1000	NM	Fl/Fr
	blv9	DiLoMaKIb39/S, H	Filo del Pelado	25°25.799' 65°54.208'	3247	NM	~100	NM	Fr
	blv10	DiLoMaKIb41/H	Filo del Pelado	25°25.841' 65°53.993'	3275	NM	~40	NM	V
	blv11	DiLoMaKIb42/S, H	Valle del Cajón	25°25.621' 65°53.583'	3160	NM	~80	NM	Fl/Fr
	blv12	DiLoMaKIb45/S, P, H	Valle del Cajón	25°25.474' 65°53.166'	3100	NM	>100	NM	Fl

[a] Germplasm collected for *ex situ* conservation in the active gene bank (BAL): S: seeds; T: tubers; P: plants; H: herbarium. [b] Number of plants. NM: Not monitored. [c] V: vegetative stage (plants without flower nor fruits); Fl: flowering; Fr: fruit development; Wi: withering. [d] Number of different cultivars present in farmers' field.

[a] Germoplasma recolectado para conservación *ex situ* en el banco activo (BAL): S: semillas; T: tubérculos; P: plantas; H: herbario. [b] Número de plantas. NM: No monitoreada. [c] V: estado vegetativo (plantas sin flores ni frutos); Fl: floración; Fr: frutos en desarrollo; Wi: marchitez. [d] Número de cultivares presentes en las huertas de los agricultores.

Table 1 (cont.). Collected and monitored populations of wild and cultivated potato species in Los Cardones National Park during two years (2017-2018).

Tabla 1 (cont.). Poblaciones de especies silvestres y cultivadas de papa recolectadas y monitoreadas en el Parque Nacional Los Cardones durante dos años (2017-2018).

Species	Population code	Collector number/ Germplasm collection[a]	Localization			Population size[b]		Phenological stage[c]	
			Site	Latitude (S) Longitude (W)	Altitude (m a. s. l.)	2017	2018	2017	2018
Solanum brevicaule	brc1	GaSAMaIb25/S, H	Valle Encantado	25°11.089' 65°51.135	3282	>100	>200	V/Fr	Fl/Fr
	brc2	GaSAMaIb5/T, P, H	Valle Encantado	25°11.128' 65°50.559'	3095	~20	NM	V	NM
	brc3	GaSAMaIb13/S, P, T, H	road to Cerro Negro	25°07.543' 65°56.383'	3208	>1000	>1000	Fl/Fr	Fl/Fr
	brc4	GaSAMaIb14/P, H	Cerro Negro	25°05.949' 65°52.251'	3756	~80	~80	Fl	Fr
	brc5	DiLoMaKIb34/S, H	Cerro Negro	25°05.361' 65°52.373'	3864	NM	~80	NM	Fr
	brc6	GaSAMaIb16/S, P, H	Cerro Negro	25°04.912' 65°52.567'	3926	~70	>100	Fr	Fl
	brc7	GaSAMaIb21/S, P, H	Ovejería	25°20.276' 65°54.782'	3213	>100	>100	Fr	Fl/Fr
	brc8	GaSAMaIb22/S, P, H	Valle del Tonco	25°20.366' 65°55.497'	3156	>100	>600	Fr	Fl/Fr
	brc9	GaSAMaIb23/P, H	Valle del Tonco	25°22.602' 65°55.216'	3046	>100	>100	V	Fr
	brc10	DiLoMaKIb19/S, H	Valle del Tonco	25°20.295' 65°55.795'	3142	NM	>100	NM	V
	brc11	DiLoMaKIb20/S, H	Valle del Tonco	25°20.543' 65°56.236'	3141	NM	>1000	NM	Fl/Fr
	brc12	DiLoMaKIb22/S, P, H	Valle del Tonco	25°24.625' 65°56.213'	2873	NM	~100	NM	Fr
	brc13	DiLoMaKIb38/S, P, H	Filo del Pelado	25°25.799' 65°54.208'	3247	NM	~30	NM	Fr
	brc14	DiLoMaKIb40/H	Filo del Pelado	25°25.842' 65°53.993'	3275	NM	~20	NM	V
	brc15	DiLoMaKIb42/S, H	Valle del Cajón	25°25.621' 65°53.583'	3160	NM	~30	NM	Fr
	brc16	GaSAMaIb24/P, H	El Churcal	25°11.319' 65°59.843'	2856	~30	NM	V	NM
	brc17	DiLoMaKIb9/P, H	El Churcal	25°11.908' 65°59.956'	2851	NM	~50	NM	V/Fl
	brc18	DiLoMaKIb10/P, H	El Churcal	25°11.580' 66°00.453'	2819	NM	~15	NM	Wi
Solanum vernei	vrn1	GaSAMaIb1/S, P, T, H	Valle Encantado	25°11.846' 65°50.498'	3087	>100	>100	Fr	Fr
	vrn2	GaSAMaIb4/S, T, H	Valle Encantado	25°11.784' 65°50.449'	3089	>100	>200	Fr	V/Fl
	vrn3	GaSAMaIb8/S, P, T, H	Valle Encantado	25°11.617' 65°50.434'	3100	>100	>100	Fr	V
	vrn4	DiLoMaKIb2/S, P, H	Valle Encantado	25°11.838' 65°50.244'	3168	NM	~50	NM	V/Fl
	vrn5	DiLoMaKIb5/P, T, H	Valle Encantado	25°11.690' 65°51.010'	3186	NM	>200	NM	Fl
	vrn6	DiLoMaKIb46/P, H	Valle del Cajón	25°25.474' 65°53.166'	3100	NM	~15	NM	V
	vrn7	GaSAMaIb 11/P, T, H	Valle Encantado	25°11.512' 65°50.386'	3110	NM	~40	V	NM
Solanum tuberosum group *Andigenum*	adg1	DiLoMaKIb8/T	Valle Encantado	25°11.666' 65°50.097'	3189	NM	4[d]	NM	-
	adg2	DiLoMaKIb29/T	Cerro Negro	25°06.408' 65°53.251'	3517	NM	8[d]	NM	-

[a] Germplasm collected for *ex situ* conservation in the active gene bank (BAL): S: seeds; T: tubers; P: plants; H: herbarium. [b] Number of plants. NM: Not monitored. [c] V: vegetative stage (plants without flower nor fruits); Fl: flowering; Fr: fruit development; Wi: withering. [d] Number of different cultivars present in farmers' field.

[a] Germoplasma recolectado para conservación *ex situ* en el banco activo (BAL): S: semillas; T: tubérculos; P: plantas; H: herbario. [b] Número de plantas. NM: No monitoreada. [c] V: estado vegetativo (plantas sin flores ni frutos); Fl: floración; Fr: frutos en desarrollo; Wi: marchitez. [d] Número de cultivares presentes en las huertas de los agricultores.

In total, 50 populations were sampled as true seeds, plants or tubers depending on the phenological stage of the populations. Phenological stages were defined as: shoot development (plant without flowers nor fruits), flowering, fruit development and withering (table 1, page 465-466). In 2017, 25 populations located in five different vegetation units and seven sites (Valle Encantado, Cerro Negro, road to Cerro Negro, Ovejería, Filo del Pelado, Valle del Tonco and El Churcal) were sampled. In 2018, populations were monitored and re-sampled and a new site, Valle del Cajón, was prospected and populations of the four wild potato species were found and collected.

In each collection site photographs were taken and the following information was recorded: date, geographical coordinates, altitude (m a.s.l.), number of plants per population, phenological stage, evidence of cattle grazing, trampling and dunging. Also, the target species (*Solanum acaule*, *S. boliviense, S. brevicaule, S. vernei*) were identified and collected. Depending on the phenological stage of the plants in each population, the type of plant material collected was: berries (B), tubers (T), and whole plant (P) (table 1, page 465-466). In each site all the plant species that grows together with the target species were identified, considering them as accompanying flora. The species were identified in the Agricultural Botany Laboratory (Faculty of Agricultural Sciences/ Mar del Plata National University) through observation under magnifying glass and the use of specific bibliography. Besides, herbarium specimens were made from potato wild relatives and accompanying flora, and finally were deposited at the Herbarium BAL - Integrated Unit Balcarce (Agricultural Experimental Station Balcarce/ National Institute of Agricultural Research and Faculty of Agricultural Sciences/ Mar del Plata National University). Farmers in Cerro Negro and Valle Encantado were visited. We carried out qualitative interviews focused on cultivation and conservation practices and inquiring about knowledge of wild potato species.

To characterise each year (2017 and 2018), environmental data from the nearest weather station of National Meteorological Service (SMN in Spanish) was used. "Salta Aero" meteorological station is located 24°50.565'S 65°28.972'W, approximately 50 kilometers northeast from the LCNP. Average temperature, accumulated precipitation and relative humidity from October to March were estimated for 2016- 2017 and 2017-2018 seasons. The biogeographic provinces and vegetation units present in LCNP were described according to Cabrera (1994) and Sánchez *et al.* (2015).

Results and discussion

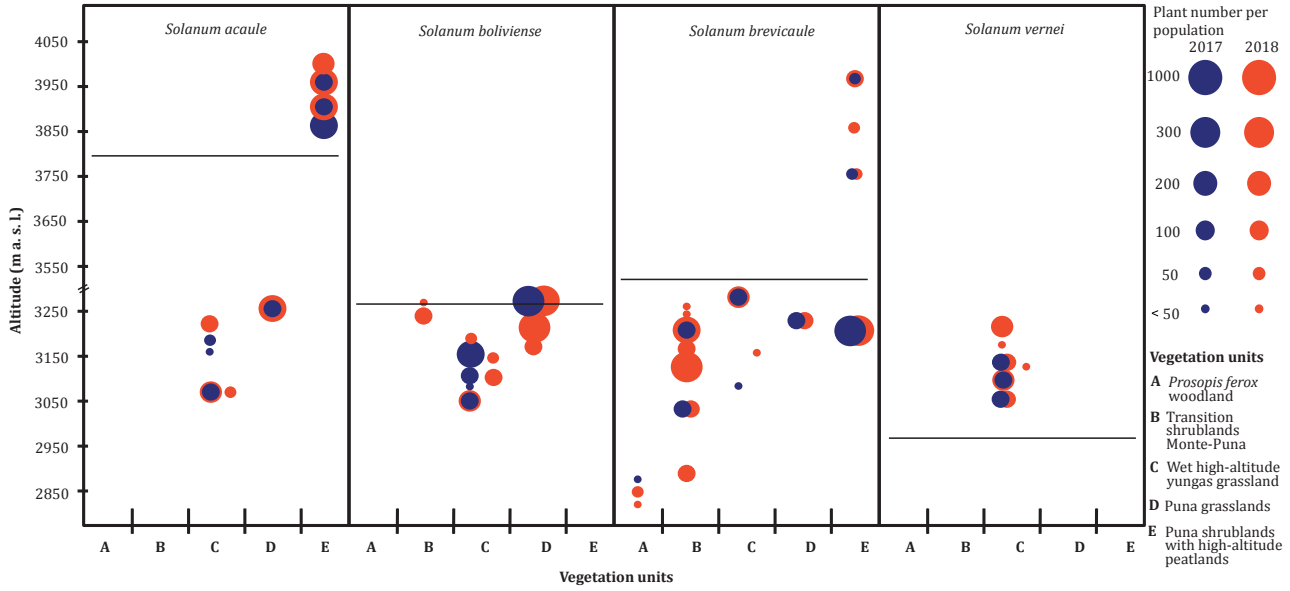
In the two prospecting and collecting expeditions to LCNP, four wild and one cultivated potato species were *in situ* identified: *Solanum acaule* Bitter (acl), *S. boliviense* Dunal (blv), *S. brevicaule* Bitter (brc), *S. vernei* Bitter & Wittm (vrn) and *S. tuberosum* group *Andigenum* (adg), respectively.

In wet high-altitude Yungas grasslands the greatest number of wild potato species was found, growing together with a high cover of herbaceous monocot and dicot species on the misty mountains of Valle Encantado and Valle del Cajón (figure 1, page 464). Evidence of cattle grazing, trampling and dunging was observed. Sympatric populations of the four species were found in Valle Encantado on both expeditions, whereas in Valle del Cajón very small distances separated populations (maximum 200 meters). In Valle Encantado Hugo Zerpa's farm was visited. He and his family cultivated Andean potatoes (*S. tuberosum* group *Andigenum*) and oca (*Oxalis tuberosa*) (table 1, page 465-466). Near the farm, populations of *S. vernei, S. boliviense* and *S. acaule* were growing.

In Cerro Negro we found two species: *S. acaule* in high altitudes above 3867 meters (in Puna shrublands with high altitude peatlands) and *S. brevicaule* in a wide range, from 3208 to 3926 meters [transition shrublands Monte-Puna and Puna shrublands with high altitude peatland (figure 2, page 468)]. Remarkable phenotypic differences (*i.e.* corolla color and shape and leaf pubescence) were detected among *S. brevicaule* populations. Populations in lower altitude showed pubescent leaf, light-purple and stellate corollas whereas populations from high altitude had no pubescent leaf and presented purple and pentagonal corollas. Populations of *S. acaule* were found mainly in peatlands (*i.e.* patches of herbaceous vegetation with permanent waterlogging).

In Cerro Negro, a farm was visited where Elsa and Elba Colque cultivate Andean potatoes in a small orchard; also commercial cultivated potatoes (*Solanum tuberosum* L.) were present in the field. In the Andean landraces, open pollinated seeds were observed. The farmers indicated that they do not use open pollinated seed for generated new germplasm, an ethnobotanical practice documented in other Andean farmer systems (40). Also, they evaluated as unlikely that some seeds produced by open-pollination of the potato landraces will germinate and introduce new genotypes to their farmer system. A common and ancestral practice in the region is the tuber exchange in local markets. The farmers exchange and often purchase tubers in markets in different localities of Salta province with the aim of increasing the diversity in their farms. Participatory exchanges of tubers among farmers is important to help them cope with environmental adversity and to avoid the loss of crop diversity. Adjacent to the orchard a *S. brevicaule* population (brc5) was found, a wild species that could contribute with its pollen in the origin of the open pollinated seed observed in landraces. Previous studies had demonstrated gene flow in the Andes between wild species and potato cultivars (11, 46). In order to evaluate this possibility in LCNP, berries from Andean landraces were collected in 2018. By molecular markers, gene flow between Andean landraces and *S. brevicaule* population will be determined.

In Puna grasslands, populations of *S. acaule*, *S. boliviense* and *S. brevicaule* were identified. In this vegetation unit, in the site named Ovejería, the largest population of *S. boliviense* (blv7) was found during both expeditions (figure 1, page 464; table 1, page 465-466). In 2018, most notoriously, the prospected areas in Ovejería were covered by *S. boliviense* in combination with grasses. Within this continuous of *S. boliviense*; *S. acaule* patchy populations were identified.



Horizontal lines show the mean altitude described for each species (26).

Overlapped circles show the same population surveyed in 2017 and 2018.

Líneas horizontales indican la altitud media descripta para cada una de las especies (26).

Círculos solapados muestran la misma población censada en 2017 y 2018.

Figure 2. Altitudinal range of the wild potatoes species *Solanum acaule, Solanum boliviense*, *Solanum brevicaule* and *Solanum vernei* within Los Cardones National Park, Salta province, Argentina. Circle area is a schematic representation of the population size surveyed in five vegetation units.

Figura 2. Rango altitudinal de las especies silvestres de papas *Solanum acaule*, *Solanum boliviense*, *Solanum brevicaule* y *Solanum vernei* dentro del Parque Nacional Los Cardones, provincia de Salta, Argentina. El área de los círculos es una representación esquemática del tamaño de las poblaciones censadas en cinco unidades de vegetación.

Several of the plants *in situ* evaluated, showed intermediate phenotypes between these two wild potato sympatric species, observation that allow inferring the presence of hybrids. Natural hybrids between *S. acaule* and *S. boliviense* had been already reported in northwest Argentina (38), and the documented scenario in Ovejería represents an ideal opportunity to study the natural hybridization as a source of variability in potatoes (7, 8, 31, 32).

On the way from Valle del Tonco to Valle del Cajón in a site named Filo del Pelado (vegetation units Puna grasslands) *S. boliviense* and *S. brevicaule* populations were found (figure 1, page 464). No morphological evidence about the presence of hybrids between these two species was observed in the field.

In transition shrublands Monte-Puna (Valle del Tonco) only *S. brevicaule* populations were found. An increase in aridity and a decrease in vegetation cover were observed while further south of the valley was reached. Evidence of plants having been eaten by some rodent species was detected. The most southern population sampled in this site showed strong symptoms of water deficit stress (brc12). In 2017 only one population of *S. brevicaule* (brc16) was sampled in the *Prosopis ferox* woodland whereas in 2018 this population was monitored and two new ones of the same species were sampled (brc17 and brc18).

Solanum vernei presented the narrowest distribution, natural populations were found in one vegetation unit within LCNP (wet high-altitude Yungas grasslands), from 3087 to 3186 m a.s.l. *Solanum boliviense* natural populations presented a narrow limit distribution and were found in three vegetation units (Puna grasslands, wet high-altitude Yungas grasslands and transition shrublands Monte-Puna), from 3083 to 3275 m a.s.l. *Solanum acaule* natural populations were found in three vegetation units (Puna grasslands, wet high-altitude Yungas grasslands and Puna shrubland with high altitude peatland) from 3083 to 4024 m a.s.l. The species with widest distribution was *S. brevicaule*, which was found in five vegetation units and from 2819 to 3926 m a. s. l. (figure 2, page 468). Natural populations of three species (*S. acaule*, *S. boliviense* and *S. brevicaule*) were found near the mean altitudinal range previously described (26). However, *S. vernei* was found in higher sites compared with previous reports (26) (figure 2, page 468).

During the two expeditions it was not possible to find populations of *S. microdontum* nor *S. venturii*. Clausen *et al.* (2018), based on data from an expedition performed on March 2010, mentioned these two species as present in two populations of LCNP. In 2018, which was as a favorable year for the growth of wild potato species due to the abundant precipitations, the absence of *S. microdontum* and *S. venturii* populations in the same site collected in 2010, indicates that the distribution of these species within LCNP is very limited and with small population sizes.

It has been informed that most often potato populations are very small (<100 plants) (15). These observations seem not to reflect the situation in LCNP, where above the 50% of the monitored population were composed by more than 100 plants. The potato population sizes are highly influenced by rainfall amount and timing (34). During the two expeditions, slight differences in sizes were observed for some populations (table 1, page 465-466 and figure 2, page 468). Climatological data were obtained from the LCNP nearest weather station in order to generate a baseline for future correlations with population sizes. Between January 2017 and January 2018 there was a difference of 100 mm in rainfall and 6.3% in RH (figure 3, page 470). Higher rainfall and humidity could play a role in the higher population sizes observed in 2018. However, to have reliable and comprehensive information about the influence of climatological variables on population size and phenology, population monitoring must be performed for a longer period (34). Considering the climatic heterogeneity in LCNP environmental data from meteorological stations in the park will be also necessary.

Plant community's heterogeneity and probably richness of wild potato species in LCNP are related with environmental conditions. According to Noé *et al.* (2012), central and western areas of LCNP are semiarid with mean annual precipitations of 200 mm approximately and mean annual temperature of 12°C, whereas in the eastern area increases in relative humidity and precipitation and decreases in temperature were reported (annual precipitation from 300 to 500 mm with a mean temperature of 9°C).

Livestock grazing is common in every site where we found wild potato species. Ugent (1981), reported the presence of seeds of *S. acaule* in animal droppings and also seedling emerging from fecal pellets in the Andes of Bolivia. It would be interesting to evaluate the contribution of domesticated animals to seed dispersal of wild potatoes species in LCNP. The current Management Plan for LCNP (1) contemplates total exclusion of cattle in certain areas like Cerro Negro and Valle Encantado; situation that could provide a good opportunity to study whether the presence of cattle represents a risk for wild potato populations or, in the other hand, contributes to their dispersion within the LCNP.

A widely used criterion for the CWR's conservation prioritizing is the gene pool concept defined by Harlan and de Wet (1971), which allows classifying species in primary, secondary, and tertiary "genepools" based on the degree of crossability of germplasm with cultivated species. Considering LCNP as a Genetic Reserve, three species of the primary gene pool (*S. acaule*, *S. brevicaule* and *S. vernei*) and one of the secondary gene pool (*S. boliviense*) are being *in situ* conserved (9, 14). In addition, several characters of breeding interest have been described for the four species (figure 4, page 471). Species of the primary gene pool can be directly crossed with cultivated species facilitating introgression of desirable traits. Whereas in species from the secondary gene pool certain reproductive barriers tend to difficult crosses.

Regarding to the *ex situ* conservation, the BAL active genebank contains germplasm of the four species found in LCNP. BAL active genebank initiated its activities in 1970 and its main objectives were to prospect, collect, conserve, characterize, evaluate and distribute germplasm of all the tuberous *Solanum* grown in Argentina, including CWR and cultivated species; and also germplasm from neighbouring countries. There are conserved 215 accessions of *S. acaule*, 27 of *S. boliviense*, 245 of *S. brevicaule* and 25 of *S. vernei*, most of them collected in Argentina, mainly from Northwest provinces.

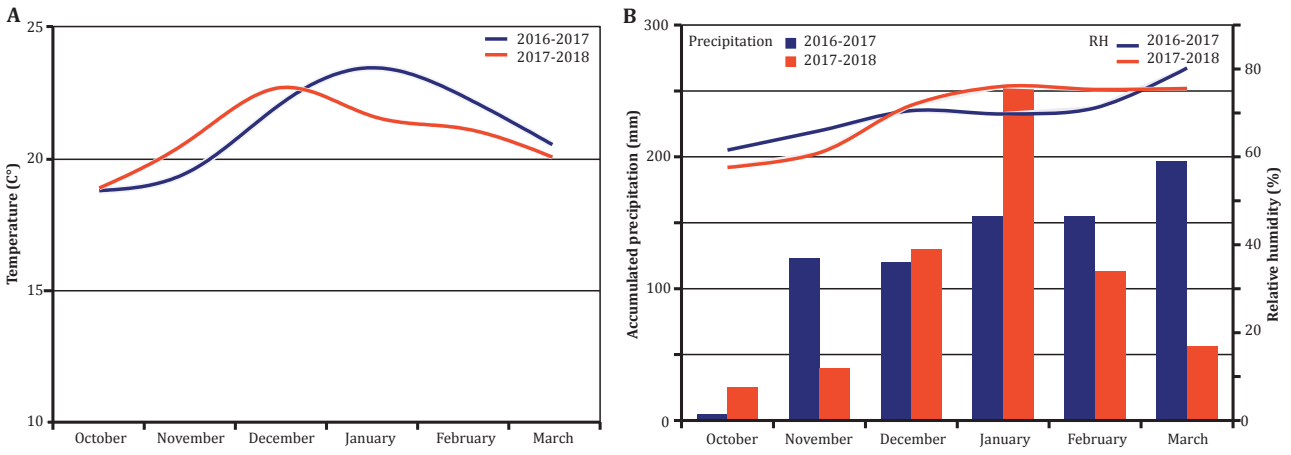


Figure 3. Expedition year characterization based on environmental data. **A.** Mean temperature from October to March for 2016-2017 and 2017-2018. **B.** Accumulated precipitation and relative humidity (RH) from October to March for 2016-2017 and 2017-2018.

Figura 3. Caracterización de los años de expedición sobre la base de datos climáticos**.** **A.** Temperatura media desde octubre a marzo para 2016-2017 y 2017-2018. **B.** Precipitación acumulada y humedad relativa (RH) desde octubre a marzo para 2016-2017 y 2017-2018.

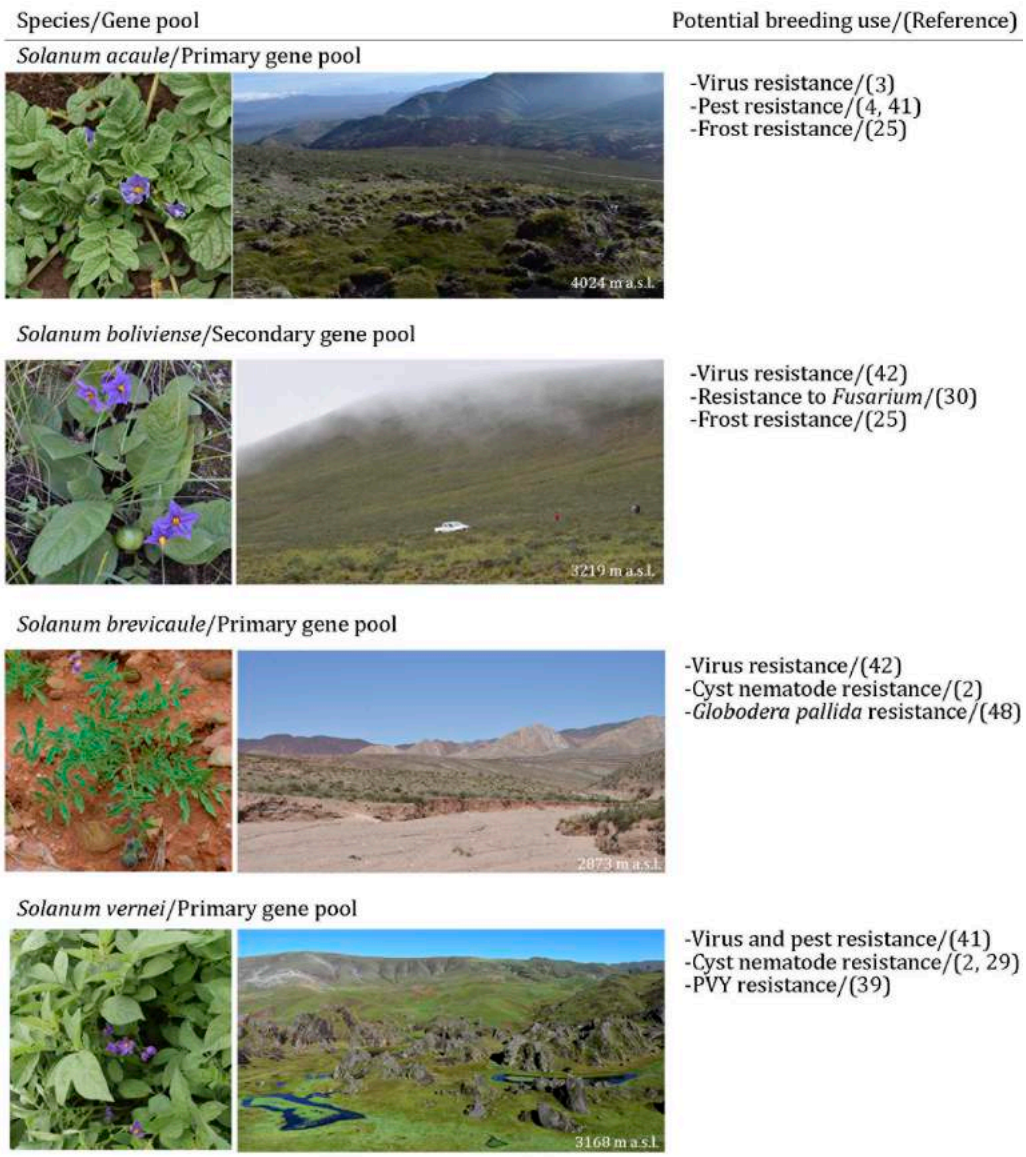


Figure 4. Representative plants and habitats of the four wild potato species monitored in Los Cardones National Park. *Solanum acaule*, Puna shrublands with high altitude peatlands; *Solanum boliviense*, Puna grassland; *Solanum brevicaule*, transition shrubland Monte-Puna; *Solanum vernei*, wet high-altitude Yungas grassland. According to the crossability with that cultivated potato the species are classified within the primary and secondary crop gene pool and in the four species several breeding interest characters have been described.

Figura 4. Fotos representativas de plantas y hábitats de las cuatro especies de papas silvestres monitoreadas en el Parque Nacional Los Cardones. *Solanum acaule*, arbustal puneño con vegas de altura; *Solanum boliviense*, pastizal puneño; *Solanum brevicaule*, arbustal de transición Monte-Puna; *Solanum vernei*, pastizal de neblina yungueño. De acuerdo con la posibilidad de cruzamiento con la papa cultivada, las especies se clasifican dentro del acervo genético primario y secundario del cultivo y en las cuatro especies se han descrito varios caracteres de interés para el mejoramiento genético.

For the cultivated species like *Solanum tuberosum* group *Andigenum*, clonal maintenance is required and *in vitro* conservation is implemented (12). There are 400 accessions of Andean potato landraces present in the genebank, mainly collected in the valleys and gorges of the Puna and Prepuna biogeographical provinces and also from local markets.

Local communities play a key role in the sustainability of any conservation program (20). Community based conservation programs apply different strategies to encourage participation and engagement of local communities and society in general, in order to achieve desired conservation goals. For example, some initiatives include the creation of socio-economic incentives for conservation and giving communities control over local natural resources (5). Changing individual or community behaviour is very complex but it is essential to achieve success in a conservation programme (36). In this sense, promoting a strategy of co-management which includes collaborative activities and interactions among local community of LCNP, Park Rangers, APN, INTA, and IBAM is fundamental to reach conservation goals in Protected Areas of Argentina, particularly in LCNP.

Development of this project has consolidated an interdisciplinary and intersectoral team. During 2017 and 2018 actions of communication, education, participation and awareness activities have been carried out. Two workshops were performed in the northwest branch office of Administration of National Parks (APN) in Payogasta, Salta. During the workshops advances in the project were exposed. A fruitful exchange of ideas and opinions was generated.

Currently, the groups are working together in initiatives related to environmental education. One of them is to develop an interpretive trail in Valle Encantado (a public and tourist site in the LCNP). Trail planning and layout were already done. Installation of interpretive panels is the next step. Workshops in local schools will be organized to promote knowledge and valorisation of wild and domesticated potatoes, which are part of our natural and cultural heritage. The interpretive trail in Valle Encantado will be used by tourists and also by the local school community.

Among the conservation values defined in the Management Plan for LCNP (1), the "useful or potentially useful plant genetic resources" were identified, including *Solanum* species. The presence of wild potato populations was known based on surveys carried out by INTA technicians (13). Years later, the implementation of the present program allowed increasing knowledge regarding the presence, diversity and geographic distribution of potato populations, thus cementing the basis for a specific conservation plan for this gene pool. It is important to emphasize that the existing populations are currently conserved in an indirect or passive way, through the protection of the habitats that contain them, and no concrete conservation actions on them are being undertaken.

Conclusions

The results of the two-year monitored populations represent a baseline for further strategy for long-term monitoring, which could require identifying threats and to develop mitigation measures, Only through a comprehensive conservation plan will be possible to consolidate the *in situ* Genetic Reserve of these populations.

The coming tasks may be summarized as follows: to study the morphological variability in the collected populations, pollen viability and sexual compatibility within and among collected populations and their genetic structure by molecular markers. Once all these issues are resolved, a monitoring and conservation strategy will be proposed and implemented for the *in situ* conservation of potato wild relatives in LCNP. Also, an adequate strategy for sampling populations for *ex situ* conservation in BAL genebank will be designed. Following this procedure, we hope to concretize one of the great challenges of the phytogenetic resources conservation: to reach the complementation between the *in* and *ex situ* conservation strategies.

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Male sterility and somatic hybridization in plant breeding

Androesterilidad e hibridación somática en el mejoramiento vegetal

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Abstract

Plant male sterility refers to the failure in the production of fertile pollen. It occurs spontaneously in natural populations and may be caused by genes encoded in the nuclear (genic male sterility; GMS) or mitochondrial (cytoplasmic male sterility; CMS) genomes. This feature has great agronomic value for the production of hybrid seeds, since it prevents self-pollination without the need of emasculation which is time-consuming and cost-intensive. CMS has been widely used in crops, such as corn, rice, wheat, citrus, and several species of the family Solanaceae. Mitochondrial genes determining CMS have been uncovered in a wide range of plant species. The modes of action of CMS have been classified in terms of the effect they produce in the cell, which ultimately leads to a failure in the production of fertile pollen. Male fertility can be restored by nuclear-encoded genes, termed restorer-of-fertility (*Rf*) factors. CMS from wild plants has been transferred to species of agronomic interest through somatic hybridization. Somatic hybrids have also been produced to generate CMS *de novo* upon recombination of the mitochondrial genomes of two parental plants or by separating the CMS cytoplasm from the nuclear *Rf* alleles. As a result, somatic hybridization can be used as a highly efficient and useful strategy to incorporate CMS in breeding programs.

Keywords
incompatibility • plant mitochondria • somatic hybrid • genetic recombination

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Resumen

La androesterilidad es una falla en la producción de polen fértil. Aparece espontáneamente en poblaciones naturales y es causada por genes codificados en el núcleo (androesterilidad génica; GMS) o en la mitocondria (androesterilidad citoplasmática; CMS). La CMS tiene un gran valor agronómico en la producción de semillas híbridas, ya que evita la autopolinización sin la necesidad de emascular, una técnica poco eficiente en términos de costos. Ha sido ampliamente utilizada en cultivos como maíz, arroz, trigo, cítricos y diversas especies de Solanáceas. Los genes mitocondriales que determinan CMS han sido clasificados de acuerdo con los efectos que producen en la célula y que impiden la producción de polen fértil. La fertilidad puede ser restaurada por genes codificados en el núcleo, llamados factores restauradores de la fertilidad (*Rf*). La CMS ha sido transferida desde especies silvestres a especies de interés agronómico a través de la hibridación somática. Esta técnica también permite generar CMS *de novo* mediante la separación de la mitocondria causante de CMS de los factores restauradores del núcleo o mediante la recombinación del genoma mitocondrial de dos plantas parentales, constituyéndose así en una estrategia altamente eficiente y útil para incorporar CMS en programas de mejoramiento.

Palabras claves
incompatibilidad • mitocondria de plantas • híbridos somáticos • recombinación genética

Male sterility in breeding programs

Androsterility, in the broadest sense, refers to the failure in the production of dehiscent anthers, functional pollen, or viable male gametes. Although Darwin acknowledged the evolutionary importance of male sterility (13), its utility was initially ignored in breeding programs. When the potential of hybrid vigor as a breeding tool was identified, male sterility was incorporated in crop species and represented a significant step in genetic improvement programs towards the study of the influence of cytoplasm on plant development (60). The concept of hybrid vigor or heterosis is related principally to yield gains of hybrid lines or cultivars given their superiority in characters like biomass, adaptability, fertility, and biotic or abiotic stress tolerance compared to their parental lines (7). A 'hybrid' can be defined as any offspring of a cross between two genetically unlike individuals. For example, the yield of hybrids obtained by crossing different lines of *Brassica napus* (rapeseed) is 30% higher than the average of their parental lines (44). However, the creation of hybrid crops is not a simple procedure from a technical point of view since producing hybrid seeds of self-pollinating plants requires emasculation (*i.e.*, removing functional pollen grains to prevent self-pollination). Until the mid-twentieth century, this technique involved manual work or chemical treatments, making it costly, inefficient, and harmful to the environment. In this sense, the use of male sterility reduces the cost of hybrid seed production for several reasons. It avoids hand emasculation and pollination, accelerating the hybrid breeding programs and allowing the large-scale production of hybrid seeds and the commercial exploitation of hybrid vigor (12).

The male sterile condition includes both genic (GMS) and cytoplasmic (CMS) male sterility (figure 1, page 477). The first one is caused only by genes encoded in the nuclear genome (12, 39). The second one is caused by mitochondrial genes that directly or indirectly affect nuclear gene functions. In GMS, nuclear *Male sterility* (*Ms*) genes control the male sterility condition without the influence of cytoplasmic sequences (figure 1A, page 477). In the simplest genetic model, there are three possible genotypes for the nuclear locus *Ms*, in which the male sterile phenotype is conditioned by recessive *ms* alleles. A Mendelian inheritance pattern can be observed, in which the offspring of a male sterile genotype (female line) could be entirely male fertile or segregate 50% male sterile: 50% male fertile depending on whether the parental line (male fertile) is homozygous or heterozygous, respectively (figure 1A, page 477). The use of GMS in plant breeding and hybrid seed production involves three different lines: i) a male sterile (female parent), ii) a maintainer, and iii) a restorer (male parent) line. The male sterile line is maintained using pollen of a maintainer line, which presents identical genotype (isoline), except for the presence of a dominant *Ms* allele.

However, the perpetuation of the male sterile (female parent) presents a difficulty: the segregation obtained in the cross with the maintainer line implicates an additional step of selecting the male sterile phenotype (identification and removal of heterozygotes) for hybrid seed production (figure 1). The inefficiency in maintaining the male sterile line had initially restricted the use of GMS in hybrid seed production of crop species in which CMS had not been found or engineered (18). At present, the discovery of environment-sensitive genic male sterility (EGMS) has overcome this drawback by eliminating the need of a maintainer line (12, 69). In this system, the male sterile phenotype is reversible in response to changes in environmental cues like day length and temperature; and two conditions can be differentiated: i) restrictive, in which the *msms* genotype exhibits male sterility, and ii) permissive, in which this genotype is male fertile (12). By cultivating under permissive conditions, the male sterile *msms* line can be propagated by self-pollination.

In CMS, the production of non-functional pollen is maternally inherited and conditioned by cytoplasmic (mitochondrial) genes coupled with nuclear genes (figure 1B). The CMS condition has been reported in more than 300 plant species (76). In natural populations, CMS could be responsible for the existence of gynodioecy, a breeding system in which females (male sterile) and hermaphroditic individuals coexist in a population (14). Thus, two or more different mitotypes exist within the same species. There are commonly two alternative mitotypes in a single population, one normal (usually designated N) and the inductor of male sterility (designated S). The S mitotype interacts with a pair of nuclear alleles: a *restorer-of-fertility* (if dominant usually designated *Rf)* and a sensitive (if recessive usually designated *rf)* allele. In the simplest genetic model, six possible mitotype-genotype combinations are possible, only one of which leads to a male sterile phenotype (figure 1B).

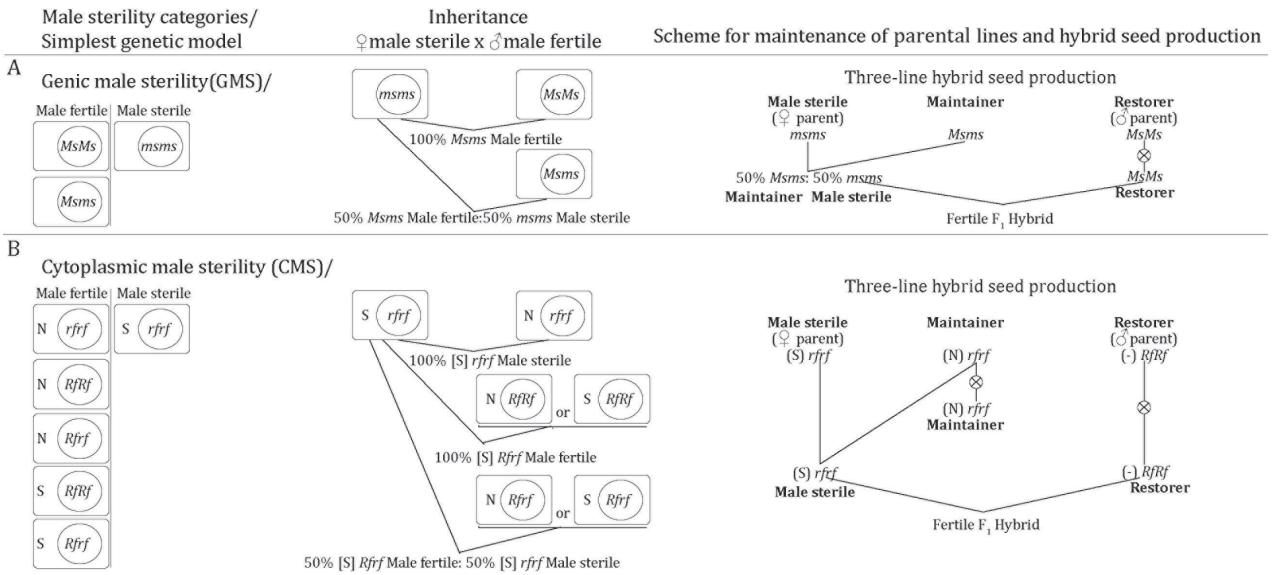


Figure 1. Genetic models for male sterility in plants and its utilization in breeding programs. Letters within circles indicate nuclear genes; letters within rectangles indicate cytoplasmic genes. **A.** Genic male sterility (GMS) is conditioned by nuclear recessive *ms* alleles. **B.** Cytoplasmic male sterility (CMS) is expressed when the sterile cytoplasm S is coupled with recessive non-functional nuclear *restorer of fertility rf* alleles. A dash (-) indicates that the cytoplasm can be N (normal) or S (inductor of male sterility).

Figura 1. Modelos genéticos para la androesterilidad en plantas y su utilización en el mejoramiento. Las letras dentro de círculos indican genes nucleares; las letras dentro de rectángulos indican genes citoplasmáticos. **A.** La androesterilidad génica (GMS) está condicionada por alelos *ms* recesivos. **B.** La androesterilidad citoplasmática (CMS) se expresa cuando el citoplasma inductor de esterilidad S se combina con alelos restauradores nucleares no funcionales recesivos *rf*. Un guión (-) indica que el citoplasma puede ser N (normal) o S (inductor de androesterilidad).

The offspring of the male sterile line (female line) could be entirely male sterile, entirely male fertile, or segregate 50% male sterile: 50% male fertile, depending on whether the male fertile parent is homozygous recessive, homozygous dominant, or heterozygous for the nuclear *restorer-of-fertility* locus, respectively (figure 1B, page 477). Similar to GMS, the breeding value of CMS depends on the management of three different lines: i) male sterile (female parent), ii) maintainer, and iii) restorer (male parent). The male sterile line is perpetuated through crosses with the maintainer line, which is isogenic and differs only in the presence of the N-cytoplasm. In contrast to GMS, the cross between the male sterile and the maintainer lines produces only male sterile offspring (figure 1B, page 477). Furthermore, the maintainer line can be propagated by self-pollination. Finally, for those crops whose seeds are harvested and commercialized, the male fertility needs to be restored in F_1 hybrids. The restorer line has dominant *restorer-of-fertility* alleles *Rf* and produces fertile F_1 hybrids. As the cytoplasm is maternally inherited, the mitotype of the restorer line is irrelevant (figure 1B, page 477).

The use of CMS lines to generate hybrids was first known in maize and it has been increasingly applied to major food crops such as wheat and rice, and also in others important cereals, vegetables, legumes, oilseeds, industrial, and ornamental species like sorghum, Brassicaceae, onion, carrot, sugar beet, sunflower, soybean, pear millet, common bean, cotton, pepper and petunia (8, 29, 36, 50, 65). It is important to acknowledge that, in general, very few sources of CMS have been used in plant breeding, situation that conduces to the development of hybrids with a narrow genetic diversity. This limitation can be illustrated by the episode of the Southern Corn Leaf Blight of 1970 in United States. Upon the discovery of CMS-T (CMS-Texas) in maize in 1952, this genetic system was widely adopted by the hybrid seed corn industry of the United States during the 1960s. By 1970, the CMS-T was part of the genetic background of 75-90% hybrid cultivars grown in this country (9). This CMS-T cytoplasm conditioned the susceptibility to Southern corn leaf blight, disease that destroyed 15% of the maize production in 1970-1971 (9). After this epidemic, CMS-T was no longer used in maize hybrid breeding programs and today other tools are preferred by breeders for maize hybrid seed production (8, 50). This example verifies the need to diversify stable sources of CMS, by identifying a variety of cytoplasmic genes producing male-sterility phenotypes along with their corresponding nuclear-encoded restorer-of-fertility genes and by improving our understanding of the co-evolution of these genetic systems. Alternate CMS/Rf systems were established in rice, maize, sunflower, wheat, and *Brassica* in search of genetic variability and resistance to pathogens and abiotic stresses (8). For instance, more than 70 CMS lines were reported in wheat and sunflower (46, 48). Modern genetic tools for studying mitochondrial genome dynamics and its interaction with nuclear genes are offering new experimental frameworks to move forward on these challenges (8, 19, 62).

In addition to the agronomic importance of CMS in hybrid seed production, it is also used in *Citrus* to achieve seedless fruit production (16, 21, 22, 81). Furthermore, CMS is a feature governed by nuclear-cytoplasmic interactions and it constitutes a valuable model to increase our understanding of the cross-talk between both genomes (24). In fact, the mutations responsible for CMS provided means to demonstrate the role of the mitochondrion in reproductive development (24).

Molecular mechanisms responsible for CMS

The CMS phenotype has arisen spontaneously many times in natural populations. It originates through spontaneous mutations that involve rearrangements of the mitochondrial genome (mtDNA). In general, these mutations result from intragenomic homologous or non-homologous recombination events that create new open reading frames (ORFs) (14). Shandu *et al.* (2007) managed to reproduce the appearance of CMS in fertile plants after repressing the expression of the nuclear gene *Msh1* that is involved in recombination surveillance in plant mitochondria. The rearrangements that cause CMS may be in low stoichiometry in plant mitochondria but can increase their concentration through substoichiometric shifting allowing the expression of the CMS phenotype (56, 63). A few studies indicated the existence of the CMS ORF in fertile lines though at extremely low concentrations (3, 43).

The molecular mechanisms that explain the condition of CMS are far from being fully understood, mainly because each CMS system seems to be unique in terms of the mitochondrial genes associated with the male sterility condition (12, 24, 26). In fact, there are CMS lines highly used in breeding programs, in which a specific restorer line has been developed, but the identity of the gene responsible of the male sterile condition remains unknown (4, 29, 41, 79, 80). To date, several modes of action for CMS genes have been described, namely, energy deficiency, cytotoxic proteins (27), aberrant programmed cell death (59, 68) and retrograde signaling from mitochondria that affects nuclear pathways (12). Retrograde signals from the mitochondria can involve nuclear miRNAs that provoke CMS as they are regulators of pollen development (67). However, the exact relationship between the candidate CMS gene and the observed phenotype has not been assessed in the majority of the cases. In addition, the mechanisms of action of the restorer-of-fertility loci are poorly understood, but most *Rf* genes encode pentatricopeptide repeat (PPR) proteins involved in diverse mitochondrial pathways. For instance, Rf systems can act by modifying CMS transcripts or decreasing the accumulation of toxic proteins (8).

Identifying the molecular basis of CMS requires the use of different strategies. One of them is to search for the gene or genes responsible for CMS by comparing mtDNAS directly. Proposing a candidate CMS gene through the examination of plant mtDNA sequences is particularly challenging in these highly rearranged genomes. However, there are cases in which the gene proposed as a candidate is almost the only difference between the mitochondrial sequences of the normal and the CMS lines (2, 43). In these cases, the mutation or rearrangement that gave rise to the CMS phenotype has been likely a very recent event in the mtDNA (43, 49). Alternatively, a fertile and a CMS lines can be combined through somatic hybridization producing a male-sterile plant with a chimeric mitochondrial genome. If limited homologous recombination gives rise to a mtDNA with few regions from the CMS parent, candidate genes for CMS could be proposed (2). When rearrangements of the mtDNA create numerous new ORFs, the identification of the CMS candidate requires a differential expression assay and/or a segregation analysis (42, 51). In general, the mitochondrial ORFs identified as CMS candidates share some characteristics: i) all causal genes for CMS are encoded in the mtDNA; ii) most ORFs are chimeric formed by a region of a known mitochondrial gene and an new sequence as a result of recombination (41, 43, 49, 51); iii) new ORFs are co-transcribed with known mitochondrial genes and iv) the resulting proteins generally have transmembrane domains (43, 68).

One of the most deeply studied cases is the CMS-Wild Abortive line (CMS-WA) that has been widely exploited in rice breeding. Through the examination of transcripts by RNA-blotting, the CMS-associated transcript was identified (47) revealing that it is a chimeric ORF that encodes a protein of 352 residues (*wa352c*) with three transmembrane domains (49). Its implication in CMS and its mitochondrial localization was confirmed by transforming the nuclear genome of rice and *Arabidopsis thaliana* with a construct that carries the candidate ORF and a mitochondrial transit signal provoking a CMS phenotype. In addition, its interaction with the mitochondrial COX11 protein, encoded in the nucleus, was confirmed by yeast two-hybrid assays. The mitochondrial *wa352c* is constitutively expressed in rice CMS-WA, but it accumulates specifically in the mitochondria of anther cells where it interacts with COX11 to prevent its function in the degradation of hydrogen peroxide, leading to programmed cell death and pollen abortion (49). Its origin and evolution were studied in detail by comparing different wild and cultivated lines. The formation of *wa352c* involved homologous and non-homologous recombination and substoichiometric shifting giving rise to protogenes that finally resulted in the ORF responsible of CMS (68). A restorer line for this CMS system was developed a long time before characterizing the gene responsible for male sterility (79, 80).

Another example comes from the male sterile somatic hybrid *Brassica juncea* + *Moricardia arvensis*, in which the mitochondrial *orf108*, identified as responsible for CMS, is co-transcribed with the gene *atp1*. In the presence of the restorer-of-fertility allele, the transcript of *atp1* is monocistronic, after separating the gene *atp1* from the *orf108* (4, 72). The mechanism by which the *orf108* causes male sterility has not been accurately confirmed. It is possible that the *orf108* translates into a cytotoxic protein or it prevents the normal translation of *atp1* (4). Another case of CMS in *B. juncea* involves the *hau* line.

The CMS mitochondrial *orf288* was identified by expression assays and it was analyzed at the protein level. The CMS protein represses the growth of *E. coli*, pointing to a possible cytotoxic effect (33). Subsequent analysis using *A. thaliana* transformants could detect the exact stage in which the *orf288* is involved, and the sites responsible for cytotoxicity. Transcript analysis detected differences in the expression of nuclear genes involved in the development of the anther and, thus, proposed a mechanism of retrograde regulation for *orf288* (27).

CMS and mitochondrial RNA editing

Gene expression in plant organelles is substantially affected by post-transcriptiona processing events, like intron splicing and RNA editing. In RNA editing, cytidines are changed to uridines (C-to-U) in specific RNA positions, called editing sites. These editions more frequently take place in diverse positions of mitochondrial mRNAs that are well conserved across angiosperms (15). Since these C-to-U changes can generally alter organellar protein products, through the creation of novel start/stop codons (71) or changing the membrane-bound properties of the proteins translated from edited RNA precursors (32, 77), RNA editing is essential for plants because it allows the synthesis of functional organellar proteins that are crucial for plant and seed development (25, 75).

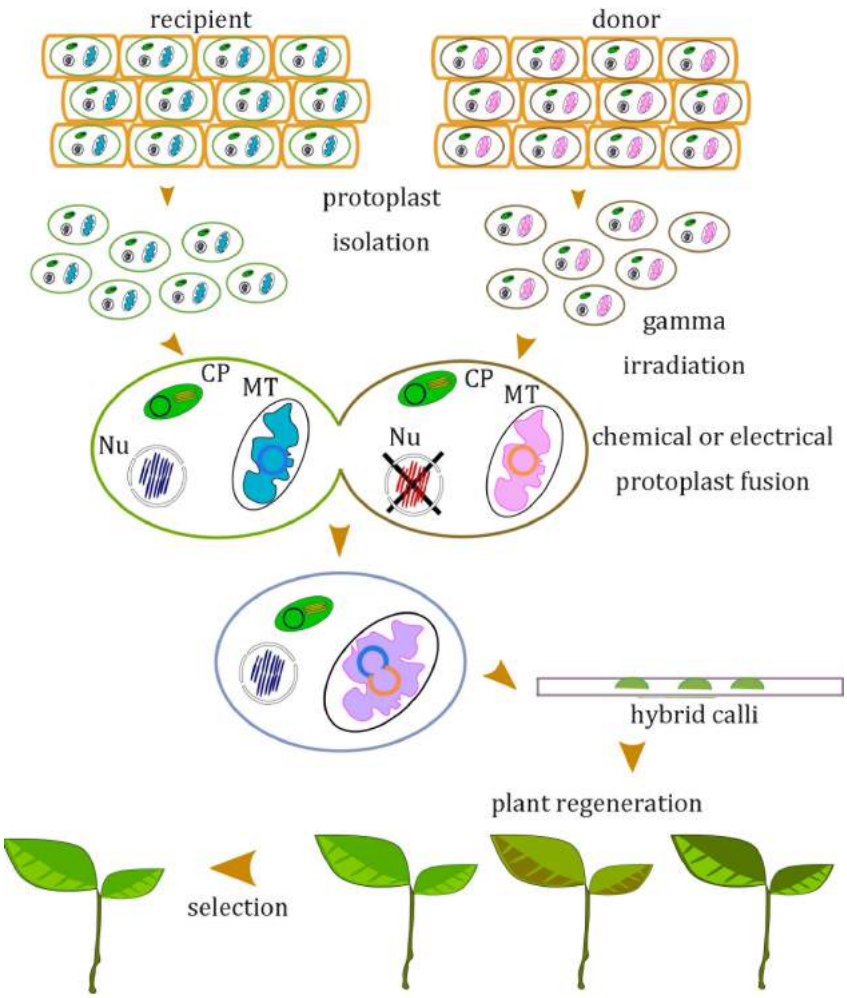
Deficient RNA editing in plant mitochondria can induce male-sterile phenotypes because abnormal proteins are synthesized, impairing mitochondrial function (24). RNA editing has been associated with some CMS systems (28, 32, 71). In one of the best studied rice CMS-systems, two *atp6* genes are present in the mitochondria of CMS-Boro II, N-*atp6* and B-*atp6.* Whereas N-*atp6* is a normal *atp6* gene, B-*atp6* is similar to N-*atp6* but fused to an additional downstream ORF named *orf79* (31). The accumulation of B-*atp6* products in microspores affects pollen fertility because it impairs ATP synthase activity in mitochondria (31, 41, 70). However, two nuclear-encoded restorer-of-fertility factors, RF1 and RF2 (40), are responsible for suppressing the expression of B-*atp6* transcripts, which are processed into two smaller transcripts that are efficiently edited and translated into normal polypeptides (31). Conversely, when such nuclear restorer genes are absent in the nuclear background, unprocessed B-*atp6* transcripts are poorly edited and associated with male sterility (31).

In addition, RNA editing has the potential to be used as a tool for male-sterility induction. For instance, CMS has been induced in tobacco plants by introducing a nuclear transgene, an unedited *atp9* from wheat targeted to mitochondria (28). In this case, ATP synthases were impaired due to the competition between mitochondrial-encoded ATP9 and nuclear-encoded mitochondrial-targeted ATP9 synthesized from unedited *atp9* transcripts, since the editing machinery only acts in plant organelles. The male fertility of transgenic tobacco plants was restored by suppressing the expression of the transgenic *atp9* through an antisense strategy (78). With a similar approach, male-sterile phenotypes have been induced using transgenic and unedited *orfB* and *nad3* genes (11, 66).

Cybridization as a tool to build CMS plants

As the CMS phenotype is very useful in plant breeding, it is often transferred to the crop of interest from natural populations or created *de novo* in the laboratory. CMS can be experimentally induced through intraspecific, interspecific or intergeneric crosses, protoplast fusions, or genetic engineering (39, 65, 72). Somatic hybridization by protoplast fusion is a technique that combines somatic cells from two different cultivars, species, or genera of plants with the aim of regenerating novel germplasm (22). It basically consists of four steps (figure 2, page 481): i) protoplast isolation of two parental species by lysis of the cell wall; ii) fusion of both cells aided by an electrical or chemical impulse; iii) regeneration of hybrid calli and plants; and iv) selection of the somatic hybrid lines of interest (45).

The fusion of protoplasts can be symmetric or asymmetric depending on the nature of the genetic contribution (nuclear and cytoplasmic) of the parents involved. It is symmetric when the contribution of both parental genomes is equivalent. That is, both nuclei are involved in the fusion and are part of the nuclear genome of the resulting somatic hybrid. In order to limit the genetic contribution of one of the parents, the nucleus of one of them (the donor) can be inactivated using radioactivity. This gives rise to an asymmetrical protoplast fusion that results in a somatic hybrid with the complete genome of the receptor and fragments of the donor's genome (22, 34, 64).



First, protoplasts are isolated from mesophyll cells by enzymatic reactions and donor protoplasts may be irradiated to inactivate the nucleus (indicated by a cross). Second, chemical or electrical protoplast fusions give rise to somatic hybrid cells and calli. Third, cybrid plants are regenerated *in vitro*. Fourth, cybrid plants of interest are selected. CP, chloroplast; MT, mitochondria; Nu, nucleus.

Primero, los protoplastos son aislados de células del mesófilo por reacciones enzimáticas y los protoplastos de la planta donante pueden ser irradiados para inactivar su núcleo (indicado con una cruz). Segundo, los protoplastos se fusionan por métodos químicos o eléctricos y dan lugar a híbridos somáticos. Tercero, los cíbridos son regenerados in vitro. Cuarto, las plantas cíbridas de interés son seleccionadas. CP, cloroplasto; MT, mitocondria; Nu, núcleo.

Figure 2. Schematic production of a cybrid plant by protoplast fusion of donor and recipient plants.

Figura 2. Esquema de la producción de plantas cíbridas mediante fusión de protoplastos entre plantas donantes y receptoras.

A cybrid (cytoplasmic hybrid) is a special type of asymmetric somatic hybrid in which its nuclear genome comes from a single parent while the cytoplasmic genomes are inherited from both parents but follow different fates (figure 2). After successive cell divisions, the chloroplast tends to be uniparental (53) with some exceptions (52) while the mitochondrial genome is recombinant, containing segments of both parental mtDNAs (1, 58). Due to the composition of the nuclear genome, cybrids are the most attractive in breeding programs. The fact that the nuclear genome is completely from a parent guarantees the integrity of the cultivar (22). In addition, cytoplasmic hybrids often show CMS and are a valuable tool in plant breeding.

Somatic hybridization represents a powerful tool for transferring genomes or genomic fragments of wild plants with useful agronomic characteristics to commercial crops (34). Protoplast fusion eludes the drawbacks of pre- and post-zygotic barriers of sexual hybridization and combines sexually incompatible germplasms between crops and even between phylogenetically distant plants (64). It also allows the transfer of desirable traits encoded by the plastid or mitochondrial genomes of an uncultivated variety to a commercial crop. Examples of the use of somatic hybridization to transfer desirable mitochondrial-encoded features include resistance to citrus canker caused by *Xanthomonas citri* (54), improved tolerance to salinity (6) and CMS (see below). In addition, somatic hybridization allows the replacement of the cytoplasm of a cultivar in a single step, which is extremely efficient, taking into account the traditional method requiring several backcrosses

to introduce exogenous cytoplasm in to crops (34). Finally, somatic hybridization has been used in fundamental science for studying nuclear-cytoplasm composition and DNA methylation patterns (10), as well as to investigate the recombination pathways that take place between donor and recipient mtDNAs (20, 62).

Cybrid production: strategies to produce CMS lines through protoplast fusion

Using somatic hybridization to transfer or create *de novo* the CMS feature has many advantages when compared to sexual reproduction. The classic transfer of characters through sexual hybridization is not always favorable because other genes than those responsible for CMS are simultaneously transmitted, leading to unwanted results (73). Cybridization by protoplast fusion has become a highly valuable method to introduce or generate the CMS condition by using different strategies.

In the first strategy, cybridization *per se* is the process by which the CMS phenotype is induced. During cybrid production, the mitochondrial genome results recombinant, containing segments of both parental mtDNAs while the nuclear content is engineered to be of a single parent (64). In some cases, the donor parent in the cybridization experiment is male fertile but presents a mitochondrial ORF responsible for CMS and a restorer-of-fertility allele in its nuclear genome. In the resulting cybrid, the CMS ORF is now in a different nuclear background that lacks the restorer allele and the cybrid exhibits the CMS phenotype (24). For instance, the intergeneric somatic hybrid between *M. arvensis* and *B. juncea* is male sterile due to the mitochondrial-encoded *orf108* obtained from *M. arvensis* (see above) However, *M. arvensis* is male fertile because of a nuclear restorer factor that cleaves the transcript containing the *orf108* and *atp1* (4, 72).

Alternatively, CMS can be generated *de novo* by the formation of chimeric ORFs through intergenomic recombination of the mtDNA in somatic hybrids (72). For example, the fusion of protoplasts from *B. napus* and *Isatis indigota* gave rise to a plant with low pollen viability. Through the analyses of the parental and hybrid mitochondrial genomes, a recombinant *cox2* gene was identified as the candidate gene for CMS (37, 38). Also, a CMS phenotype was created through a protoplast fusion experiment between the Solanaceae *Nicotiana tabacum* and *Hyoscyamus niger* (82). This feature has likely originated from the homologous recombination events that took place between the parental mtDNAS (20, 62).

Another strategy involves the transfer of CMS from wild plants into cultivars. As mentioned above, valuable features encoded in the organellar genomes can be transferred directly to crops through cybridization assays. Breeding programs have taken advantage of the fact that CMS has arisen spontaneously in wild species, such as in *B. napus* 'Polima' (17), in cultivars of radish (*Raphanus sativus cv.* Ogura and *cv.* Kosena (30, 55), in *Citrus inshui cv.* Satsuma (74) and in *Nicotiana suaveolens* (23). The CMS feature has been incorporated directly or indirectly into breeding programs via somatic hybridization (17, 22, 35, 57, 61, 73). For instance, the CMS phenotype observed in the wild plant *R. sativus cv.* Kosena was transferred to *B. napus* through asymmetric protoplast fusion (61). The mitochondrial genome of the somatic hybrid SW18 was sequenced and compared to the parental mtDNAs. Through comparative genomics, the mitochondrial-encoded *orf125* derived from *R. sativus cv.* Kosena was identified as responsible for the CMS condition (2). Alternatively, the CMS could be transferred indirectly. First, the CMS condition is transferred to a crop of interest through sexual intergeneric hybridization followed by several backcrosses (5) and it is incorporated into breeding programs using protoplast fusion experiments (57). For example, CMS was transferred from *R. sativus cv.* Ogura to *B. napus* by sexual intergeneric hybridization. The resulting cultivar showed CMS but also chlorosis due to nuclear-chloroplast incompatibility. To overcome this, the chloroplast of *R. sativus* was replaced by that of *B. napus* through an intraspecific somatic hybridization between a CMS and a fertile line of *B. napus* (35, 57). Finally, breeding programs of the genus *Citrus* took advantage of valuable features of two cultivars through symmetric somatic hybridization experiments. The cultivar *C. inshui cv.* Satsuma that exhibits CMS and lacks seeds was combined with the cultivar *Citrus grandis* HBP of high commercial quality but with abundant seeds (22). Even though it is not clear whether CMS is a cytoplasmic-based feature in *C. inshui cv.* Satsuma, its mtDNA in the nuclear background of *C. grandis* resulted in CMS (22). Transcriptomic analyses showed that miRNA regulatory networks may be involved in the citrus floral development and retrograde regulation in nuclear-cytoplasmic interactions in *Citrus* CMS (16).

Final thoughts

Plant breeding programs are in constant need of new sources of CMS lines to avoid a narrow, susceptible genetic background. Often, wild plants contain CMS cytoplasms but are male fertile due to the presence of restorer-of-fertility genes in their nuclear genomes. Therefore, CMS cytoplasms are usually discovered by genetic crossing or somatic hybridization that separates the CMS cytoplasm from the nuclear *Rf* alleles. Interestingly, the fully sequenced mitochondrial genomes of most angiosperms contain ORFs with typical features described for CMS genes. Those ORFs may be able to induce the CMS phenotype but are likely suppressed by nuclear regulators. Thus, it is probable that diverse angiosperm mitochondria may reveal the presence of CMS genes when moved to a novel nuclear background through somatic hybridization assays. This relatively simple experimental procedure is a powerful tool to uncover CMS/*Rf* systems to incorporate in plant breeding programs.

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