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JUAN BRUNO CAVAGNARO (1943-2021)

Se graduó de Ingeniero Agrónomo en 1966 en la Facultad de Ciencias Agrarias de la Universidad Nacional de Cuyo y en el año 1985 como Master of Science en el Departament of Agronomy and Range Sciences, University of California, Davis, USA. Su tesis sobre: "Effect of water stress on growth and dry matter partitioning in four species of *Amaranthus*", fue desarrollada bajo la dirección del Dr. Subodh Jain. En 1970 ingresó a la Carrera del Investigador Científico del Consejo Nacional de Investigaciones Científicas (CONICET), donde se desempeñó en los últimos años como Investigador Independiente. Fue Profesor titular por concurso Dedicación Exclusiva de la Cátedra de Fisiología Vegetal y luego de su jubilación se desempeñó como Profesor Emérito.

También desarrolló en 1967 estudios superiores en el Instituto Interamericano de Cooperación para la Agricultura (*IICA*) y la Organización de los Estados Americanos (*OEA*) en Costa Rica. Fue Profesor Adjunto en la Universidad Católica, Córdoba (1969) y en 1983 se desempeñó como Associate Researcher del College of Agriculture; University of California, USA.

Tempranamente inició su tarea docente en la Cátedra de Fisiología Vegetal de nuestra Facultad de Ciencias Agrarias (UNCuyo), donde trabajaban destacados profesores que fueron sus primeros orientadores en esta área del conocimiento: el Prof. Ricardo M. Tizio; Prof. Sinibaldo Oscar Trione y la Prof. Beatriz R. de Lis. Muchos de nosotros lo conocimos en esta época cuando se desempeñaba como Jefe de Trabajos Prácticos. A mediados de los años setenta sufre la persecución política e ideológica de parte de la dictadura cívico-militar argentina y fue expulsado de la UNCuyo. Por seguridad tuvo que radicarse en Córdoba, hasta que pudo volver a su provincia.

Sobre el año 1978 se incorpora como Investigador del CONICET al Instituto Argentino de Investigación de las Zonas Áridas (IADIZA) para formar un grupo de Ecofisiología Vegetal de especies nativas del desierto Argentino. Allí lideró proyectos de gran envergadura como el “Banco de germoplasma de especies nativas” (BID-CONICET) que realizó las primeras investigaciones en forrajeras nativas y su funcionamiento bajo condiciones de estrés; de esa manera fue incorporando en sus estudios a la Ecología y la Fisiología de vegetales de ambientes naturales. Entre los años 1985 y 1986, fue director del IADIZA.

En el año 1985, al normalizarse el sistema universitario argentino se abrieron nuevamente los concursos docentes lo que permitió “por concurso” el regreso a su querida facultad de la que fuera sacado y así retornar a sus primeros temas de investigación sobre especies cultivadas.

La visión que dejó su paso por el IADIZA respecto de la gran influencia que los factores ambientales tienen sobre las plantas; determinó que Bruno iniciara las primeras investigaciones en Mendoza sobre el funcionamiento de la vid frente a los desafíos que impone el cambio climático; producto de ello generó numerosas publicaciones y fundamentalmente formación de jóvenes investigadores.

Fue desde sus inicios miembro del Instituto de Ciencias Básicas (ICB) y uno de los impulsores en la posterior creación del Doctorado en Ciencias Biológicas (PROBIOL) dependiente de dos unidades académicas de nuestra universidad: las facultades de Ciencias Agrarias y Ciencias Médicas.

Participó activamente en la incorporación de conceptos de ecología en la formación de Ingenieros agrónomos, y consecuencia de ello en el armado de la asignatura Ecología Agrícola y Protección Ambiental, luego la instrumentación de la carrera de Ingeniería en Recursos Naturales Renovables y sobre los últimos años otorgando su apoyo a la creación de la Cátedra de Ecología.

Fue integrante del Comité Organizador del Instituto de Biología Agrícola Mendoza (IBAM), dependiente de CONICET y la UNCuyo, con sede en la Facultad de Ciencias Agrarias.

Bruno fue ampliamente reconocido por sus pares y recibió importantes premios y distinciones, fue jurado de numerosas y prestigiosas revistas científicas, además director de varios proyectos de investigación.

Debido a sus relaciones interinstitucionales entre INTA, CONICET y la Universidad Nacional de Cuyo, logró ser puente para una fluida cooperación entre estos organismos a fin de materializar "en conjunto" proyectos de investigación; nuevas ofertas de posgrados o el uso conjunto de instrumentos comúnmente usados en ecofisiología vegetal.

Bruno fue muy generoso con su tiempo y sus conocimientos que brindaba gustosamente y con gran dedicación a sus alumnos, discípulos y amigos. Por vocación y pasión en el ejercicio diario de su profesión, fue un educador de tiempo completo, que transmitía gran entusiasmo por lo que hacía, motivando a muchos en el ejercicio de la agronomía y la investigación científica, incluidos su propio hijo y su sobrino. Prueba de vocación como educador y formador de recursos humanos es que fue director de 11 tesis en posgrados nacionales y extranjeros y de 22 becas para la formación científica financiadas por CONICET, CONICOR, INTA, SECyT y CIUNC.

Investigador nato, agudo en sus consideraciones y persistente en el trabajo supo integrar la investigación a la docencia (Docente - Investigador). Incorporó tempranamente a los alumnos en sus proyectos. Sus clases eran permanentemente enriquecidas con datos y conclusiones de sus propias investigaciones o las de otros grupos de la Cátedra. Sus proyectos integraron a la Fisiología Vegetal a las condiciones locales y ambientales, resultando así el abordaje desde la Ecofisiología Vegetal en estudios en laboratorio pero especialmente "a campo" con especies nativas del desierto de Argentina o con plantas cultivadas; analizando el efecto de la sequía; la salinidad o el aumento de la temperatura (Calentamiento Global) en la producción y calidad del principal cultivo de Mendoza, la vid.

Producto de sus estudios y los de su grupo en el año 2013 logró -como obtentor- registrar en el Instituto Nacional de Semillas (INASE) cuatro variedades de *Trichloris crinita,* una de las principales gramíneas forrajeras de las zonas áridas de Argentina, tres de ellas con título de propiedad intelectual.

En el año 2003 Bruno materializó lo que para él era un desafío, "tener su propio vino" y lo hizo en forma excelente presentando un SIRAH que luego de pasar por roble fue embotellado con el nombre de Sapiens, fue una pequeña partida que compartió gustosamente con sus seres queridos.

Hacer una semblanza del ingeniero, amigo y hermano Bruno es complejo pues se mezclan los afectos y la razón. Más que un excelente docente-investigador, ejemplo de ética y pensamiento crítico, ecofisiólogo vegetal, persona de apreciaciones estrictas, investigador científico, profesor para ser recordado; amigo entrañable; compañero seguro y confiable; por sobre todo fue él, en efecto, un hombre de bien, que marcó a quienes lo conocimos y tuvimos el privilegio de compartir con nuestro querido Bruno momentos que imborrablemente quedarán en nuestra memoria.

Dr. Carlos Passera

JORGE LÓPEZ
(1962-2021)

Homenaje

> *Licenciado y director de nuestra Biblioteca. De trato afable y cordial, curioso por las innovaciones y los cambios, supo acompañar al personal de la Revista en eventos de actualización, capacitación e institucionales. Nos abrió las puertas de la Biblioteca de la Facultad y sus instalaciones para que trabajáramos cuando hiciera falta, dedicó tiempo para asesorarnos, estuvo atento a nuestros progresos y compartió con afecto los logros obtenidos.*

El 7 de junio de 2021 despedimos a un querido Amigo de la Revista científica de la Facultad de Ciencias Agrarias.

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Revisión

Path analysis under multiple-trait BLUP: application in the study of interrelationships among traits related to cotton *(Gossypium hirsutum)* fiber length

Análisis de ruta bajo BLUP de rasgos múltiples: aplicación en el estudio de las interrelaciones entre los rasgos relacionados con la longitud de la fibra de algodón *(Gossypium hirsutum)*

Rodrigo Silva, Alves [1], João Romero do Amaral Santos de Carvalho Rocha [2], Larissa Pereira Ribeiro Teodoro [3], Paulo Eduardo Teodoro [3], Luiz Paulo de Carvalho [4], Francisco José Correia Farias [4], Marcos Deon Vilela de Resende [1], Leonardo Lopes Bhering [2]

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Abstract

Multiple-trait best linear unbiased prediction (BLUP) is generally the most appropriate method to predict genetic values because it considers the genetic and residual correlations among traits and leads to higher selective accuracy. Thus, the aim of the present study was to analyze the interrelationships among traits related to cotton fiber length through path analysis under multiple-trait BLUP. To that end, 36 elite lines were evaluated in three environments and phenotyped for fiber quality and agronomic traits. Variance components were estimated via residual maximum likelihood (REML). The genetic correlation coefficients among traits were obtained through the mixed model output, and a correlation network was constructed to graphically express these results. Subsequently, path analysis was performed considering fiber length as the main dependent variable. Genetic parameters indicate that the phenotypic variance for most traits is mainly composed of residual effects, which reinforces the need for more accurate statistical methods, such as multiple-trait BLUP. The results for genetic correlations and path analysis reveal the difficulty of selection based on important fiber quality traits, especially fiber length, since most traits show a low cause-and-effect relationship, while other important traits have an undesirable cause-and-effect relationship. Path analysis under multiple-trait BLUP proved to be suitable and useful for studying interrelationships among traits related to cotton fiber length.

Keywords
mixed models • genotype × environment interaction • genetic correlation • genetic selection • *Gossypium hirsutum*

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Resumen

La mejor predicción lineal insesgada de rasgos múltiples (BLUP) es, en general, el método más apropiado para predecir valores genéticos porque considera las correlaciones genéticas y residuales entre rasgos y conducta con mayor precisión selectiva. Por lo tanto, el presente estudio tuvo como objetivo estudiar las interrelaciones entre los rasgos relacionados con la longitud de la fibra de algodón a través del análisis de ruta en BLUP de rasgos múltiples. Con este fin, se evaluaron treinta y seis líneas élite en tres ambientes y se fenotipificaron para muchos rasgos relacionados con la calidad de la fibra y los rasgos agronómicos. Los componentes de la varianza se estimaron mediante la máxima verosimilitud residual (REML). Los coeficientes de correlación genética entre rasgos se obtuvieron a través de la salida del modelo mixto, y para expresar gráficamente estos resultados se construyó una red de correlación. Posteriormente, realizamos el análisis de ruta considerando la longitud de la fibra como variable dependiente principal. Los parámetros genéticos indican que la variación fenotípica para la mayoría de los rasgos se compone principalmente de efectos residuales, lo que refuerza la necesidad de utilizar métodos estadísticos más precisos, como BLUP de rasgos múltiples. Los resultados encontrados para las correlaciones genéticas y el análisis de ruta revelan la dificultad de la selección basada en rasgos importantes de la calidad de la fibra, especialmente la longitud de la fibra, ya que la mayoría de los rasgos muestran una relación de causa y efecto muy baja, y otros rasgos importantes presentan una relación de causa y efecto no deseada. El análisis de ruta en BLUP de rasgos múltiples resultó ser adecuado y útil para el estudio de las interrelaciones entre los rasgos relacionados con la longitud de la fibra de algodón.

Palabras clave

modelos mixtos • interacción genotipo x ambiente • correlación genética • selección genética • *Gossypium hirsutum*

Introduction

Upland cotton (*Gossypium hirsutum* L.r. latifolium Hutch) produces one of the most important natural fibers in the world (4). Due to its wide adaptability and yields (5, 22), it contributes to the world economy as a leading fiber crop in the textile industry (21). To meet industrial demand, cotton breeding programs aim to select genotypes with fibers that are longer and that have greater strength for spinning and baling and greater uniformity of length, as well as genotypes with decreased short fiber content and more consistent micronaire and fiber fineness (2). All these traits are desirable to increase processing speed in the textile industry (3, 20).

To efficiently increase fiber length (FL), it is necessary to know the genetic interrelation of this trait with other agronomic and fiber quality traits, since undesirable changes in other traits may occur when selecting cotton genotypes with higher FL. Some studies have reported a negative relationship between FL and traits such as micronaire (MIC) and fiber percentage (FP) (4, 27). Nevertheless, in a study mapping quantitative trait loci (QTL) for fiber quality traits, Tan *et al.* (2015) found complex significant correlations among fiber quality traits, wherein FL was positively correlated with fiber strength (FS) and MIC, and negatively correlated with fiber elongation (FE). Given this scenario, methods that can make reliable estimates of genetic correlations will help in selection of genotypes (12) with agronomic traits desirable to farmers and that are technological requirements of the textile industry.

In this scenario, the multiple-trait best linear unbiased prediction (BLUP) proposed by Henderson and Quaas (1976) is generally the most appropriate method for genetic evaluation because it considers the genetic and residual correlations among traits, considering pleiotropism and/or gametic phase imbalance. Moreover, it can be fitted to experiments with heterogeneous residual variance and correlated errors within blocks or environments, leading to higher selective accuracy (17).

For genetic selection, it is necessary to identify variables that have a cause-and-effect relationship with the main trait, given that these traits can be used in indirect selection or to compose selection indices. To understand the association of different traits, Wright (1921) proposed path analysis, which partitions the genetic correlation coefficients into direct and indirect effects on a main trait. Estimates of these effects are obtained by regression equations, which use previously standardized variables. Selection of auxiliary traits should be performed by analyzing the magnitude of their direct effect on the main trait. Traits with a high direct effect (negative or positive) in the same direction as their correlation with the main trait can be used in selection indices aiming to increase the main trait and to keep the other traits within the standards accepted by farmers and the textile industry.

Despite the importance of the multiple-trait BLUP approach, there are still no studies using it for path analysis. Thus, the aim of the present study was to analyze the interrelationships among traits related to cotton fiber length via path analysis under multiple-trait BLUP.

Materials and methods

Genetic material, experimental design, and sites

Phenotypic data was obtained from evaluation of 36 elite lines derived from crosses between two commercial cotton lines that have long fiber. To obtain the elite lines, the plants from these crosses were manually self-pollinated from the F_2 to F_4 generation in a greenhouse. The pedigree selection method was adopted from the F_4 generation on; in the F_4 generation, 271 plants were obtained, of which 51 $F_{4:5}$ plants with FL greater than or equal to 31 mm were selected. These lines were evaluated by Carvalho *et al.* (2015) in a greenhouse, and 36 plants with FL greater than or equal to 32 mm were selected, which were evaluated in the next generation ($F_{4:6}$). Of these, 36 lines with FL greater than or equal to 32 mm were also selected.

These lines were evaluated in three field trials in the municipality of Apodi, RN, in 2013 and 2014 and in Santa Helena, GO, in 2013 in a randomized block design with two replications. The trials in Apodi were set up under irrigation, and the trail in Santa Helena was set up in the rainy season. Plots consisted of two 5-m rows, spaced at 0.80 m, with 60 plants per row. Crop practices followed the recommendations for cotton growing. The herbicides trifloxysulfuron-sodium at 750 g kg^{-1} for dicotyledons and clethodim at 240 g L^{-1} for monocotyledons were used for weed control. Pyraclostrobin and metconazole fungicides were applied at a rate of 65 g ha^{-1} and 80 g ha^{-1}, respectively. Pest control consisted of applying deltamethrin insecticide at a rate of 10 g ha^{-1} and was carried out according to the integrated pest management recommended for the crop in the region.

Evaluated traits

The agronomic traits evaluated were plant height (PH) (cm), cotton boll mass (CBM) (g), and fiber percentage (FP) in five plants from each plot. The PH was measured with a ruler, from soil level to the tip of the plant. CBM was obtained by the average of the bolls, which were harvested manually. The FP was determined by the relationship between the weight of the fiber and the total weight of the sample (fiber plus cottonseed). Twenty bolls were randomly collected from the middle third of the plants from each experimental unit and the following fiber quality traits were evaluated: fiber length (FL, mm), fiber uniformity (FU, %), short fiber index (SFI), fiber strength (FS, gf/tex), fiber elongation (FE, %), and MIC.

Statistical analyses

Single-trait analyses were performed to test the significance of genotype effects and genotype × environment (G×E) interaction effects considering the following model:

$$y = Xb + Zg + Wi + e \qquad [1]$$

where:

y = the vector of phenotypic data,

b = the vector of replication-environment combinations (assumed to be fixed), which consists of the effects of environment and replication within environment, added to the overall mean,

g = the vector of genotype effects (assumed to be random),

i = the vector of the G×E interaction effects (random),

e = the vector of residuals (random).

Uppercase letters represent the incidence matrices for these effects. The significance of the random effects was tested by the likelihood ratio test (LRT) (25) using the chi-square statistic with one degree of freedom and significance level equal to 0.05 (16).

According to the LRT, the traits that exhibit genetic variability and the non-significant G×E interaction will be used in subsequent analyses. Variance components were estimated via residual maximum likelihood (REML). Multiple-trait analysis was performed considering the following model:

$$y = Xb + Zg + e \quad [2]$$

In this model, $g \sim N(0, \sum_g \otimes I)$ and $e \sim N(0, \sum_e \otimes I)$, where $\sum_g$ is the genetic unstructured covariance matrix among the traits, $\sum_e$ is the residual covariance matrix, I is an identity matrix with an appropriate order for the respective random effect, and $\otimes$ denotes the Kronecker product. In addition, $\sum_e$ is composed of diagonal blocks of unstructured covariance structure ($\sum_{e1}$, $\sum_{e2}$, and $\sum_{e3}$) among the traits for each environment, in which

$$\sum_e = \begin{bmatrix} \sum_{e1} & 0 & 0 \\ 0 & \sum_{e2} & 0 \\ 0 & 0 & \sum_{e3} \end{bmatrix}$$

Selective accuracies ($r_{\hat{g}g}$) were estimated based on the following expression (17):

$$r_{\hat{g}g} = \sqrt{(\sigma_{\hat{g}}^2 - PEV)/\sigma_g^2} \quad [3]$$

where:

σ_g^2 = the genetic variance,

PEV = the prediction error variance, extracted from the diagonal of the generalized inverse of the coefficient matrix of the mixed model equations.

The genetic correlation coefficients between traits were obtained by multiple-trait BLUP output, based on the following expression:

$$\rho_{xy} = \frac{\sigma_{g(xy)}}{\sqrt{\sigma_{g_x}^2 \ \sigma_{g_y}^2}} \quad [4]$$

where

$\sigma_{g(xy)}$ = the genetic covariance between *x* and *y* traits,

$\sigma_{g_x}^2$ = the genetic variance of trait *x*,

$\sigma_{g_y}^2$ = the genetic variance of trait *y*.

A correlation network was used to graphically express the functional relationship between the estimates of genetic correlation coefficients among the traits, in which the proximity between the nodes (traces) is proportional to the absolute value of the correlation between these nodes. The thickness of the edges was controlled by applying a cut-off value of 0.60, which means that only $|\rho_{xy}| \geq 0.60$ have their edges highlighted. Finally, positive correlations were highlighted in green, whereas negative correlations were represented in red.

Subsequently, a multicollinearity diagnosis was performed on the genetic correlation matrix, according to the recommendations of Montgomery and Peck (2001). Thus, a condition number less than 100 indicates the absence of multicollinearity.

Path analysis, considering FL as the main dependent variable, was performed using the following equation:

$$FL = \beta_1 FP + \beta_2 CBM + ... + \beta_8 PH + p_{\epsilon'} \quad [5]$$

where:

$\beta_1, \beta_2, ..., \beta_8$ = the direct effect estimators of the traits FP, CBM, FU, SFI, FS, FE, MIC PH on FL, and $p_{å}$ = the residual effect of the analysis.

Thus, the normal equation system was used to estimate the direct and indirect effects of each explanatory variable on FL, according to the following expression:

$$\begin{bmatrix} 1.0 & \cdots & r_{FP,PH} \\ \vdots & \ddots & \vdots \\ r_{PH,FP} & \cdots & 1.0 \end{bmatrix} \begin{bmatrix} \hat{\beta}_1 \\ \vdots \\ \hat{\beta}_8 \end{bmatrix} = \begin{bmatrix} r_{FP,FL} \\ \vdots \\ r_{PH,FL} \end{bmatrix} \quad [6]$$

The coefficient of determination (R^2) of the path analysis was obtained by the following equation:

$$R^2 = \hat{\beta}_1 r_{FP,FL} + \cdots + \hat{\beta}_8 r_{PH,FL} \quad [7]$$

The residual effect ($\hat{p}_\varepsilon$) of the path analysis was obtained by the following equation:

$$\hat{p}_\varepsilon = \sqrt{1 - R^2} \quad [8]$$

Software

Statistical analyses were performed using the ASReml 4.1 (7) and R (13) software.

Results

Initially, the LRT was applied to test the random effects of the single-trait model. This test revealed a significant effect of genotypes for all traits evaluated (figure 1), indicating the existence of genetic variability among the cotton lines; and non-significant G×E interaction for all traits evaluated (figure 1).

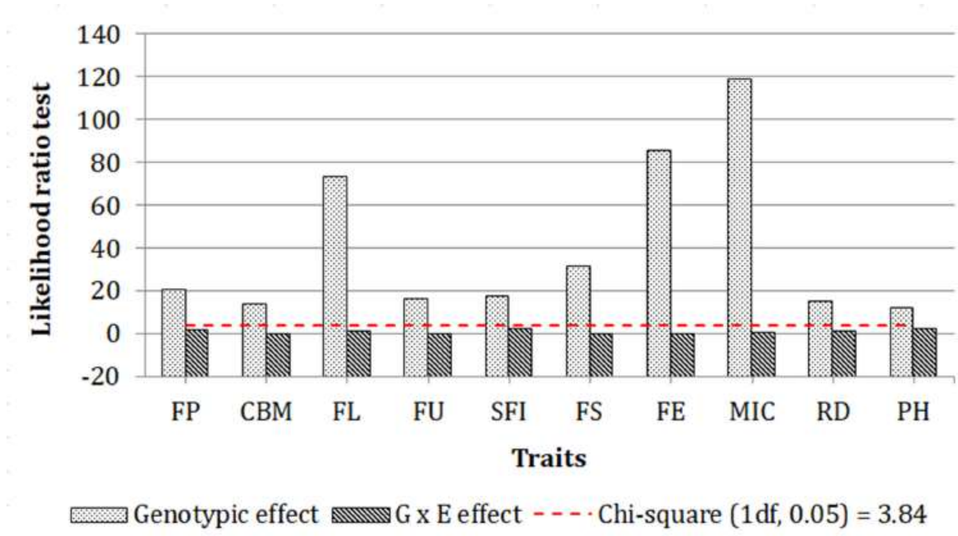

Figure. 1. Likelihood ratio test considering single-trait analyses for the fiber percentage (FP), cotton boll mass (CBM), fiber length (FL), fiber uniformity (FU), short fiber index (SFI), fiber strength (FS), fiber elongation (FE), micronaire (MIC), and plant height (PH) traits. All bars above the dashed red line are significant by chi-square test at 5% probability.

Figura 1. Test de razón de probabilidad considerando análisis de un único carácter para porcentaje de fibra (FP), masa de cápsulas de algodón (CBM), longitud de la fibra (FL), uniformidad de la fibra (FU), índice de fibra corta (SFI), fuerza de la fibra (FS), elongación de la fibra (FE), micronaje (MIC) y altura de la planta (PH). Todas las barras por encima de la línea roja discontinua son significativas por la prueba de chi-cuadrado con una probabilidad del 5%.

In general, the estimates of residual variance differed from environment to environment and did not follow any pattern. According to the scale presented by Resende (2015), heritability estimates were of low ($h^2 \leq 0.15$) or moderate ($0.15 < h^2 < 0.50$) magnitude, except for the FL, FE, and MIC traits, which were of high ($h^2 \geq 0.50$) magnitude. The coefficients of experimental variation (CV_e) were lower than 15% for all the traits evaluated, similar to the values obtained in other studies on the cotton crop (4). The coefficient of genetic variation (CV_g) indicates genetic variability among the cotton lines for all the traits evaluated. In addition, the estimates of average selective accuracy were of high ($0.70 < r_{\hat{g}g} < 0.90$) to very high ($r_{\hat{g}g} \geq 0.90$) magnitude, according to the scale presented by Resende (2015) (table 1, page 6).

Table 1. Estimates of genetic and non-genetic parameters for the fiber percentage (FP), cotton boll mass (CBM), fiber length (FL), fiber uniformity (FU), short fiber index (SFI), fiber strength (FS), fiber elongation (FE), micronaire (MIC), and plant height (PH) traits obtained via REML.

Tabla 1. Estimaciones de los parámetros genéticos y no genéticos para los caracteres porcentaje de fibra (FP), masa de cápsulas de algodón (CBM), longitud de fibra (FL), uniformidad de fibra (FU), índice de fibra corta (SFI), fuerza de fibra (FS), elongación de fibra (FE), micronaje (MIC) y altura de planta (PH) obtenidos a través de REML.

Genetic or non-genetic parameter	FP	CBM	FL	FU	SFI	FS	FE	MIC	PH
σ_g^2	8.67	0.16	1.99	0.42	0.18	1.50	0.44	0.29	28.26
σ_{e1}^2	4.21	0.38	1.53	1.44	0.56	3.32	0.21	0.08	42.08
σ_{e2}^2	4.48	1.05	0.98	1.16	0.28	2.18	0.16	0.06	181.02
σ_{e3}^2	22.60	0.41	0.51	0.79	0.12	1.79	0.29	0.05	61.01
h_{e1}^2	0.67	0.30	0.56	0.23	0.24	0.31	0.67	0.78	0.40
h_{e2}^2	0.66	0.13	0.67	0.27	0.39	0.41	0.74	0.84	0.14
h_{e3}^2	0.28	0.28	0.80	0.35	0.60	0.46	0.60	0.85	0.32
$r_{\hat{g}g}$	0.95	0.87	0.96	0.91	0.92	0.95	0.96	0.97	0.88
μ_1	36.53	5.41	28.28	84.42	7.07	30.23	5.95	5.23	87.26
μ_2	35.19	6.98	30.03	85.63	6.64	31.49	5.67	4.93	113.76
μ_3	35.12	6.80	31.22	85.80	6.48	30.48	5.64	4.32	119.74
CV_{e1}	5.62	11.39	4.38	1.42	10.60	6.02	7.76	5.50	7.43
CV_{e2}	6.01	14.71	3.30	1.26	7.95	4.69	7.00	4.85	11.83
CV_{e3}	13.54	9.47	2.28	1.04	5.35	4.39	9.59	5.21	6.52
CV_{ge1}	8.06	7.45	4.99	0.77	5.96	4.05	11.18	10.38	6.09
CV_{ge2}	8.37	5.77	4.70	0.76	6.35	3.88	11.73	11.00	4.67
CV_{ge3}	8.38	5.93	4.52	0.76	6.50	4.01	11.79	12.57	4.44

σ_g^2: genetic variance; σ_{e1}^2, σ_{e2}^2, and σ_{e3}^2: residual variance for environments 1, 2, and 3, respectively; h_{e1}^2, h_{e2}^2, and h_{e3}^2: individual broad-sense heritability in environments 1, 2, and 3, respectively; $r_{\hat{g}g}$: average selective accuracy; μ_1, μ_2, and μ_3: overall mean in environments 1, 2, and 3, respectively; CV_{e1}, CV_{e2}, and CV_{e3}: coefficient of experimental variation in environments 1, 2, and 3, respectively; and CV_{ge1}, CV_{ge2}, and CV_{ge3}: coefficient of genetic variation in environments 1, 2, and 3, respectively.

σ_g^2: variación genotípica; σ_{e1}^2, σ_{e2}^2 y σ_{e3}^2: variación residual para los ambientes 1, 2 y 3, respectivamente, h_{e2}^2 y h_{e3}^2: heredabilidad amplia individual considerando los ambientes 1, 2 y 3, respectivamente; $r_{\hat{g}g}$: precisión de selección media ;μ_1, μ_2 y μ_3: media general para los entornos 1, 2 y 3, respectivamente; CV_{e1}, CV_{e2} y CV_{e3}: coeficiente de variación experimental para los ambientes 1, 2 y 3, respectivamente; CV_{ge1}, CV_{ge2} y CV_{ge3}: coeficiente de variación genética para los ambientes 1, 2 y 3, respectivamente.

Genetic correlations were expressed graphically by the correlation network (figure 2), a procedure that indicates the interrelations among the traits evaluated. The FL trait was negatively correlated with FP, MIC, and FE, indicating that genotypes with higher FL have lower FP, MIC, and FE. The FP trait was negatively correlated with FS and positively correlated with FE. These results are also partially undesirable because they reveal that genotypes with higher FP have lower strength but higher elongation.

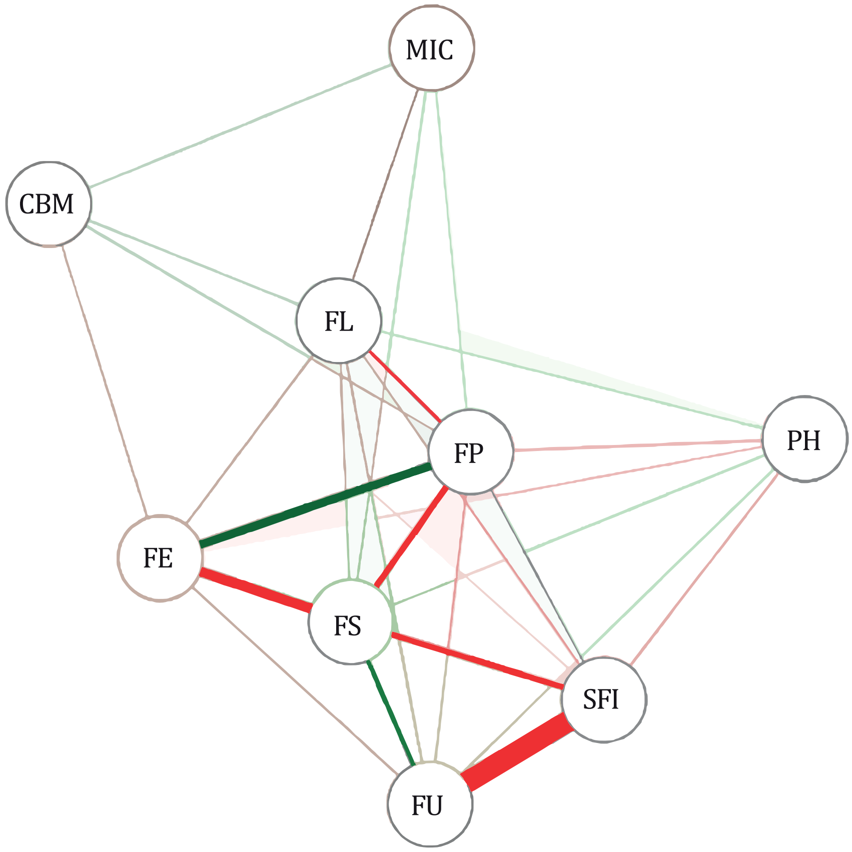

Figure 2. Correlation network among the fiber percentage (FP), cotton boll mass (CBM), fiber length (FL), fiber uniformity (FU), short fiber index (SFI), fiber strength (FS), fiber elongation (FE), micronaire (MIC), and plant height (PH) traits via multiple-trait BLUP.

Figura 2. Red de correlación entre el porcentaje de fibra (FP), masa de cápsulas de algodón (CBM), longitud de la fibra (FL), uniformidad de la fibra (FU), índice de fibra corta (SFI), fuerza de la fibra (FS), elongación de la fibra (FE), micronaje (MIC) y altura de la planta (PH), a través de BLUP multi-caracteres.

The direct and indirect effects of yield and fiber quality on FL are shown in table 2 (page 8). The estimate of the coefficient of determination was of high magnitude ($R^2 = 0.77$) and the residual effect was of low magnitude (0.23). To better understand the magnitudes of the effects and for comparison, effects whose magnitudes were above 0.70 were considered high-magnitude, whereas effects from 0.30 to 0.70 were considered moderate magnitude, and effects lower than 0.30 were considered low-magnitude (6). MIC was the fiber quality trait that obtained the highest direct effect on FL and in the same direction as its correlation. The FE trait had a moderate direct effect in the same direction as its correlations with FL, which is undesirable because an increase in FL is associated with a reduction in FE.

Table 2. Direct and indirect effects of fiber percentage (FP), cotton boll mass (CBM), fiber uniformity (FU), short fiber index (SFI), fiber strength (FS), fiber elongation (FE), micronaire (MIC), and plant height (PH) on fiber length (FL).

Tabla 2. Efectos directos e indirectos del porcentaje de fibra (FP), masa de ramitas de algodón (CBM), uniformidad de la fibra (FU), índice de fibra corta (SFI), resistencia de la fibra (FS), elongación de la fibra (FE), micronaje (MIC), y altura de la planta (PH) en la longitud de la fibra (FL).

Effect	FP	CBM	FU	SFI	FS	FE	MIC	PH
Direct	0.15	-0.18	0.05	-0.09	0.27	-0.35	-0.68	0.14
Indirect via FP	---	0.06	-0.07	0.07	-0.10	0.11	0.03	-0.07
Indirect via CBM	-0.07	---	0.01	0.02	-0.01	-0.06	-0.05	-0.02
Indirect via FU	-0.02	0.00	---	-0.05	0.03	-0.02	0.00	0.01
Indirect via SFI	-0.04	0.01	0.09	---	0.06	-0.03	0.00	0.04
Indirect via FS	-0.18	0.01	0.18	-0.18	---	-0.21	0.10	0.07
Indirect via FE	-0.25	-0.12	0.14	-0.12	0.27	---	0.03	0.07
Indirect via MIC	-0.14	-0.19	0.03	-0.01	-0.24	0.06	---	-0.02
Indirect via PH	-0.07	0.01	0.04	-0.05	0.04	-0.03	0.00	---
Total (genetic correlation)	-0.63	-0.41	0.47	-0.42	0.33	-0.55	-0.60	0.24
R^2	0.77							
Residual effect	0.23							

Discussion

It is important to note that all traits exhibited genetic variability and there was no significant G×E interaction for any of the traits (figure 1, page 5), which indicates similar expression of the genes in controlling these traits in the three environments. Consequently, the estimates of genetic correlation between the environments were equal to 1.00 for all the traits evaluated.

The results for heritability call attention to the MIC, which is often used as an indication of fiber fineness and maturity. Fiber fineness depends on FL, FS, and FU. Therefore, a low estimate of heritability was expected for this trait. However, the residual variance of this trait was very low, that is, the mean values obtained fluctuated little from one replication to another in each genotype. Ulloa (2006) also observed heritability estimates for micronaire higher than the estimates observed for fiber fineness and maturity traits. In general, the results indicate that the phenotypic variance for most traits is mostly composed of residual effects and reinforces the need for using more accurate statistical methods, such as multiple-trait BLUP, to obtain unbiased genetic correlations (1).

Similar results for genetic correlations were also reported by Zeng and Pettigrew (2015) who evaluated genetic correlations among yield traits and fiber properties in upland cotton and found a negative genetic correlation between MIC × FL and MIC × FS. These results raise concerns in plant breeders because FP is related to the amount of product to be marketed, where high values are desirable to farmers. In contrast, the negative correlation between FL x MIC is a favorable result, since genotypes that have greater fiber length and lower micronaire values are desired. Micronaire is an indicator of fiber fineness and maturity, and high micronaire or coarse fibers negatively affect the spinning process and yarn quality (19).

Cotton genotypes with higher FP are desirable because this trait affects the price paid by industry to farmers. FE is a trait related to the strength of cotton fibers and contributes to higher fiber processing speed and yarn and fabric quality (3). Lastly, a high negative correlation was found for SFI × FU, which was expected, since more uniform fibers tend to have a longer length. The other estimates of genetic correlations were of low magnitude.

Studies involving technological traits have been the focus of many cotton breeders (11). FL is among the fiber quality traits that still need to be improved. To that end, knowledge of the correlations among agronomic and technological traits in breeding programs is essential. This is because when selection is carried out based on a specific trait, changes occur in other traits of agronomic importance.

The results for genetic correlations found here reveal the difficulty of selection based on important fiber quality traits, especially FL, since selection based on this trait could consequently lead to selection of other undesirable traits, such as lower FP and FE. However, correlation estimates can produce misconceptions about the relation between two traits and are not necessarily an accurate cause-and-effect measure (14). In this respect, path analysis is a procedure that can effectively direct simultaneous selection. Teodoro *et al.* (2014) mention that this analysis provides detailed knowledge of the effect of traits and reveals positive and negative correlations of high and low magnitudes among the traits evaluated. For that reason, genetic correlation coefficients of yield and fiber quality traits were partitioned into direct and indirect effects on FL.

The use of path analysis to establish selection criteria in cotton is recent and has been restricted to considering fiber yield as the main dependent variable (14). The R^2 for path analysis indicates that practically all variation in FL was explained by the auxiliary traits considered in the path analysis. Furthermore, it is essential to highlight that path analysis was applied using the genetic values predicted via multiple-trait BLUP, which entails greater selective accuracy (17).

This higher direct effect of MIC on FL is important from the perspective of cotton breeding for FL as it reveals that it is possible to select genotypes with higher FL and lower MIC, which is one of the main interests of cotton breeding programs. The low magnitude of the indirect effects of all traits confirms the difficulty of selecting cotton genotypes superior for both yield and fiber quality traits. Some traits have an undesirable cause-and-effect relationship, or they even show a very low cause-and-effect relationship, making simultaneous selection for traits of interest difficult, thus compromising breeding program efficiency.

Therefore, the findings of this study indicate that there is no genotype carrying all desirable traits, confirming that simultaneous selection for yield and fiber quality traits is a major challenge in cotton breeding. Some studies (9, 18, 19) have already reported that this difficulty in selecting cotton genotypes superior for yield and fiber quality traits may be because there are many genes associated with undesirable traits as a consequence of linkage drag.

Conclusion

Path analysis under multiple-trait BLUP proved to be suitable and useful for the study of the interrelationships among traits related to cotton fiber length.

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Combining yield, earliness and plant height in a single genotype: a proposal for breeding in grain sorghum (*Sorghum bicolor* L.)

Combinación del rendimiento, precocidad y altura de la planta en un solo genotipo: una propuesta para mejoramiento en sorgo de grano (*Sorghum bicolor* L.)

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Abstract

Grain sorghum production has expanded during the off-season when rainfall oscillates and becomes insufficient. Aiming to obtain better adapted cultivars, breeding programs have sought new combinations of hybrids with earliness, high grain yield, and ideal plant height for harvesting. This study aimed to estimate de combining ability of grain sorghum lines, proposing a breeding strategy, to identify hybrids gathering high yield, earliness, and desired plant height. Thirty-six hybrids from crosses of 12 lines were evaluated at two sites in the Brazilian region known as Cerrado biome. The evaluated traits were: days to flowering, plant height, and grain yield. For the diallel analysis, Method 4 of Griffing adapted to partial diallel was adopted. By combining ability analysis, we identified promising lines to be used as parents to obtain more yielding, early, and ideal height hybrids. The findings allowed us to propose a breeding strategy, in which complex crosses should be performed to gather favorable alleles in new restorer and male-sterile lines. The hybrids 7, 9, 19, and 22 are the most suitable for growing in the evaluated sites.

Keywords
combining ability • diallel • hybrids • male sterility • *Sorghum bicolor* L.

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Resumen

El cultivo de sorgo granífero se ha expandido durante la temporada baja, cuando la lluvia es oscilante e insuficiente. Para obtener cultivares mejor adaptados a estas condiciones, los programas de mejoramiento han buscado nuevas combinaciones de híbridos con precocidad, alto rendimiento de grano y altura ideal de la planta para la cosecha. Este estudio tuvo como objetivo estimar la capacidad de combinación de líneas de sorgo granífero, con el objetivo de proponer una estrategia de mejoramiento e identificar híbridos con alto rendimiento, precocidad y altura de plantas adecuadas para la cosecha. Treinta y seis híbridos obtenidos del cruzamiento entre 12 líneas fueron evaluados en dos sitios en la región brasilera denominada Bioma Cerrado. Los caracteres evaluados fueron: días hasta la floración, altura de la planta y el rendimiento de grano. Para el análisis del dialelo, se adoptó el Método 4 de Griffing adaptado al dialelo parcial. Por medio del análisis de capacidad de combinación, identificamos líneas promisorias para ser usadas como parentales con el fin de obtener híbridos de mayor rendimiento, precocidad y altura ideal. Los resultados nos permitieron proponer una estrategia de mejoramiento, donde cruzamientos complejos deben ser realizados para reunir alelos favorables para los caracteres en nuevas líneas restauradoras y macho estériles. Los híbridos 7, 9, 19 y 22 son los más adecuados para el cultivo en los sitios evaluados.

Palabras clave
capacidad combinatoria • dialelo • híbridos • macho esterilidad • *Sorghum bicolor* L.

Introduction

Grain sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most cultivated cereal in the world, being a critical caloric source for human feeding in developing countries and in feed formulation for poultry, pigs, and cattle. One of the significant advantages of this crop is its high photosynthetic efficiency and high tolerance to abiotic stresses, especially to drought (1, 2, 3, 18). Grain sorghum tolerates and avoids drought better than many other cereal crops, like wheat and maize (1).

Drought tolerance makes sorghum especially important in arid and semi-arid regions of the world, such as Northeast Africa, India, and the plains of the southern United States (15). In Brazil, grain sorghum growing during the off-season has shown significant expansion in several agricultural regions, mainly in the Cerrado biome (20). At the time of grain sorghum sowing, in addition to temperature and luminosity factors, the volume and frequency of rainfall tend to oscillate and be insufficient, reducing the probability of adequate water supply for most crops. Aiming to have better adapted cultivars to these conditions, grain sorghum breeding programs have sought new combinations of hybrids with earliness, high grain yield, and ideal plant height for harvesting (10, 14, 20).

One of the crucial decisions in breeding programs is choosing the populations to be used, since it is in these populations that the favorable alleles for the traits of interest should be concentrated, allowing the obtention of superior hybrids (11, 16). Diallel analyses are crucial for deciding how to form the base population, enabling the choice of promising parents based on estimates of combining ability. Furthermore, the analysis of diallel crosses contributes to understanding the genetic effects involved in the trait determination (5, 9). Therefore, the study of combining ability provides essential information for breeding program planning.

The objectives of this study were: i) to estimate the combining ability of grain sorghum lines, aiming at proposing a breeding strategy for developing new male-sterile and fertility restorer lines with potential for obtaining superior hybrids for the traits of interest, and ii) to identify hybrids that meet high yield, earliness and adequate height for harvesting in Cerrado biome.

Material and methods

Field experiments

Thirty-six grain sorghum hybrids were assessed in experiments during the 2011/2012 harvest at two sites in the Brazilian Cerrado region. The experiments were carried out at Embrapa Agrossilvipastoril, in the municipality of Sinop-MT, and at Embrapa Maize and Sorghum, municipality of Sete Lagoas-MG. The climatic characteristics of the sites are shown in table 1.

Table 1. Soil and climatic characteristics of the evaluated sites.

Tabla 1. Características del suelo y clima de los sitios evaluados.

Site	Altitude (m)	Latitude (S)	Longitude (W)	Annual rainfall (mm)	Average annual temperature (°C)	Climate †
Sinop-MT	370	11°51'43''	-55°36'45''	2250	25	Aw
Sete Lagoas-MG	761	19°27'57''	44°14'48'	1403	23	Aw

†: according to Köppen classification.

†: según la clasificación de Köppen.

For conducting these experiments, it was used 12 grain sorghum lines (six A lines: F1-A, F2-A, F3-A, F4-A, F5-A E F6-A; and six R lines: M1, M2, M3, M4, M5 e M6) from the sorghum breeding program of the Embrapa Maize and Sorghum, divided into two groups (I and II) according to the presence or absence of cytoplasmic-genetic male sterility. Group I was composed of six fertility restorative lines (R lines), *i.e*., fertile males. Group II was composed of six male-sterile lines (A lines), used as females (table 2).

Table 2. Source and code of the 36 hybrids from the cross between 12 sorghum lines, evaluated in Sinop-MT and Sete Lagoas-MG.

Tabla 2. Fuente y código de los 36 híbridos del cruzamiento entre 12 líneas de sorgo, evaluados en Sinop-MT y Sete Lagoas-MG.

Source	Code			Source	Code		
	Hybrid	R Line	A Line		Hybrid	R Line	A Line
1173-015	1	1	1	1173-375	19	4	1
1173-001	2	1	2	1173-361	20	4	2
1173-003	3	1	3	1173-363	21	4	3
1173-013	4	1	4	1173-373	22	4	4
1173-049	5	1	5	1173-409	23	4	5
1173-051	6	1	6	1173-411	24	4	6
1173-135	7	2	1	1173-615	25	5	1
1173-121	8	2	2	1173-601	26	5	2
1173-123	9	2	3	1173-603	27	5	3
1173-133	10	2	4	1173-613	28	5	4
1173-169	11	2	5	1173-649	29	5	5
1173-171	12	2	6	1173-651	30	5	6
1173-255	13	3	1	1173-735	31	6	1
1173-241	14	3	2	1173-721	32	6	2
1173-243	15	3	3	1173-723	33	6	3
1173-253	16	3	4	1173-733	34	6	4
1173-289	17	3	5	1173-769	35	6	5
1173-291	18	3	6	1173-771	36	6	6

The crosses for obtaining hybrids were conducted manually and controlled according to the model of partial diallel crosses between the groups, in which the six females of Group II were crossed with the six males of Group I, obtaining 36 hybrids. The sowing of the female lines was carried out ten days after the sowing of the male lines, in order to coincide with the flowering time of male lines. Cross rows were composed of 50 plants, distributed in five meters, and spaced in 0.7 meters.

The sowing of the female and male lines was performed alternately in the field, where each female line was sown six times and alternating with the six males, making it possible to perform the crosses according to the partial diallel. After flowering, panicles of the parents were adequately protected with a paper bag, and crosses were performed according to the maturation of male and female reproductive systems. Manual pollinations consisted of the collection of pollen from male lines (Group I) using a paper bag for cross, followed by the pollen melt on stigmas of male-sterile lines (Group II). Crosses were performed in the morning, when the pollen grains were more abundant and the stigmas receptive.

The 36 hybrids from the crosses between the 12 sorghum lines were evaluated in experiments under rainfed conditions, with sowing performed on March 10, 2012. At 15 days after the seedling emergence, thinning was carried out for maintaining a population of 180,000 plants ha^{-1}. The experimental design was a randomized complete block with two replicates. Each plot was composed of two rows of five meters spaced 0.50 m between them. Seed and cover fertilization were carried out based on chemical soil analysis, and cultural and phytosanitary treatments occurred according to the needs of the crop.

The evaluated traits were: days to flowering (DF), measured by counting the days from sowing to flowering 50% of the plants belonging to the plot area; plant height (PH), measured in meters, from the neck of the plant to the tip of the panicle; and grain yield (GY), corrected to 13% moisture and extrapolated to t ha^{-1}.

Genetic-statistical analysis

Initially, a joint analysis of variance was performed according to the statistical model described below:

$$Y_{ijk} = \mu + B/E_{jk} + G_i + E_j + GxE_{ij} + e_{ijk} \quad (1)$$

where:

Y_{ijk} = the observation in the k-th block, evaluated in the i-th genotype and j-th environment
μ = the overall mean of the experiments
B/E_{jk} = the effect of k block within the j environment
G_i = the effect of the i-th genotype considered as fixed
E_j = the effect of the j-th environment considered as random
GxE_{ij} = the random effect of the interaction between i genotype and j environment
e_{ijk} = the random error associated with observation Y_{ijk}

Subsequently, the effect of genotypes and G x A interaction was unfolded at each site according to the partial diallel structure. Method 4 of Griffing (1956) adapted to partial diallel was adopted, which estimates the general (GCA) and specific (SCA) combining ability in partial diallel involving only F_1s hybrids, according to the model below:

$$Y_{ij} = \mu + g_i + g_j + s_{ij} + e_{ij} \quad (2)$$

where:

Y_{ij} = the mean of the cross between the i-th line of Group I and the j-th line of Group II
μ = the overall mean of the diallel; g_i is the general combining ability of the i-th line of Group I
g_j = the general combining ability of the j-th line of Group II
s_{ij} = the specific combining ability of the lines of the Groups I and II
e_{ij} = the experimental error

The GCA estimates of each group of lines and SCA estimates were plotted on a graph where the axes corresponded to the two sites analyzed. Subsequently, a mean clustering (overall and across sites) of the 36 crosses was performed by the Scott-Knott test (1974). All statistical analyses were performed with the Genes software (5) and followed the procedures recommended by Cruz *et al.* (2012).

Results

Combining ability of grain sorghum lines

The joint diallel analysis is shown in table 3. The hybrid effect was significant for all traits evaluated. For GCA effects for R lines of the Group I (GCA I), there was significant only for plant height. Regarding the GCA for A lines of Group II (GCA II), there were significant effects for days to flowering and plant height. There was a significant SCA effect only for plant height. The GCA mean squares were higher than the SCA mean squares for all traits.

Table 3. Joint analysis of variance with the sources of variation and their respective mean squares, regarding a partial diallel analysis composed of 36 hybrids of grain sorghum from the cross among six male lines (R lines – Group I) and six male-sterile lines (A lines - Group II) for the traits days to flowering, plant height (m) and grain yield (t ha^{-1}) evaluated at Sinop-MT and Sete Lagoas-MG.

Tabla 3. Análisis conjunto de la varianza con las fuentes de variación y sus respectivos cuadrados medios, con respecto a un análisis dialélico parcial compuesto de 36 híbridos de sorgo de grano del cruzamiento entre seis líneas masculinas (líneas R - Grupo I) y seis líneas masculinas-estériles (líneas A - Grupo II) para los caracteres días a floración, altura de planta (m) y rendimiento de grano (t ha^{-1}) evaluados en Sinop-MT y Sete Lagoas-MG.

Source of variation	DF	Mean Squares		
		Days to flowering	Plant height	Grain yield
Hybrids (H)	35	37.7831*	0.1225*	2.3060*
GCA I - R lines	5	88.9903^{ns}	0.3032*	8.1720^{ns}
GCA II - A lines	5	87.6736*	0.3184*	3.6477^{ns}
SCA	25	17.5636^{ns}	0.0472*	0.8644^{ns}
Sites (E)	1	6307.0069*	1.0438*	19.3453*
H x E	35	20.6355*	0.0162*	1.1901*
GCA I x E	5	19.4569*	0.0530*	2.3678*
GCA II x E	5	17.3403*	0.0094^{ns}	2.9838*
SCA x E	25	21.5303*	0.0102^{ns}	0.5959^{ns}
Error	70	2.4046	0.0077	0.4230
Mean		66.92	1.44	2.38
CV (%)		2.31	6.07	27.26

* Significant difference (P<0.05) by F test.
ns = not significant.
DF = degrees of freedom.
CV = coefficient of variation.

Diferencia significativa (P<0,05) por la prueba de F.
ns = no significativa.
DF = grados de libertad.
CV = coeficiente de variación.

The hybrid and site interaction (H x E or G x E) was significant for all evaluated traits, reflecting on significant GCA I x E interaction for all traits and significant GCA II x E for days to flowering and grain yield. However, the SCA x E interaction was significant only for days to flowering. The coefficient of variation (CV) was lower than 10% for days to flowering and plant height and lower than 30% for grain yield.

Significant GCA x E interaction for days to flowering was represented in figure 1 (page 16). The M5 line presented a negative GCA estimate at the two sites. This contributed to the low means of the hybrids (25, 26, 27, 28, 29, and 30) that had this line as male parent in both sites (table 4, page 17). The F1-A, F3-A and F4-A lines presented the lowest GCA estimates in both sites and also contributed to a decreased mean of the hybrids (1, 3, 4, 7, 9, 10, 13, 15, 16, 19, 21, 22, 25, 27, 28, 31, 33 and 34) for this trait in both sites.

Despite there was no significant interaction between GCA II x E for plant height, it was chosen to make a biplot graph with the GCA estimates for R and A lines at the two sites evaluated (figure 1, page 16). The M2 line stands out among the restorer lines of Group I by having the lowest GCA estimates at Sinop and Sete Lagoas, contributing to favorable alleles for decreasing plant height at these sites, except for the hybrid 10 at Sete Lagoas. Among the male-sterile lines, those with the lowest GCA estimates were F6-A and F5-A, both at Sinop and Sete Lagoas. These lines generated smaller plants at both sites, except the hybrids 5, 23, and 30, which were allocated to the highest mean Group at Sete Lagoas.

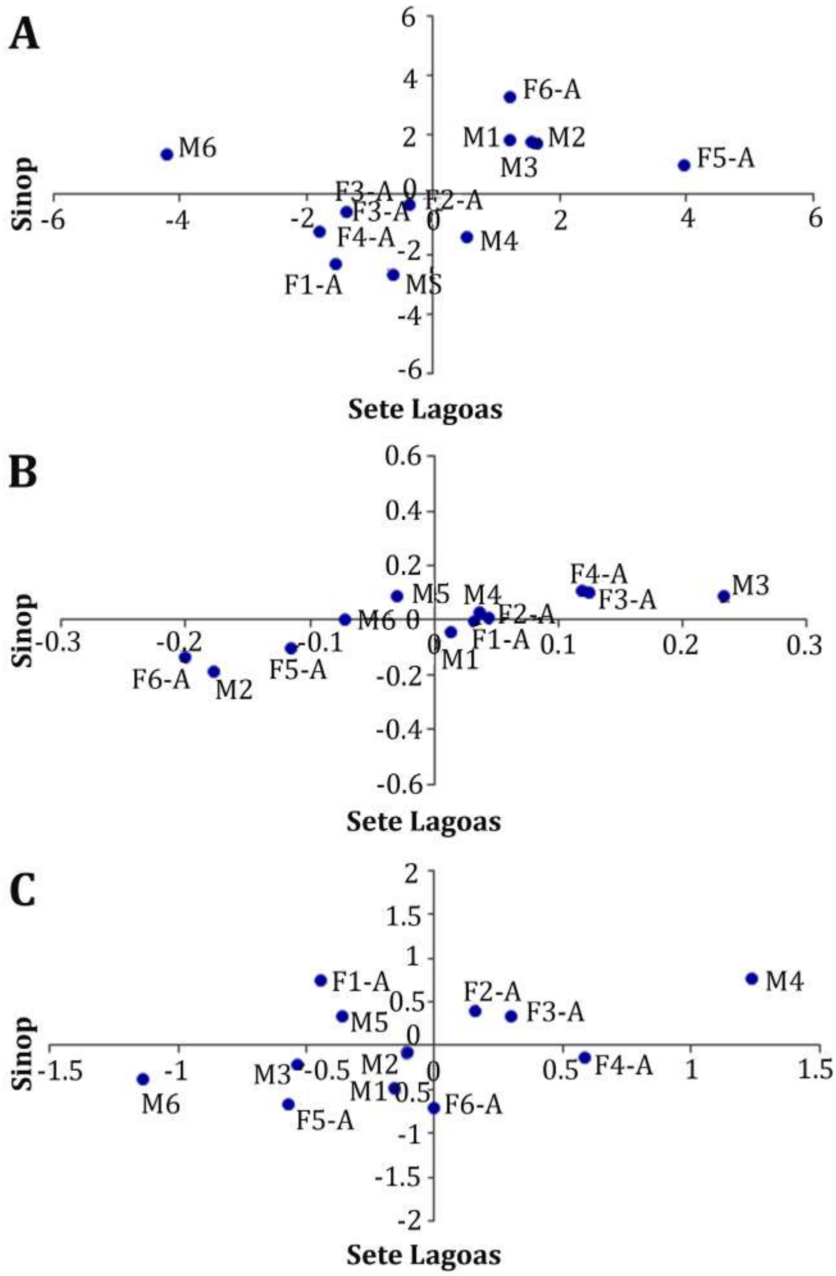

Figure. 1. Estimates of general combining ability (GCA) for days to flowering (A), plant height (B) and grain yield (C) evaluated in 12 grain sorghum lines grown at Sinop-MT and Sete Lagoas-MG.

Figura 1. Estimaciones de capacidad general de combinación (CGC) para los caracteres días a floración (A), altura de planta (B) y rendimiento de grano (C) evaluados en 12 líneas de sorgo de grano cultivados en Sinop-MT y Sete Lagoas-MG.

Table 4. Means clustering for the traits days to flowering, plant height (m) and grain yield (t ha^{-1}) evaluated in 36 grain sorghum hybrids grown at Sinop-MT and Sete Lagoas-MG.

Tabla 4. Agrupación de medias para los caracteres días a floración, altura de planta (m) y rendimiento de grano (t ha^{-1}) evaluados en 36 híbridos de sorgo de grano cultivados en Sinop-MT y Sete Lagoas-MG.

Parents	Hybrids	Days to flowering		Plant height		Grain yield	
♂ x ♀		Sinop	Sete Lagoas	Sinop	Sete Lagoas	Sinop	Sete Lagoas
M1 x F1-A	1	59.5 c	73.5 c	1.50 c	1.23 b	2.56 b	1.71 b
M1 x F2-A	2	60.0 c	75.5 b	1.49 c	1.22 b	2.52 b	1.40 b
M1 x F3-A	3	61.0 c	73.5 c	1.77 a	1.58 a	3.62 a	2.67 a
M1 x F4-A	4	59.0 c	73.0 c	1.45 c	1.24 b	2.50 b	1.56 b
M1 x F5-A	5	66.0 a	74.0 c	1.61 b	1.39 a	1.73 b	0.90 b
M1 x F6-A	6	63.5 b	83.0 a	1.37 c	1.19 b	2.61 b	1.08 b
M2 x F1-A	7	60.0 c	70.5 d	1.43 c	1.04 c	2.76 a	3.02 a
M2 x F2-A	8	61.0 c	75.0 c	1.47 c	1.28 b	3.20 a	2.46 a
M2 x F3-A	9	60.5 c	77.0 b	1.45 c	1.29 b	2.82 a	2.32 a
M2 x F4-A	10	60.5 c	73.0 c	1.57 b	1.38 a	4.21 a	1.93 b
M2 x F5-A	11	66.5 a	74.0 c	1.05 d	0.85 d	1.45 b	1.04 b
M2 x F6-A	12	63.0 b	82.0 a	1.1 d	1.16 b	1.37 b	0.92 b
M3 x F1-A	13	60.5 c	70.0 d	1.70 b	1.41 a	2.84 a	2.74 a
M3 x F2-A	14	60.5 c	71.5 d	1.89 a	1.58 a	3.61 a	2.19 a
M3 x F3-A	15	60.5 c	74.0 c	1.90 a	1.51 a	4.27 a	2.86 a
M3 x F4-A	16	60.0 c	77.0 b	1.90 a	1.58 a	3.62 a	0.75 b
M3 x F5-A	17	66.5 a	80.0 a	1.61 b	1.28 b	1.84 b	1.03 b
M3 x F6-A	18	63.0 b	79.5 a	1.51 c	1.31 b	3.54 a	1.23 b
M4 x F1-A	19	60.5 c	71.0 d	1.53 b	1.35 a	3.29 a	3.09 a
M4 x F2-A	20	59.5 c	73.0 c	1.68 b	1.48 a	4.11 a	3.30 a
M4 x F3-A	21	58.0 c	73.5 c	1.63 b	1.49 a	3.99 a	2.46 a
M4 x F4-A	22	58.5 c	69.0 d	1.70 b	1.51 a	5.01 a	2.97 a
M4 x F5-A	23	66.0 a	64.5 e	1.48 c	1.45 a	3.62 a	2.85 a
M4 x F6-A	24	62.5 b	82.0 a	1.31 c	1.01 c	3.84 a	2.01 b
M5 x F1-A	25	57.0 d	72.0 c	1.60 b	1.52 a	1.11 b	3.14 a
M5 x F2-A	26	59.5 c	72.0 c	1.43 c	1.43 a	1.93 b	3.56 a
M5 x F3-A	27	59.0 c	70.0 d	1.57 b	1.43 a	1.86 b	2.24 a
M5 x F4-A	28	58.5 c	72.0 c	1.65 b	1.55 a	3.15 a	1.86 b
M5 x F5-A	29	62.0 b	77.0 b	1.44 c	1.29 b	3.26 a	1.39 b
M5 x F6-A	30	62.0 b	62.5 e	1.24 d	1.45 a	2.99 a	2.00 b
M6 x F1-A	31	55.0 d	70.5 d	1.55 b	1.52 a	1.23 b	2.88 a
M6 x F2-A	32	59.0 c	72.5 c	1.41 c	1.16 b	2.02 b	1.62 b
M6 x F3-A	33	54.5 d	70.0 d	1.53 b	1.45 a	1.71 b	1.57 b
M6 x F4-A	34	54.5 d	70.0 d	1.57 b	1.51 a	1.49 b	2.21 a
M6 x F5-A	35	58.5 c	78.0 b	1.22 d	1.26 b	1.13 b	0.86 b
M6 x F6-A	36	55.0 d	72.0 c	1.39 c	1.23 b	2.07 b	0.70 b
Mean		60.31	73.54	1.52	1.35	2.75	2.01

Mean groups followed by the same letters in the same column do not differ by the Scott-Knott test at 0.05 significance.

Grupos de media seguidos por las mismas letras en la misma columna no difieren por la prueba de Scott-Knott a 0,05 de significación.

A significant interaction between GCA x E for grain yield was also represented in figure 1 (page 16). The M4 line stands out for presenting the highest GCA estimates among the restorers at both sites, contributing to an increased grain yield for the hybrids 19, 20, 21, 22, 23, and 24. Male-sterile lines F2-A and F3-A obtained the highest GCA estimates considering the two sites, where most hybrids (3, 8, 9, 14, 15, 20, and 21) generated by using these lines as female parents obtained high means.

Potential of grain sorghum hybrids

SCA estimates for days to flowering and plant height are shown in figure 2 (page 18). Regarding days to flowering, the hybrids 4, 7, 14, 22, 33, and 36 presented negative estimates, besides the lowest means (table 4) for this trait at both sites. Furthermore, it is interesting to highlight the hybrids 2, 20, 21, 25, 29, and 35, which obtained low SCA estimates at Sinop, representative site for the sorghum crop.

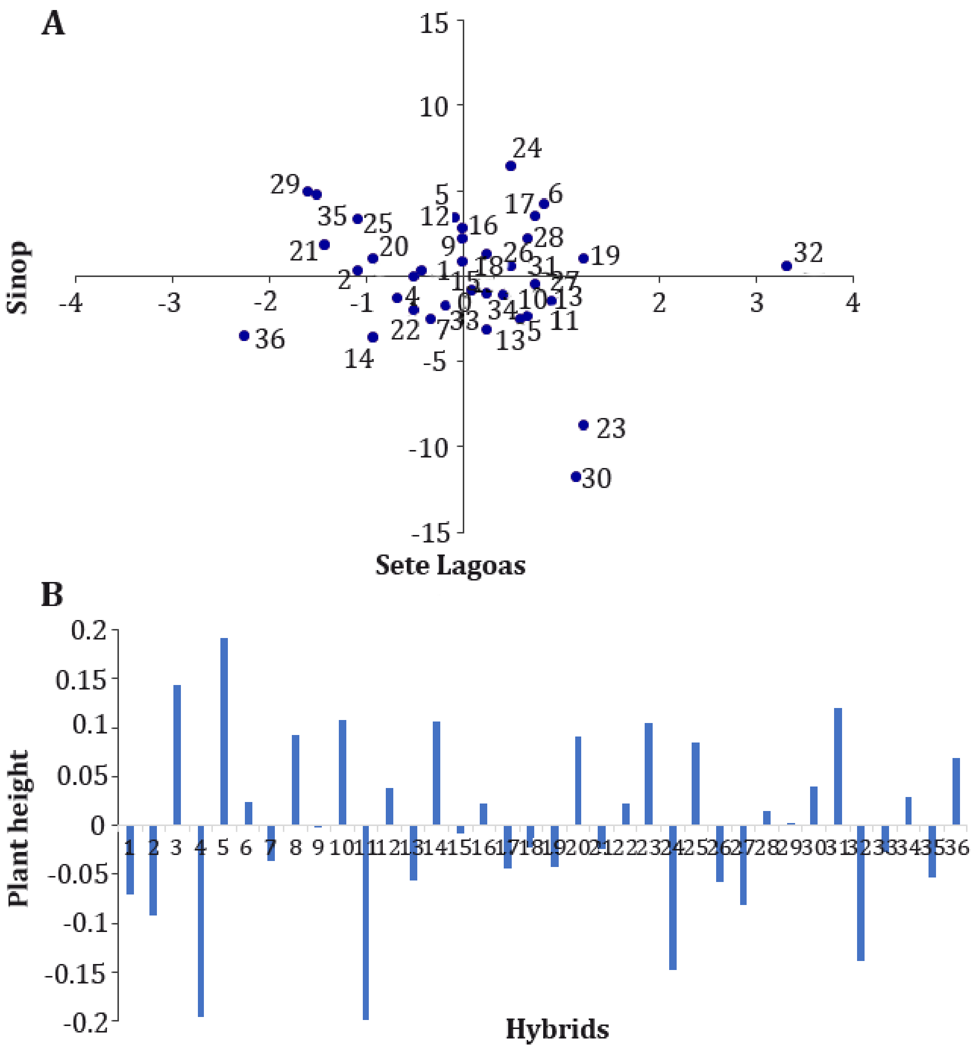

Figure 2. Estimates of specific combining ability (SCA) for days to flowering (A) and plant height (B) evaluated in 12 grain sorghum lines grown at Sinop-MT and Sete Lagoas-MG.

Figura 2. Estimaciones de capacidad específica de combinación (CEC) para los caracteres días a floración (A) y altura de planta (B) evaluados en 12 líneas de sorgo de grano cultivados en Sinop-MT y Sete Lagoas-MG.

Regarding plant height, it was showed the SCA estimates considering the mean of the two sites since the SCA x E interaction for this trait was not significant. The hybrids 1, 2, 4, 7, 11, 17, 18, 19, 21, 24, 26, 27, 32, 33, and 35 obtained the lowest estimates considering the two sites. Furthermore, hybrids 1, 2, 4, 7, 32, 33, and 35 stood out for obtaining greater earliness at both sites.

Discussion

Impact of combining ability on the future of the grain sorghum breeding

Significant effect for hybrids for all traits reveals the existence of genetic variability among the genotypes. In cases where GCA is significant, it can be inferred that, at least, one parent differs from the others regarding the concentration of favorable alleles (6). The significance of GCA I for plant height and GCA II for days to flowering and plant height indicates the existence of parents contributing with a higher number of favorable alleles for these traits transmitted to offspring (17). On the other hand, significant SCA effects evidenced that there are deviations from hybrids behavior concerning what was expected based on the GCA of parents (5). This allows inferring that, for days to flowering and grain yield, whose SCA estimates were not significant, the hybrid populations behaved as expected based on the GCA of parents.

Flowering is highly correlated with the crop cycle and is an essential measure for the off-season sorghum, since the flowering is a susceptible stage to water stress, and the sooner the plant to complete its cycle in off-season crops, the better chance it will have to escape the dry period (12). Furthermore (20) reported that plant height between 1.0 and 1.5 m is desirable for facilitating the harvesting with adapted soybean or maize harvesters. These characteristics are important to consolidate the grain sorghum cultivation in the off-season in the Brazilian Cerrado. For this reason, negative GCA and SCA estimates for days to flowering and plant height are desirable, aiming at reducing the mean values of these traits in sorghum genotypes.

The superiority of GCA effects regarding SCA reveals that additive effects are predominant in controlling these traits. Additive gene effects are cumulative over generations and are the main sources of genetic variation exploited by most breeding programs (8), since it is responsible for setting the characteristics of interest. Menezes *et al.* (2014), Menezes *et al.* (2015) and Mohammed *et al.* (2015) also found predominance of additive gene effects in controlling these traits. However, Premalatha *et al.* (2006) reported that both additive and non-additive effects govern the inheritance of agronomic traits in sorghum.

The presence of significant G x E interaction can be attributed to predictable factors, such as soil, pests and disease management, supplementary irrigation, base fertilization, among others, such as rainfall, temperature, relative air humidity, and solar radiation over the crop cycle at these sites.

The traits here studied are governed by many small-effect genes on the phenotype and highly influenced by the environment. The CV values show experimental accuracy and are inferior to other studies with sorghum genotypes (10, 11, 12, 20).

Currently, grain sorghum breeding programs seek to develop hybrids with earliness, shorter height, and high grain yield (12, 14). These characteristics are desirable to consolidate off-season growing of this crop in Brazilian Cerrado. Thus, the presence of significant GCA x E interaction for some traits indicates that the additive allele effect donated by the parents is changed depending on the environment.

Grain sorghum breeding should be done individually in each line group, in which male-sterile lines breeding is performed in their isogenic lines (B lines) and, subsequently, cytoplasmic-genetic male sterility is introduced. Hence, the identification of parents from each Group (R and A) that donate alleles exerting desirable effects at both sites is a promising strategy for developing future hybrids with broad adaptability for these traits.

In this study, lines from each group contributing to the desired phenotype in the respective hybrids generated were identified. It was found that favorable alleles for each trait are contained in different parents. No parent is a good donor of favorable alleles for all traits at the two sites. A strategy to be adopted to obtain hybrids simultaneously gathering earliness, plant height suitable for harvesting, and high yield is the gene pyramiding, which allows the concentration of a set of genes in a single individual.

Thus, this study presents a proposal for the next steps of the grain sorghum breeding program, aiming at pyramiding of favorable genes for all traits evaluated in lines of each group (figure 3, page 20). To improve the R lines, it is proposed to cross M2 (good donor for shorter height at both sites) x M5 (good donor for earliness at both sites). The hybrid resulting from this cross can be crossed with the M4 line (good donor for high grain yield at both sites). From this, a population with favorable alleles will be obtained for all traits, in which the selection of the most promising lines must be carried out.

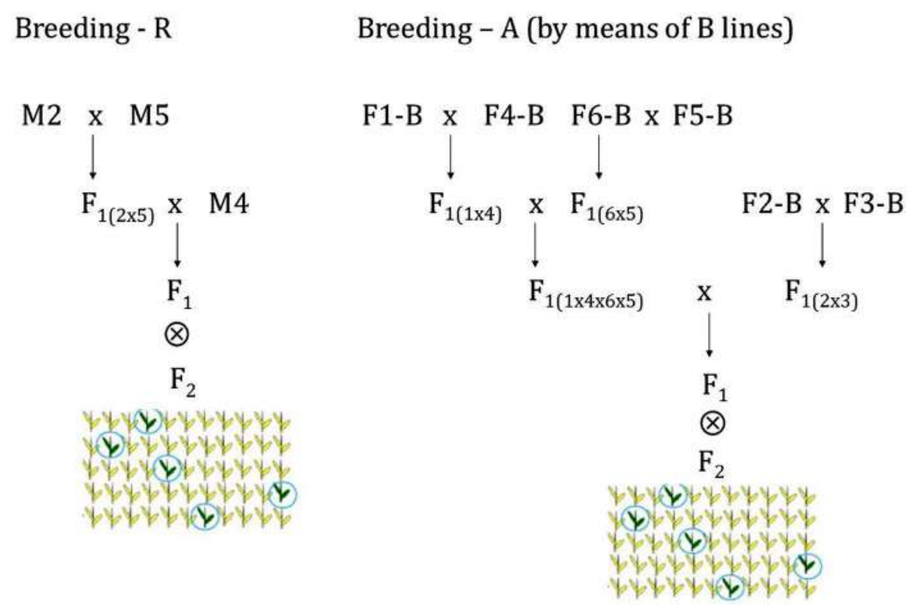

Figure 3. Breeding proposal for obtaining restorative (R) and male-sterile (A) lines gathering earliness, shorter height and high grain yield for the two sites evaluated.

Figura 3. Propuesta de mejoramiento para obtención de líneas restauradoras (R) y machos-estériles (A) reuniendo precocidad, menor altura de planta y alta productividad de granos para los locales evaluados.

Owing to the presence of cytoplasmic-genetic male sterility in the A lines, the cross among male-sterile lines (A) is unfeasible. Hence, the A lines breeding should occur through their maintainers, B isogenic lines, which has fertile cytoplasm. In this case, complex crosses should be performed to gather favorable alleles for all traits in a single population. Maintainer lines (B) crosses in pairs, based on selected A lines, should be performed. These crosses can be carried out manually by emasculating one of the lines and using the pollen from the other.

Therefore, we proposed that the hybrid generated by the cross among the maintaining lines F1-B x F4-B (high earliness) should be crossed with the hybrid derived from maintainers F6-B x F5-B (shorter height). In turn, the new hybrid should be crossed with the hybrid generated by F2-B x F3-B (high grain yield). Thus, there will be the transmission of favorable genes, which are dispersed among the parents, to a single population in which the selection should be carried out. At last, the B lines selected in this population would be crossed with a cytoplasmic male sterile source and then, by using backcrossing, to obtain new pairs of A and B lines.

Conclusions

The findings provided by the combining ability analysis allowed the identification of promising lines to be used as parents in a grain sorghum breeding program. As the traits of interest are found in different lines, complex crosses should be performed to gather favorable alleles for the traits evaluated in new restorative and male-sterile lines.

For improving R lines, we proposed the crossing M2 x M5 (donors for shorter height and earliness, respectively). The hybrid resulting from this cross must then be crossed with M4, which is a good donor for high grain yield. Another breeding strategy should be carried out on the maintainer lines, in which complex crosses involving the hybrids resulting from F1-B x F4-B, F6-B x F5-B, and F2-B x F3-B should be carried out in order to transfer favorable genes dispersed among the parents to a single population in which the selection should be carried out.

Regarding hybrids, we identified that 7, 9, 19, and 22 are the most suitable hybrids for growing in the evaluated environments since they have earliness, adequate height to harvesting, and high grain yield, as well as high phenotypic stability for these traits.

Taking into account that this study assessed 36 grain sorghum hybrids at two sites in one crop season, we understand that the findings are accurate for the traits flowering and plant height, but may not be ideal for yield assessment, which is one of the possible reasons for the high data variability verified for this trait. In this sense, further studies assessing more hybrids and environments (sites and crop seasons) should be carried out to support our findings, especially regarding grain yield.

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Genetic diversity in *Coffea canephora* genotypes for leaf nutrient concentration

Diversidad genética en genotipos de *Coffea canephora* para la concentración de nutrientes en la hoja

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Abstract

This study analyzed the genetic diversity in *Coffea canephora* genotypes by univariate and multivariate statistical analysis, based on concentrations of macro- and micronutrients in coffee leaves in the stages of pre-flowering and grain filling. The experiment was arranged in randomized blocks with three replications, in a 42x2 factorial design, in which factor one represented the evaluated genotypes and factor two the periods of leaf sampling, *i.e.*, pre-flowering and grain filling. The data of leaf nutrient concentrations were subjected to analysis of variance by the F test ($p < 0.01$), and genetic parameters were estimated. For the study of genetic diversity, the genotypes were grouped by the hierarchical unweighted pair-group method using arithmetic averages (UPGMA). The relative importance of a trait to predict genetic diversity was also studied. There is genetic divergence for leaf nutrient concentration in *C. canephora* genotypes. With a maximum limit of 60% of dissimilarity between genotypes, four groups were also formed by UPGMA. For the 42 evaluated genotypes, leaf S concentration was the most important trait for genetic diversity; this genotypic variability should be investigated to enhance the efficiency of nutritional diagnosis.

Keywords
Conilon coffee • sampling period • mineral nutrition • multivariate analysis

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Resumen

Este estudio analizó la diversidad genética en los genotipos de *Coffea canephora* mediante análisis estadístico univariado y multivariado, basado en las concentraciones de macro y micronutrientes en las hojas de café en las etapas de pre-floración y llenado de granos. El experimento se organizó en bloques aleatorios con tres repeticiones, en un diseño factorial de 42x2, donde el factor uno representaba los genotipos evaluados y el factor dos los períodos de muestreo de hojas, es decir, pre-floración y llenado de granos. Los datos de las concentraciones nutricionales de las hojas se sometieron a un análisis de varianza mediante la prueba F ($p < 0{,}01$), y se estimaron los parámetros genéticos. Para el estudio de la diversidad genética, los genotipos se agruparon por el método jerárquico de pares de grupos no ponderados, utilizando medios aritméticos (UPGMA). También se ha estudiado la importancia relativa de un rasgo para predecir la diversidad genética. Existe divergencia genética para la concentración de nutrientes en las hojas en los genotipos de *C. canephora*. Con un límite máximo del 60% de disimilitud entre los genotipos, el método UPGMA formó cuatro grupos. Para los 42 genotipos evaluados, la concentración de S en las hojas fue la característica más importante para la diversidad genética. La variabilidad genotípica debe investigarse para mejorar la eficiencia del diagnóstico nutricional.

Palabras clave

Café conilon • periodo de muestreo • nutrición mineral • análisis multivariante

Introduction

Two species of the genus *Coffea* are commercially produced, *i.e.*, *C. arabica* and *C. canephora*. Brazil is the world's largest coffee producer, and in the last 10 years, *C. canephora* yields have increased over 90% (6). Among the technologies applied to raise yields, *e.g.*, irrigation, superior genotypes, higher planting density and phytosanitary control (23), an appropriate nutritional management is also relevant (35).

The nutritional status of plants can be determined by the nutrient content in plant tissues, and the leaves are physiologically active organs that are used for the nutritional diagnosis. A correct interpretation of the leaf analysis is a fundamental tool for an adequate supply of nutrients in coffee plantations. To this end, reference values such as critical levels and sufficiency ranges are used (30, 34, 35). However, these reference values to determine the nutritional status do not take the genetic diversity for leaf nutrient concentrations in Conilon coffee genotypes into account, which is an inherent feature of self-incompatible allogamous species (19).

The high genetic variability in Conilon coffee allows the identification of plants with different characteristics within the species (17, 18, 28). The genotypes used in commercial crops differ from each other in nutrient and dry matter accumulation (24, 33), vegetative growth (32) and nutrient uptake and use efficiency (2, 26, 27). Thus, genotypic variation is one of the main factors causing differences in species nutrition (10).

Multivariate analysis is a technique that has been widely used to quantify genetic divergence and allows integrating the multiple information of a set of traits extracted from experimental units. This increases the possibilities of choosing divergent parents in breeding programs (15). For the study of genetic diversity in *C. canephora*, multivariate approaches have been used to evaluate morpho-agronomical (17, 22), morphological (7) and leaf morpho-anatomical traits (18).

Since nutrient uptake, transport and redistribution in plants are genetically controlled, genotypes can be improved and/or selected for a more efficient nutrient use (16), using multivariate techniques as analysis method. Thus, the characterization of genetic variability for leaf nutrient concentration in *C. canephora* species may contribute to more accurate diagnoses of the nutritional management of the crop and generate important information for the planning of breeding programs.

The hypothesis of the work is that the genotypes have different concentrations of nutrients in the leaves, contributing to the genetic variability of the species *C. canephora*.

Therefore, the objective of this study was to identify the genetic diversity of coffee leaf concentrations of macro- and micronutrients in the phenological stage of pre-anthesis and grain filling in *C. canephora* genotypes by univariate and multivariate statistical analysis.

Material and methods

The experiment was conducted on a rural property in northern Espírito Santo (18° 39' 43" S, 40° 25' 52" W; 199 masl) where the mean annual temperature is 23 °C. According to Köppen, the predominant climate in the region is Aw (tropical with a dry season) (1). The soil at the site is a Latossolo Vermelho-Amarelo, distrófico, with clayey texture and a wavy relief (38). The chemical and physical characteristics are described in table 1.

Table 1. Chemical and granulometric analysis of soil in the experimental area. Nova Venécia, ES - Brazil.

Tabla 1. Análisis químico y granulométrico del suelo en el área experimental. Nova Venécia, ES - Brasil.

Chemical attributes	Depth (cm)					
	0-10	10-20	20-30	30-40	40-50	50-60
K (mg dm^{-3})	110	95	74	57	52	46
S (mg dm^{-3})	15	11	29	15	15	17
Ca (cmol dm^{-3})	3.8	3.4	1.9	1	0.7	0.6
Mg (cmol dm^{3})	1	0.9	0.4	0.3	0.1	0.1
Al (cmol dm^{-3})	0	0	0.3	0.7	0.8	0.8
H+Al	1.6	1.8	2.4	2.9	3.1	3.1
pH-H_2O	6.6	6.5	5.3	4.8	4.8	4.8
Organic matter (dag dm^{-3})	2.1	1.7	1.1	0.8	0.7	0.5
Fe (mg dm^{-3})	140	138	126	94	88	87
Zn (mg dm^{-3})	10.2	4.5	2.9	1.1	0.6	0.5
Cu (mg dm^{-3})	3.4	4.3	3	1.9	1.2	1
Mn (mg dm^{-3})	207	174	104	46	44	40
B (mg dm^{-3})	0.81	0.83	0.58	0.55	0.56	0.61
Na (mg dm^{-3})	11	37	8	6	5	4
Granulometry (g kg^{-1})						
Sand	434	352	188	368	366	376
Silt	86	168	212	32	74	124
Clay	480	480	600	600	560	500

In 2014, Conilon coffee was planted, consisting of 42 *C. canephora* genotypes, grown under full sun, in rows spaced 3 m and plants spaced 1 m apart, *i.e.*, at a plant density of 3333 plants per hectare. The cultural treatments were applied according to the technical guidelines for the crop, basically with herbicide weed control and manual cutting, preventive phytosanitary management, liming, fertilization and drip irrigation.

The treatments received 500, 100, and 400 kg ha^{-1} $year^{-1}$ of N, P_2O_5, and K_2O, respectively, applied depending on plant requirements and phenological stages. Soil micronutrients were corrected by applying 2 kg ha^{-1} $year^{-1}$ Zn, 1.0 kg ha^{-1} $year^{-1}$ B, 2.0 kg ha^{-1} $year^{-1}$ Cu, and 10 kg ha^{-1} $year^{-1}$ Mn.

The experiment was arranged in randomized blocks with three replications, in a 42x2 factorial design, in which factor one represented the evaluated genotypes (table 2, page 25) and factor two the sampling periods (pre-flowering and grain filling). Each experimental plot consisted of seven plants, considering the five central plants for evaluation.

Table 2. Identification of 42 *Coffea canephora* genotypes. Nova Venécia, ES - Brazil.
Tabla 2. Identificación de 42 genotipos de *Coffea canephora*. Nova Venécia, ES - Brasil.

Code	Name	Code	Name	Code	Name
1	Verdim R	15	Bamburral	29	Tardio C
2	B01	16	Pirata	30	A1
3	Bicudo	17	Peneirão	31	Cheique
4	Alecrim	18	Z39	32	P2
5	700	19	Z35	33	Emcapa 02
6	CH1	20	Z40	34	Emcapa 153
7	Imbigudinho	21	Z29	35	P1
8	AD1	22	Z38	36	LB1
9	Graudão HP	23	Z18	37	122
10	Valcir P	24	Z37	38	Verdim D
11	Beira Rio 8	25	Z21	39	Emcapa 143
12	Tardio V	26	Z36	40	Ouro negro 1
13	AP	27	Ouro Negro	41	Ouro negro 2
14	L80	28	18	42	Clementino

Genotype 33 belongs to cv. Emcapa 8111 and genotypes 34 and 39 to cv. Emcapa 8131 (4). Genotypes 1, 11, 15, 16 and 30 belong to cv. Tributun (18, 37) and 30 and 35 to cv. Andina (28, 36).

In June, coffee leaf samples were collected in the pre-flowering period and in December during grain filling. In both periods, leaf samples were collected on either side of each tree, from the middle third of the plant, taking the third or fourth pair of leaves from the apex of the plagiotropic branches. The leaves were placed in paper bags and dried in a forced air circulation oven at 65 °C to constant weight.

The collected material was sent to a plant tissue analysis laboratory to determine the leaf concentrations of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron. (Fe), zinc (Zn), copper (Cu), manganese (Mn) and boron (B), according to the methodology described by Silva (2009).

The values for leaf nutrient concentration were subjected to analysis of variance by the F test ($p < 0.01$) for each trait separately to detect genetic variation between the genotypes. For the leaf concentration of each nutrient, the coefficient of experimental variation (CVe), coefficient of genetic variation (CVg) and coefficient of genotypic determination (H^2) were estimated. The Scott Knott algorithm ($p \leq 0.05$) was used to group the genotypes according to the mean leaf nutrient concentrations.

For the study of genetic diversity, Mahalanobis' generalized distance matrix (D^2) was established as similarity measure and the genotypes were grouped by the Unweighted Pair Group Method using Arithmetic Means (UPGMA). The study of the relative importance of leaf nutrient concentrations to predict genetic diversity was also applied, as proposed by Singh (1981). All statistical analyses were performed using Genes software (8).

Results and discussion

According to the analysis of variance, the differences in leaf concentration of all nutrients between the evaluated periods and genotypes were significant at 1% and 5% probability by the F test, except for Zn, which was not significant for either source of variation (table 3, page 26). Significant interactions between evaluated periods and genotypes were only observed for Ca, S and Cu. The significant results indicate differentiated responses of the evaluated genotypes, suggesting the existence of variability in leaf nutrient concentrations, which makes studies related to genetic diversity possible.

Table 3. Summary of variance analysis, estimates of experimental coefficient of variation (CVe), coefficient of genetic variation (CVg) and coefficient of genotypic determination (H^2) for leaf concentration of macro- and micronutrients in 42 *Coffea canephora* genotypes during pre-flowering and grain filling. Nova Venécia, ES - Brazil.

Tabla 3. Resumen del análisis de varianza, estimaciones del coeficiente de variación experimental (CVe), coeficiente de variación genética (CVg) y coeficiente de determinación genotípica (H^2) para la concentración foliar de macro y micronutrientes en 42 genotipos de *Coffea canephora* durante la floración previa y relleno de grano. Nova Venécia, ES - Brasil.

Nutrients	Mean square				Mean	CVe (%)	CVg (%)	H^2 (%)
	Stage (St)	Genotypes (G)	St x G	Error				
N	724.71**	5.47*	2.47 ns	3.23	26.63	6.75	2.29	40.81
P	2.11**	0.02**	0.01 ns	0.01	1.08	8.39	4.71	65.41
K	1352.42**	7.26**	3.50 ns	2.81	11.63	14.42	7.40	61.23
Ca	1067.45**	32.14**	16.72**	8.33	18.34	15.73	10.85	74.07
Mg	290.87**	5.73**	1.42 ns	1.32	5.86	19.58	14.61	76.96
S	10.50**	0.70**	0.16**	0.08	2.05	14.13	15.72	88.12
Fe	35145.14**	638.16**	402.20 ns	282.11	90.65	18.52	8.49	55.79
Zn	6.34 ns	4.93 ns	4.32 ns	3.53	7.79	24.11	6.19	28.39
Cu	80.01**	36.13**	17.35**	8.06	16.21	17.51	13.33	77.67
Mn	4053847.00**	76890.91**	22610.86 ns	31852.94	596.43	29.92	14.52	58.57
B	5040.19**	489.81**	110.32 ns	92.93	63.25	15.24	12.85	81.02

ns, ** and *, not significant, significant at 1 and 5% probability, respectively, by the F test.

ns, ** y *, no significativo, significativo a 1 y 5% de probabilidad, respectivamente, por la prueba F.

For most leaf concentrations, the experimental coefficient of variation (CVe) was < 20%, which is an acceptable value for experiments with perennial crops such as coffee (11). The lowest leaf concentration was that of N and the highest of Mn, which suggests the lowest environmental influence on N and the highest on Mn (table 3). Lower CVe values for leaf concentration of N and higher values for Mn were also found by Partelli *et al.* (2007, 2016, 2018) and Gomes *et al.* (2016), who evaluated the leaf concentration of these nutrients during pre-flowering and grain filling.

Leaf concentrations from 2.29% for N to 15.72% for S were observed for the coefficient of genetic variation (CVg) (table 3). This is an important parameter that allows conclusions about the range of variability contained in the population for different traits, allowing a comparison of the levels of genetic variability in different genotypes (11). Since the parameter is directly linked to genetic variability, it can provide breeders with an idea of the relative magnitude of changes accessible by selection (14).

Considering the genotype effect as fixed, the genotypic coefficient of determination (H^2), *i.e.*, the limit of heritability, was estimated at 28.39% for Zn (minimum), and 88.12% for S (maximum). Values of H^2 close to 100% indicate a high genetic control for the traits in question, along with a low environmental influence (table 3). Together with S, the leaf concentrations of Cu and Mg were the highest for H^2. Evaluating macronutrient concentrations in plant tissues of a *C. canephora* genotype, Starling *et al.* (2019) also observed that the S and Mg concentrations stood out among the macronutrients with highest H^2 values.

During grain filling, the leaf concentrations of the macronutrients N, P and K of the genotypes were 13.59%, 18.18% and 49.67% higher, respectively, than in the pre-flowering period (table 4, page 27). These results agree with those of Partelli *et al.* (2016; 2018), in that the leaf concentrations of N, P and K are higher during grain filling. In this period, the growth rate of *C. canephora* trees is higher (31), and these nutrients play a fundamental role in plant metabolism and are essential for the functioning of the photosynthetic apparatus. Nitrogen is a constituent of many plant cell components such as chlorophyll, amino acids and nucleic acids, P is a component of respiration and photosynthesis intermediates, as well as nucleotides used in plant energy metabolism such as ATP, and K plays an important role in regulating the osmotic potential of plant cells, with direct participation in stomatal opening (42).

Conversely to the primary macronutrients (N, P and K) the leaf concentrations of Mg, Fe, Mn and B were higher during pre-flowering, namely 44.88%, 29.95%, 54.01%. and 15.22% higher, respectively, than during grain filling (table 4). Higher concentrations of these nutrients during pre-flowering were also reported by Partelli *et al.* (2016; 2018). All of these nutrients are important for plant development and their concentrations should be maintained at appropriate levels in plant tissues, according to the metabolic demand. Magnesium is a constituent of the chlorophyll molecule and required by a series of enzymes involved in phosphate transfer, Fe plays an important role as a component of enzymes involved in electron transfer, B is involved in cell elongation and nucleic acid metabolism., and Mn is required for some enzyme activities, such as decarboxylases and dehydrogenases involved in the Krebs cycle (42).

Table 4. Mean leaf concentrations of N, P, K, Mg, Fe, Mn and B in 42 *Coffea canephora* genotypes during pre-flowering and grain filling. Nova Venécia, ES - Brazil.

Tabla 4. Concentraciones medias de hojas de N, P, K, Mg, Fe, Mn y B en 42 genotipos de *Coffea canephora* durante la floración previa y el llenado de grano. Nova Venécia, ES - Brasil.

Period	——— Macronutrients ($g.kg^{-1}$) ———				— Micronutrients ($mg.kg^{-1}$) —		
	N	P	K	Mg	Fe	Mn	B
Pre-flowering	24.93 b	0.99 b	9.32 b	6.94 a	102.46 a	723.26 a	67.72 a
Grain filling	28.32 a	1.17 a	13.95 a	4.79 b	78.84 b	469.60 b	58.77 b

Distinct letters in a column differ from each other by the F test at 5% probability.

Las letras distintas en una columna difieren entre sí por la prueba F con una probabilidad del 5%.

Based on the mean grouping by the Scott-Knott test, the genotypes were divided into two dissimilar groups for leaf concentrations of N, P, K, Fe and Mn. For Mg and B concentrations of the genotypes, the variability was highest and three and four dissimilar groups, respectively, were formed (table 5, page 28). The same genotypes identified in the group with highest means for a given nutrient also appear in the group with the lowest means for another nutrient. However, genotype 3 stood out for appearing more frequently in the group with highest means and was grouped in the cluster of highest means for six nutrients (N, K, Mg, Fe, Mn, B). On the other hand, genotypes 5, 8, 14, 17 and 30 were not found in the group with highest means for leaf concentrations of any evaluated nutrient.

Significant differences between leaf nutrient concentrations of *C. canephora* genotypes were also reported by Gomes *et al.* (2016) and Martins *et al.* (2019b). These differences between genotypes may be related to nutrient uptake affinity, compartmentalization in roots or other plant organs, mobility in xylem and phloem vessels and changes in the rhizosphere during growth (25). Another important factor is that the biomass accumulation rates of *C. canephora* genotypes differ from each other (32), *i.e.*, nutrient dilution effects may occur in genotypes with higher and effects of nutrient concentration in genotypes with lower biomass accumulation rates. These genetic variations cause the differences in leaf nutrient contents, indicating a higher or lower efficiency of nutrient uptake, translocation or use by the plant between cultivars or lines (12), and consequently, the possibility of improving and/or selecting more efficient cultivars for nutrient use (13).

The significance of interactions for Ca, S and Cu concentrations indicated a differential response of genotypes to the two sampling periods. For pre-flowering, the genotypes were clustered into three groups for leaf concentrations of Ca, S and Cu. For grain filling, two groups were formed for Ca and Cu and four for S (table 6, page 29).

For nutrient Ca, the leaf concentration of none of the evaluated genotypes was higher during grain filling than pre-flowering, but was mostly statistically equal in both periods or lower during grain filling, indicating a tendency to higher Ca concentrations during pre-flowering (table 6, page 29). Higher Ca concentrations during pre-flowering were also reported by Partelli *et al.* (2016; 2018). Genotypes 1, 6, 10, 11 and 16 stood out for leaf Ca concentration for being clustered in the group with the highest means for both periods. This nutrient fulfills two distinct functions in plants - a structural and a signaling, *i.e.*, it serves as a secondary messenger that triggers plant responses to environmental stimuli (9).

Table 5. Mean leaf concentration of N, P, K, Mg, Fe, Mn and B in 42 *Coffea canephora* genotypes. Nova Venécia, ES - Brazil.

Tabla 5. Concentración media de hojas de N, P, K, Mg, Fe, Mn y B en 42 genotipos de *Coffea canephora*. Nova Venécia, ES - Brasil.

Means followed by the same letter in a column do not differ from each other by the Scott-Knott test at 5% probability.

Las medias seguidas de la misma letra en una columna no difieren entre sí en la prueba de Scott-Knott con una probabilidad del 5%.

Genotype	——— Macronutrients ($g.kg^{-1}$) ————				— Micronutrients ($mg.kg^{-1}$) —		
	N	P	K	Mg	Fe	Mn	B
1	28.23 a	1.06 b	10.52 b	6.29 b	86.33 b	675.16 a	82.16 a
2	28.71 a	1.05 b	11.87 a	5.53 c	84.33 b	832.33 a	70.33 b
3	28.71 a	1.05 b	12.29 a	7.26 a	111.16 a	647.50 a	88.83 a
4	25.28 b	1.08 b	12.39 a	5.25 c	84.50 b	519.83 b	50.50 d
5	25.41 b	1.03 b	11.35 b	5.77 c	87.50 b	550.16 b	61.00 c
6	26.13 b	0.97 b	9.89 b	8.53 a	92.66 b	680.50 a	83.16 a
7	27.53 a	1.15 a	10.21 b	7.55 a	83.50 b	757.83 a	65.50 c
8	26.85 b	1.01 b	10.21 b	6.58 b	89.16 b	553.66 b	58.50 c
9	26.25 b	1.16 a	11.56 a	5.82 c	88.16 b	748.50 a	63.66 c
10	26.21 b	0.99 b	11.56 a	6.44 b	105.83 a	535.33 b	59.33 c
11	25.18 b	1.02 b	11.77 a	6.00 b	101.16 a	628.83 a	70.16 b
12	26.42 b	1.14 a	12.81 a	5.63 c	88.16 b	719.16 a	53.83 d
13	28.66 a	1.11 a	12.08 a	6.33 b	85.00 b	572.66 b	65.00 c
14	24.94 b	1.05 b	10.00 b	6.82 b	85.00 b	530.00 b	61.66 c
15	26.32 b	1.03 b	11.56 a	6.41 b	87.16 b	630.33 a	66.50 c
16	26.62 b	1.05 b	10.94 b	7.72 a	97.50 a	675.66 a	78.16 a
17	25.66 b	1.04 b	10.73 b	5.64 c	84.83 b	485.50 b	54.00 d
18	26.42 b	1.09 a	12.08 a	6.11 b	88.83 b	767.16 a	64.50 c
19	26.20 b	1.03 b	13.02 a	5.13 c	103.66 a	478.33 b	54.33 d
20	26.33 b	1.03 b	12.29 a	4.34 c	78.83 b	542.66 b	50.50 d
21	26.55 b	1.17 a	12.40 a	6.06 b	76.50 b	411.33 b	63.16 c
22	26.76 b	1.02 b	12.60 a	7.31 a	89.16 b	585.50 b	63.00 c
23	26.84 b	1.12 a	13.33 a	5.34 c	88.50 b	711.33 a	71.33 b
24	26.47 b	1.17 a	11.25 b	5.45 c	88.00 b	491.83 b	63.83 c
25	28.21 a	1.16 a	11.56 a	5.39 c	122.16 a	333.66 b	52.83 d
26	27.77 a	1.12 a	14.48 a	4.69 c	89.66 b	471.00 b	60.33 c
27	27.09 a	1.12 a	11.77 a	5.41 c	85.83 b	718.00 a	67.50 b
28	27.61 a	1.08 b	14.06 a	4.47 c	85.16 b	536.66 b	53.16 d
29	25.88 b	1.11 a	11.88 a	4.69 c	109.33 a	571.50 b	53.00 d
30	25.88 b	0.99 b	10.00 b	5.25 c	86.33 b	545.83 b	68.50 b
31	27.63 a	1.21 a	10.83 b	4.69 c	112.16 a	353.83 b	65.66 c
32	26.16 b	0.97 b	11.15 b	5.24 c	94.83 b	609.16 a	59.00 c
33	27.83 a	1.12 a	12.29 a	5.38 c	92.16 b	611.33 a	58.50 c
34	26.37 b	1.15 a	12.19 a	5.13 c	78.50 b	623.66 a	50.16 d
35	26.51 b	1.05 b	12.81 a	4.48 c	88.16 b	540.16 b	55.66 d
36	27.46 a	1.16 a	10.52 b	6.84 b	94.00 b	829.33 a	64.16 c
37	26.86 b	1.11 a	11.35 b	6.17 b	96.16 b	626.00 a	65.83 c
38	26.56 b	1.01 b	10.42 b	7.11 a	86.33 b	688.00 a	69.00 b
39	25.34 b	1.05 b	11.04 b	6.27 b	74.00 b	611.16 a	73.16 b
40	27.40 a	1.18 a	11.87 a	5.46 c	76.83 b	574.00 b	63.50 c
41	26.64 b	1.06 b	12.19 a	4.33 c	81.33 b	589.33 b	52.33 d
42	24.82 b	1.03 b	9.58 b	5.98 b	99.16 a	486.50 b	61.16 c

Table 6. Partitioning of interaction for Ca, S and Cu leaf concentrations in 42 *Coffea canephora* genotypes during pre-flowering and grain filling. Nova Venécia, ES - Brazil.

Tabla 6. Particionamiento de la interacción para las concentraciones de hojas de Ca, S y Cu en 42 genotipos de *Coffea canephora* durante la floración previa y el llenado de granos. Nova Venécia, ES - Brasil.

Means followed by equal letters, lowercase letters and uppercase letters do not differ from each other by the F and Scott-Knott tests, respectively, at 5% probability.

Las medias seguidas de letras iguales, letras minúsculas y letras mayúsculas no difieren entre sí por las pruebas F y Scott-Knott, respectivamente, con un 5% de probabilidad.

Genotypes	—— Ca ($g.kg^{-1}$) ——		—— S ($g.kg^{-1}$) ——		—— Cu ($mg.kg^{-1}$) ——	
	Pre-flowering	Grain filling	Pre-flowering	Grain filling	Pre-flowering	Grain filling
1	26.31 Aa	18.40 Ab	1.82 Ca	1.65 Da	12.66 Ca	14.66 Ba
2	17.39 Ca	17.30 Aa	1.82 Ca	2.10 Ca	9.33 Cb	14.00 Ba
3	22.06 Ba	18.90 Aa	1.71 Ca	2.17 Ca	10.33 Cb	15.33 Ba
4	17.84 Ca	13.37 Ba	1.98 Ca	2.29 Ca	16.00 Ba	16.66 Ba
5	17.57 Ca	14.78 Ba	1.55 Ca	1.96 Ca	15.66 Ba	16.00 Ba
6	27.29 Aa	18.24 Ab	1.89 Cb	2.56 Ba	15.66 Ba	16.66 Ba
7	18.01 Ca	18.95 Aa	1.78 Ca	2.20 Ca	15.00 Ca	19.00 Aa
8	17.65 Ca	20.21 Aa	1.52 Cb	2.06 Ca	9.00 Cb	17.00 Ba
9	22.11 Ba	20.84 Aa	2.13 Bb	2.88 Ba	16.66 Ba	16.66 Ba
10	24.74 Aa	19.28 Ab	1.75 Cb	2.56 Ba	15.00 Cb	25.00 Aa
11	24.80 Aa	21.35 Aa	1.88 Ca	2.20 Ca	12.00 Ca	15.00 Ba
12	22.39 Ba	14.95 Bb	1.62 Ca	2.02 Ca	17.00 Ba	18.00 Aa
13	21.26 Ba	15.93 Bb	1.88 Ca	2.20 Ca	19.33 Aa	20.66 Aa
14	20.72 Ba	14.00 Bb	1.86 Ca	1.59 Da	14.00 Cb	20.00 Aa
15	22.08 Ba	18.05 Aa	1.78 Cb	2.56 Ba	16.33 Ba	18.00 Aa
16	25.21 Aa	19.63 Ab	2.17 Ba	2.35 Ca	15.66 Ba	15.00 Ba
17	22.65 Ba	16.72 Bb	1.92 Ca	2.05 Ca	15.33 Ba	16.00 Ba
18	17.51 Ca	17.32 Aa	1.75 Ca	1.92 Da	14.33 Cb	19.00 Aa
19	19.41 Ca	16.99 Aa	1.63 Ca	2.00 Ca	17.00 Ba	16.33 Ba
20	18.80 Ca	13.93 Bb	1.62 Ca	1.57 Da	13.00 Ca	13.00 Ba
21	19.83 Ca	15.58 Ba	1.71 Ca	2.03 Ca	21.00 Aa	15.33 Bb
22	16.92 Ca	18.67 Aa	2.09 Bb	3.52 Aa	19.00 Aa	16.33 Ba
23	16.41 Ca	14.72 Ba	1.86 Ca	2.12 Ca	20.33 Aa	18.66 Aa
24	20.80 Ba	13.33 Bb	1.55 Ca	1.68 Da	15.66 Ba	16.33 Ba
25	16.04 Ca	12.22 Ba	1.59 Ca	1.74 Da	11.66 Cb	18.33 Aa
26	18.37 Ca	20.57 Aa	2.45 Bb	3.06 Ba	14.00 Ca	15.00 Ba
27	21.93 Ba	12.06 Bb	1.62 Cb	2.10 Ca	16.66 Ba	16.66 Ba
28	15.57 Ca	15.05 Ba	1.72 Cb	2.23 Ca	18.33 Aa	15.66 Ba
29	17.91 Ca	12.29 Bb	1.76 Cb	2.30 Ca	13.00 Ca	13.33 Ba
30	28.06 Aa	15.99 Bb	1.65 Ca	2.09 Ca	10.00 Ca	13.33 Ba
31	20.03 Ca	15.74 Ba	1.85 Ca	1.74 Da	13.33 Ca	15.33 Ba
32	18.86 Ca	14.32 Ba	1.89 Ca	1.85 Da	13.33 Ca	15.00 Ba
33	17.91 Ca	18.90 Aa	2.19 Bb	2.67 Ba	17.33 Ba	21.33 Aa
34	16.06 Ca	14.68 Ba	1.72 Ca	2.16 Ca	20.33 Aa	18.33 Aa
35	19.67 Ca	14.62 Bb	1.72 Cb	2.23 Ca	17.66 Ba	15.33 Ba
36	22.30 Ba	15.53 Bb	1.69 Ca	2.09 Ca	19.33 Aa	21.33 Aa
37	21.51 Ba	15.37 Bb	3.03 Ab	3.81 Aa	21.33 Aa	16.66 Bb
38	21.00 Ba	16.16 Bb	2.02 Cb	2.56 Ba	19.33 Aa	17.66 Aa
39	22.57 Ba	15.98 Bb	2.03 Ca	2.23 Ca	14.00 Ca	12.00 Ba
40	21.45 Ba	16.07 Bb	1.90 Ca	2.36 Ca	22.66 Aa	18.00 Ab
41	16.09 Ca	13.33 Ba	1.82 Cb	2.39 Ca	18.66 Aa	16.66 Ba
42	21.65 Ba	13.52 Bb	1.58 Cb	2.79 Ba	11.00 Cb	16.00 Ba
Mean	20.40	16.28	1.84	2.25	15.65	16.78

For leaf S concentration, the genotypes responded inversely to that of Ca, since the S concentrations of 16 genotypes were highest during grain filling, while those of the others were considered statistically the same in both periods, indicating a tendency to higher S concentrations during grain filling (table 6, page 29). Genotype 37 stood out for forming a separate group of high S concentration during pre-flowering, superior to the other groups. For the grain filling period, genotype 37 and 22 formed a group with the highest means. The S content in plant tissues of these lines is extremely important, as this nutrient is a constituent of coenzymes, vitamins and certain amino acids that are essential for the metabolism, while a deficiency, similarly as in the case of nitrogen, can lead to plant growth reduction (5).

Similar to S, a trend of higher Cu concentration during grain filling can be observed, except for genotypes 21, 37 and 40, for which the mean concentrations were higher during pre-flowering (table 6, page 29). The importance of adequate Cu concentrations in plants is related to the functions of the element, which is essential for mitochondrial respiration, carbon and nitrogen metabolism, for protection of oxidative stress and necessary for cell wall synthesis (3). It also participates in photosynthetic reactions. since more than half of the Cu in plants is found in chloroplasts (21). The genotypes 13, 23, 34, 36, 38 and 40 were found to have the highest leaf Cu concentrations in both evaluated periods.

The grouping of genotypes by the UPGMA hierarchical clustering, using Mahalanobis' generalized distance (D^2) as a measure of genetic dissimilarity for the macro- and micro-nutrient leaf concentrations in the periods pre-flowering and grain filling, allowed the formation of a dendrogram. By establishing a maximum limit of 60% dissimilarity between genotypes, the formation of four groups was observed (figure 1).

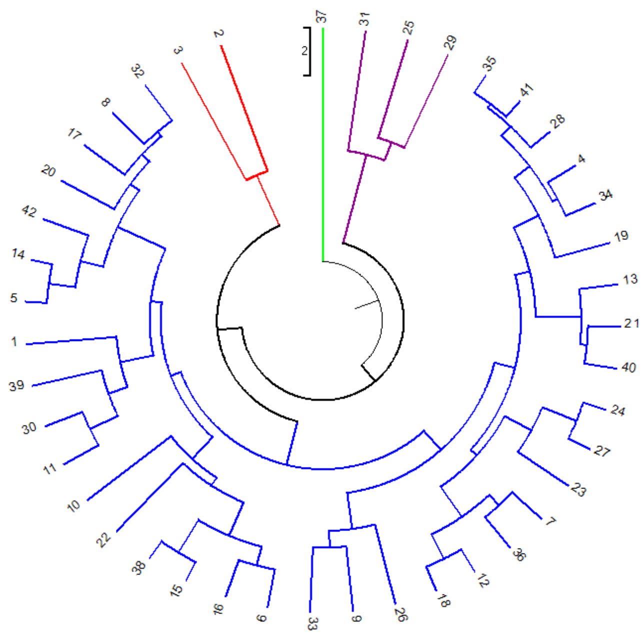

Figure 1. Representative dendrogram of genetic dissimilarity among 42 *C. canephora* genotypes, obtained by UPGMA clustering, using Mahalanobis' generalized distance (D^2) for leaf concentrations of nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, iron, copper, manganese and boron during pre-flowering and grain filling. Cophenetic correlation = 0.62.

Figura 1. Dendrograma representativo de la diferencia genética entre 42 genotipos de *C. canephora*, obtenidos por agrupamiento UPGMA, utilizando la distancia generalizada de Mahalanobis (D^2) para las concentraciones foliares de nitrógeno, fósforo, potasio, calcio, magnesio, azufre, hierro, cobre, manganeso y boro durante pre-floración y relleno de grano. Correlación cophenetic = 0,62.

The first group established by the UPGMA method consisted of only genotype 37. The second group comprised three genotypes, 31, 25 and 29. Genotypes 2 and 3 formed the third group, and the fourth group contained 36. genotypes, representing 85.71% of all evaluated genotypes. The formation of the groups, considered divergent, indicates the variability between *C. canephora* genotypes for leaf nutrient concentrations. By the hierarchical UPGMA clustering, Gomes *et al.* (2016) and Martins *et al.* (2019b) also observed the formation of divergent groups of *C. canephora* genotypes for nutritional characteristics. In breeding programs, the study of genetic diversity by multivariate techniques is useful for planning and to define work strategies (22).

According to the grouping by the UPGMA method, the mean of the evaluated characteristics was calculated for each group, thus allowing a comparison of the leaf concentrations that differentiate the groups (table 7). In the first group, with only genotype 37, the highest S and Cu and lowest K concentrations were found. The second group had the highest P and Fe and lowest Mg, Mn, B., Ca and S leaf concentrations. For the third group, highest N, K, Mg, Mn, B and Ca concentrations and lowest P and Cu concentrations were recorded. The fourth group differs from the others by not presenting highest leaf concentrations of any of the evaluated nutrients, but the lowest N and Fe concentrations.

Table 7. Means of macro- and micronutrient leaf concentrations in *Coffea canephora* for groups formed by UPGMA clustering based on Mahalanobis' generalized distance (D^2). Nova Venécia, ES - Brazil.

Tabla 7. Medias de las concentraciones de hojas de macro y micronutrientes en *Coffea canephora* para grupos formados por agrupamiento UPGMA basado en la distancia generalizada de Mahalanobis (D^2). Nova Venécia, ES - Brasil.

Group	**Macronutrients**						**Micronutrients**			
	——————— ($g.kg^{-1}$) ———————						———— ($mg.kg^{-1}$) ————			
	N	P	K	Ca	Mg	S	Fe	Cu	Mn	B
G1	26.86	1.11	11.35	18.44	6.17	3.42	96.16	19.00	626.00	65.83
G2	27.24	1.16	11.42	15.71	4.92	1.83	114.55	14.16	419.66	57.16
G3	28.71	1.05	12.08	18.91	6.40	1.95	97.75	12.25	739.92	79.58
G4	26.52	1.07	11.64	18.53	5.90	2.04	88.12	16.53	602.37	62.77

To determine the relative contribution of leaf concentration of evaluated nutrients, we used the method of Singh (1981), resulting in values from 4.69 to 22.52% (table 8). Leaf concentrations that contributed most to genetic divergence among the 42 genotypes were nutrients S (22.52%), Cu (11.54%), B (11.46%) and Mg (11.10%), which together accounted for 55.62% of the variability between the genotypes.

Table 8. Relative contribution of leaf concentration of macro- and micronutrients to genetic diversity in 42 *Coffea canephora* genotypes, according to Singh's method (1981), Mahalanobis' generalized distance (D^2). Nova Venécia, ES - Brazil.

Tabla 8. Contribución relativa de la concentración foliar de macro y micronutrientes a la diversidad genética en 42 genotipos de *Coffea canephora*, según el método de Singh (1981), distancia generalizada de Mahalanobis (D^2). Nova Venécia, ES - Brasil.

Nutrients	**S.j**	**S.j (%)**	**Cumulative S.j (%)**
S	2539.352978	22.52	22.52
Cu	1300.724814	11.54	34.06
B	1291.781259	11.46	45.52
Mg	1250.1168	11.10	56.62
P	1122.41325	9.96	66.58
Ca	1102.525267	9.78	76.36
Fe	747.869333	6.63	82.99
Mn	730.187555	6.48	89.47
K	660.416076	5.86	95.33
N	528.287387	4.69	100

S. j: value estimated by the statistic of Singh (1981).

S. j: valor estimado por la estadística de Singh (1981).

Leaf N concentration was the trait that contributed least to the genetic diversity among genotypes (table 8, page 31). Similarly, Starling *et al.* (2019) reported that the N concentration in plant tissues of *C. canephora* genotypes had the lowest relative contribution to genetic diversity by the method of Sing (1981). The study of the relative importance of traits for genetic divergence is highly relevant, since it estimates values based on which those of minor importance for genotype discrimination can be eliminated (20).

Conclusions

Genetic divergence is available among *C. canephora* genotypes for leaf nutrient concentration during the phenological stages pre-anthesis and grain filling.

Genotype 3 (Bicudo) stands out with the highest leaf concentrations of six evaluated nutrients (N, K, Mg, Fe, Mn and B).

Leaf S concentration contributed most to the genetic diversity among the 42 evaluated genotypes, followed by Cu, B and Mg concentrations.

To improve the efficiency of nutritional diagnosis, it is suggested that apart from the sampling periods of pre-flowering and grain filling, the genotypic variability for leaf nutrient concentration should also be taken into consideration.

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Methodological proposal for the characterization of accessions in Germplasm Banks using Generalized Procrustes Analysis applied to incomplete but connected trials

Propuesta metodológica para la caracterización de accesiones en Bancos de Germoplasma mediante Análisis de Procrustes Generalizado aplicado a ensayos incompletos pero conectados

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Abstract

Characterization of plant material conserved in germplasm banks allows the study and analysis of the genetic variability within a collection. When germplasm banks have a large number of accessions, field evaluation should be performed using assays with manageable accession subsets. Common checks connecting the different assays are required to compare these accession subsets. In this study, the Generalized Procrustes Analysis was proposed as a basis for obtaining a factorial plane where all individuals are projected. This technique is applied to genotypes common to all assays, iteratively generating scale factors and rotation matrices. Accessions only belonging to a given assay are considered supplementary elements. This proposal was illustrated using datasets of 54 maize accessions from the Pergamino Active Germplasm Bank of the Experimental Station at the Instituto Nacional de Tecnología Agropecuaria (INTA) in Argentina. The proposal achieved highly satisfactory results.

Keywords

three-way data • MANOVA • modeling biological process • plant genetic resources

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Resumen

La caracterización del material vegetal conservado en bancos de germoplasma permite el estudio y análisis de la variabilidad genética dentro de una colección. Cuando los bancos de germoplasma tienen una gran cantidad de entradas, la evaluación de campo debe realizarse utilizando ensayos en los cuales se evalúa un subconjunto de poblaciones manejable experimentalmente. Se requieren poblaciones testigo que conecten los diferentes ensayos para comparar estos subconjuntos de accesiones. En este estudio, se propuso utilizar el Análisis de Procrustes Generalizado como base para obtener un plano factorial donde se proyectan todos los individuos. Esta técnica se aplica a los genotipos que son comunes a todos los ensayos, para generar iterativamente factores de escala y matrices de rotación. Las accesiones que solo pertenecen a un ensayo dado se consideran elementos suplementarios. La propuesta se ilustró utilizando un conjunto de datos de 54 accesiones de maíz del Banco Activo de Germoplasma Pergamino de la Estación Experimental del Instituto Nacional de Tecnología Agropecuaria de Argentina, donde se obtienen resultados altamente satisfactorios.

Palabras clave
datos de tres vías • MANOVA • modelización de procesos biológicos • recursos fitogenéticos

Introduction

Germplasm banks play an important role in genetic resources conservation. Characterization of genetic resources is the process of describing accessions with respect to a particular set of characters (6). The evaluation process has several objectives: i) to measure the genetic variability of the studied group; ii) to establish the representativeness of the collection in relation to the genetic variability of species in a region or to intra-species genetic variability; and iii) to identify genes that can be used for research and practical purposes, such as biotic resistance (11). The evaluation of total conserved accessions is affected by limitations such as experimental plot areas, labor availability and logistics. These limitations call for trait evaluations on consecutive field assays, each of them with a subset of manageable number of accessions. Associated with the complete evaluation of a germplasm bank by agronomic and morphological descriptors, two considerations should be taken into account: a) to conduct consecutive assays for evaluating a large number of accessions, and b) to use multivariate statistical methods for analysis of set quantitative traits. Complete evaluation of accessions from a germplasm bank requires the inclusion of a set of checks or controls in all assays, performed over the seasons. These checks connect the assays and allow the comparison of the different accessions.

Taba *et al.* (1998) reported the results of field evaluations to develop a core subset of Caribbean maize (*Zea mays l.*) accessions from the maize germplasm bank of CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo). A total of 498 accessions were evaluated in two sets of 249 accessions each, and seven common checks. The authors present a combined analysis of these data sets. Firstly, they propose a mixed linear model including the following effects: assay, accession and their interaction, plus other design-associated factors. These effects were estimated and removed from the observed value of each entry for each trait, leaving only the genotype effect. Secondly, the adjusted trait means were used to cluster accessions into homogeneous groups through a multivariate analysis. Following this same approach, Reeb *et al.* (2007) developed a model to evaluate 145 maize accessions from the Active Germplasm Bank (BAP) of the Experimental Station at the Instituto Nacional de Tecnología Agropecuaria (EEA INTA) in Pergamino, Argentina. Both models have a high degree of unbalance and the interactions result in a large number of empty cells, making difficult the effects estimation. In addition, given that they are univariate models, they ignore relationships structure among variables within datasets.

On the other hand, data from every assay are arranged into matrices of accession by markers sharing, over time, a group of accessions characterized in all assays. This may be seen as a three-way data structure, where input matrices are incomplete but connected through common checks. The first way comprises the accessions, the second one, the characterization traits and the third one, the assays. Thus, a three-way data analysis should be a useful tool to deal with the multidimensional nature of the information.

Objective

To develop a multivariate methodology based on Generalized Procrustes Analysis (GPA) (8, 9, 25) to define similar accession groups in the context of the characterization assays, previously described.

Materials and methods

Plant material

Maize accessions were evaluated in three assays. Assay 1 was planted in Pergamino (Buenos Aires, Argentina) during the 2006/2007 season, and included 23 accessions of different racial forms: Cristalino Colorado (11); Cristalino Amarillo (1); Dentado Amarillo (6); and Dentado Blanco (8). Assay 2 was conducted in Ferré (Buenos Aires, Argentina) during the 2006/2007 season. This assay included 24 accessions of different racial forms: Cristalino Colorado (12); Avatí Morotí Ti (1); Dentado Amarillo (3); Dentado Blanco (8); and non-classifiable type (1). Seven common checks were included in both assays: four synthetic open pollinated (BS13p, Candelaria INTA, Payagua INTA and SP1234) and three accessions (ARZM14007 of Dentado Blanco, ARZM14023 of Dentado Amarillo and ARZM18004 of Cristalino Colorado). Assay 3 included all the 47 accessions of assays 1 and 2 and the seven common checks, and it was carried out in Ferré during the 2011/2012 season. Assay 3 was used as a reference for the comparative analysis of the methodology proposed. Random Complete Block Design with two replications was used for the three assays. Twelve morphological, agronomic and phenological traits were measured according to IBPGR descriptors (12): plant height (PL.HEIGHT-cm); ear height (E.HEIGHT-cm); kernel width (K.WIDTH-mm); kernel length (K.LENGTH-mm); number of kernel rows (N.ROW); ear diameter (E.DIAM-mm); 1000 kernel weight (W1000-gr); yield (YIELD-kg); prolificacy (PROL); root lodging (ROOT.L-%); days to anthesis (D.ANT) and days to silking (D.SILK) (3).

Principal Components Analysis on the concatenated table

Principal Component Analysis (PCA) adequately describes a set of *n* individuals and *p* variables through a small set of variables expressed as linear combinations of the original ones. In this way the information is optimally represented in a reduced dimensional space. In addition, the initial variables are usually correlated, whereas the new ones are not. This transformation facilitates data interpretation because it is possible to infer about linear relations between variables and similarities about individuals (5, 27). In this study, a single PCA matrix of 54 accessions by 12 variables was performed using the mean value of accessions over replications per assay and subsequent concatenation of the assays.

Principal Component Analysis on the matrix obtained after the estimation and elimination of model effects

A mixed linear model was adjusted for each variable according to Reeb *et al.* (2007) and Taba *et al.* (1998). This model firstly estimates the effect of each factor and then eliminates it from the value of each variable obtained for each accession:

$$y_{ikl} = \mu + G_i + E_k + B_{l(k)} + GE_{(ik)} + \varepsilon_{ikl}$$

where:

y_{ikl} = the observed value
μ = the general mean
G_i = the effect of the *i*-th accession *i*=1,..., 54
E_k = the effect of the *k*-th assay, *k*=1.2
$B_{l(k)}$ = the effect of the block-within-assay set, *l*=1.,2
ε_{ikl} = the random error.

As a result, a matrix of 54 accessions by 12 adjusted variables was used to perform a PCA.

Principal Component Analysis on the reference assay

A matrix of individuals-by-variables was obtained using the average of the replications, based on data from the reference trial conducted in the 2011/2012 season. This matrix was used to perform the PCA.

Generalized Procrustes Analysis on incomplete but connected trials

An algorithm based on Generalized Procrustes Analysis (GPA) (8, 9, 25) was developed to generate a consensus configuration of the common checks from a multivariate approach. GPA can be applied to a set of individuals described by the same variables in different conditions. This technique searches for an optimal consensus configuration of the different individuals-by-variables data matrices. The consensus is obtained through a series of iterative algebraic steps that include translation, rotation, reflection and scaling of each individual configuration, optimizing a goodness-of-fit criterion. The latter relies on maintaining the relative distances among elements of the individual configurations and on minimizing the sums of squares between analogous points, *i.e.*, points that correspond to the same element under different configurations. After the initial standardization or the translations have been done and all the configurations have been transformed, an iteration is completed. The process is repeated until the change in the residual square sums between two consecutive steps is less than a particular value. The consensus configurations are obtained from the average of all individual transformed configurations. Once the iterative process of the GPA ends, the total variability can be partitioned in the form of a table of analysis of variance (ANOVA).

Some applications of this technique are related to measuringthe consensus between the agronomic and molecular information (1, 3, 10, 18, 20, 22, 26). On the other hand, GPA can be applied in crop characterization and other agronomic objectives (4, 19, 23, 27).

The experimental situation under study can be analyzed using incomplete matrices which contain the information from q assays. In each k-th assay ($k=1 \dots q$), a set of ($n+n_k$) accessions is assessed by p variables. Each set of ($n + n_k$) accessions includes a set of n common checks measured under q conditions, and n_k other accessions, different in each k-th assay ($k = 1 \dots q$). In matrix notation, information on the conditions is represented by q matrices X_k. They have both: n rows representing the individuals measured under all conditions (hereafter referred to as individuals in common), and n_k rows representing those individuals which correspond only to the k-th condition. The latter are unique to each particular condition and differ among conditions. Therefore, each X_k data matrix can be partitioned into two submatrices: X_k^C, of order (nxp) with the coordinates of the individuals in common in the k-th condition, and X_k^R, of order (n_kxp) with the coordinates of the individuals unique to the k-th condition (figure 1). A common space where to project all the set of individuals is required for analyzing the relationships between the $N = n + \sum_{k=1}^{q} n_k$ individuals. However, an assumption of the GPA is that all objects are measured under all conditions.

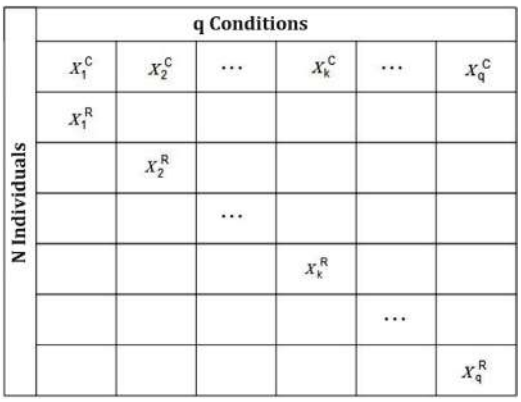

Figure 1. Data structure. In each condition, the individuals corresponding to the k-th condition are represented by the matrix X_k^R, of order ($n_k\,xp$), while the individuals in common are represented by X_k^C, of order ($n\,xp$). The conditions are juxtaposed.

Figura 1. Estructura de los datos. En cada condición k los individuos pertenecientes a dicha condición se representan en la matriz X_k^R de orden ($n_kx\,p$) y los individuos comunes en la matriz X_k^C de orden ($n\,x\,p$). Las condiciones están yuxtapuestas.

Thus, the GPA-based algorithm must be applied on the *n* individuals in common, while the individuals unique to each condition are considered as supplementary elements (17). The n_k individuals in the *k*-th condition are centered, scaled and rotated through the same transformations applied for the individuals in common, but are excluded from the calculation of the parameters involved in these operations. The individuals in common act as pivots for the rest of the accessions, being the geometric reference for the successive rotations. Therefore, it is essential to select individuals in common that satisfy some requirements for obtaining reliable and accurate results.

The assumptions of the proposal are that individuals in common are clustered into groups showing significant differences between the considered traits and that these groups remain stable across conditions. These are verified by MANOVA, checking the absence of genotype-by-assay interaction and the presence of two or more significantly different groups. If these assumptions are verified, then the relationships between individuals in each condition can be analyzed through the set of individuals in common (figure 2). That is to say, individuals that belong only to the *k*-th condition X_k^R are compared with the individuals in common that correspond to this same condition, X_k^C. Then, using the GPA performed on the individuals in common, X_k^C is compared with X_k^C through the consensus configuration *Y*. Finally, individuals in common within de *k'*-th condition are compared with individuals corresponding only to this condition, X_k^R .

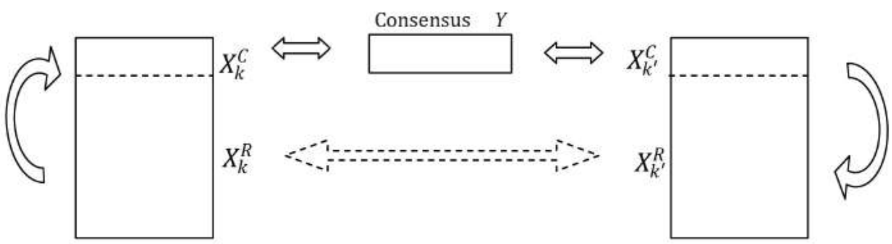

Figure 2: Flow scheme for comparing individuals in each condition through the consensus of individuals in common.

Figura 2: Esquema de flujo para comparar los individuos de cada condición a través del consenso de los individuos comunes.

In this multivariate solution, GPA is used as a basis for obtaining a factorial plane where all individuals are projected (7, 13 , 14, 15, 17). Thus, individuals are grouped according to the similarity of their multivariate behavior (2).

A brief description of the steps involved in the methodological proposal is presented here:

- Fit a MANOVA model to the individuals in common, taking into account the main effects, the interactions and the design terms. The aim is to verify the assumptions of absence of genotype-by-assay interaction effect and presence of, at least, two significant different groups of the individuals in common (significant accession effect). The multivariate model is:

$$\boldsymbol{y}_{ikl} = \boldsymbol{\mu} + \boldsymbol{G}_i + \boldsymbol{E}_k + \boldsymbol{B}_{l(k)} + \boldsymbol{GE}_{(ik)} + \boldsymbol{\varepsilon}_{ikl} \quad (1)$$

where:

$\boldsymbol{y}_{ikl}$ = the *l*-th multivariate replication of the *p* observable variables,
$\boldsymbol{y}_{ikl} = (y_{ikl1}, y_{ikl2}, ...y_{iklp})'$;
$\boldsymbol{\mu}$ = the general mean;
$\boldsymbol{G}_i$ = the effect of the *i*-th accession, *i*=1,...,7;
$\boldsymbol{E}_k$ = the effect of the *k*-th assay, *k*=1,2;
$\boldsymbol{B}_{l(k)}$ = the block-within-trial set, *l*=1,2;
$\boldsymbol{\varepsilon}_{ikl}$ = the random error.

- Generate the assay configurations X_k, $k=1...q$ by carrying out a PCA on each separate assay. Each X_k matrix is considered to be partitioned into X_k^C and X_k^R. The matrix X_k^C contains the coordinates of the individuals in common that belong to the k-th condition (controls). The matrix X_k^C contains the coordinates of those individuals belonging only to the k-th condition.
- Center each configuration X_k, $k=1...q$ on the gravity center of the individuals in common.
- Scale each matrix X_k, $k=1...q$, initialize the scale factors and calculate the initial residual sum of squares.
- Perform the rotation of the individuals in common. Compute the rotation matrix of each matrix X_k^C and use it to rotate the individuals only belonging to the X_k^R condition.
- Calculate the consensus configuration Y as the average of the configurations of the rotated individuals in common, and obtain a new residual sum of squares.
- If necessary, adjust the scaling factors and then recalculate the consensus configuration Y and the residual sums of squares.
- If the difference in the residual sum of squares between subsequent steps is greater than the set tolerance, go to step 5; else, concatenate into a single matrix X.
- Finally, perform a PCA on X to obtain the coordinates of the individuals.

The consensus configuration obtained was compared with those from the PCAs performed as described in (2.2), (2.3) and (2.4). For comparison, correlation coefficient between matrices of distances between individuals projected on the principal plane was calculated. These correlation coefficients were computed for each strategy. The proposed algorithm was implemented in an ad-hoc Matlab routine (16).

Results and discussion

Principal Components Analysis on the concatenated matrix

The principal plane obtained by applying the PCA on the concatenated matrix (2.2) explained 68.54% of total variability. The PCA plot (figure 3) shows a horizontal gradient from left to right of phenological traits, plant height, ear height, kernel width, kernel length and 1000 kernel weight, and in opposite direction: number of kernel rows. Yield and Prolificacy contributed to the vertical axis formation, establishing an upward gradient, whereas root lodging established a downward gradient.

The analysis of the individual's factorial plane (figure 4, page 41) shows that the accessions of Dentado Blanco are placed on the right end. These accessions include the plants of largest size and longest flowering cycle, with ARZM14066 and ARZM14090 standing out from the rest.

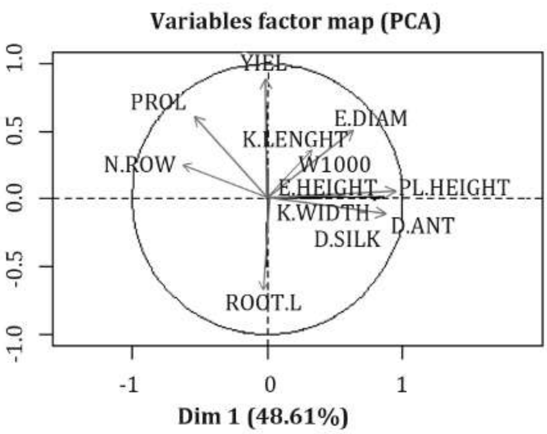

Figure 3. Projection of the variables on the correlation circle obtained performing a PCA after matrix concatenation.

Figura 3. Proyección de las variables en el círculo de correlación obtenido mediante un ACP sobre la matriz concatenada.

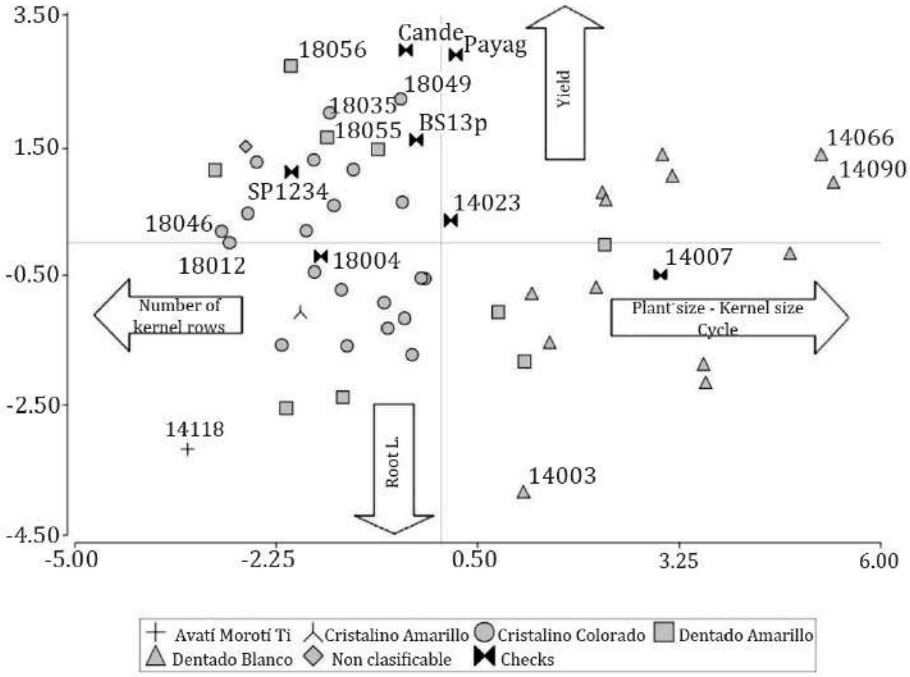

Figure 4. Configuration of individuals (maize landraces accessions) on the principal factorial plane obtained after matrix concatenation.

Figura 4. Configuración de los individuos (accesiones locales de maíz) sobre el plano principal obtenido luego de concatenar las tablas.

The accessions of Dentado Amarillo and Cristalino are placed on the left end. These accessions showed a larger number of kernel rows and a shorter flowering cycle than those of Dentado Blanco, particularly ARZM18046 and ARZM18012. The accessions having the highest yield values are situated on the upper end of the vertical axis. This group is composed by the synthetic varieties used as checks (Candelaria INTA, Payagua INTA, BS13p and SP1234), the accessions ARZM18055 and ARZM18056 of Dentado Amarillo and ARZM18035 and ARZM18049 of Cristalino Colorado. In contrast, the accessions ARZM14003 of Dentado Blanco and ARZM14418 of Avatí Morotí Ti are located at the lower end of the vertical axis, with the lowest yield and largest root lodging percentage.

The accessions of Dentado Amarillo are dispersed, showing the following distribution: the largest plants were grouped with the accessions of Dentado Blanco, those having high yield values were grouped with the synthetic varieties used as checks whereas those of low yield values are grouped with Avatí Morotí Ti. The accessions of Cristalino Colorado constitute an homogeneous group in terms of plant and kernel size and cycle duration, but they are separated into two subgroups in regard to high yield (upper left quadrant) and low yield values (lower left quadrant).

Principal Components Analysis on the matrix of adjusted means

The PCA principal plane obtained after the estimation and elimination of the model effects (2.3) explained 65.14% of the total variability. Traits behaved as in the previous analysis, except for number of kernel rows and prolificacy that contributed to a lesser extent to axis formation. In addition, ear diameter and yield contributed to form the vertical axis. Accessions were included in the same groups as in 3.1.

Principal Components Analysis on the reference trial

The PCA principal plane on data from the reference trial (2.4) explained 61.24% of total variability. Traits behaved as in previous analyses, except for number of kernel rows that contributed to the formation of the vertical axis. Accessions were grouped as before.

Generalized Procrustes Analysis on incomplete but connected trials

After verifying the assumptions, no genotype-by-environment interaction was detected and three groups of individuals in common revealed highly significant differences. Group 1: ARZM14007; Group 2: ARZM14023, ARZM18004, SP1234; and Group3: BS13p, Candelaria INTA, Payagua INTA. In step 2 of the presented methodological proposal, the replicates for each accession were averaged and a PCA was performed on each assay. Within the algorithm, and before the iterative process, both configurations were centered and scaled (Steps 3 and 4) and the initial residual sum of squares was calculated. Then, rotation and scaling were made until the convergence criterion was satisfied (Steps 5 to 8). The algorithm converged in two iterations (table 1).

Table 1. Residual sum of squares after rotation and scaling in two iterations.

Tabla 1. Sumas de cuadrados residuales luego de la rotación y el escalado en las dos iteraciones.

Iteration	SSR after rotation	SSR after scaling
0		0.316400
1	0.063110	0.063110
2	0.063108	0.063108

Figure 5 shows the configuration of individuals obtained by the methodology proposed in this research (Step 9). The accessions are grouped according to their race. Dentado Blanco landraces were separated from Dentado Amarillo and Cristalino along the first principal axis. The synthetic varieties are positioned at the upper end of the vertical axis, which is associated with yield. Accessions were grouped as in the other cases already described.

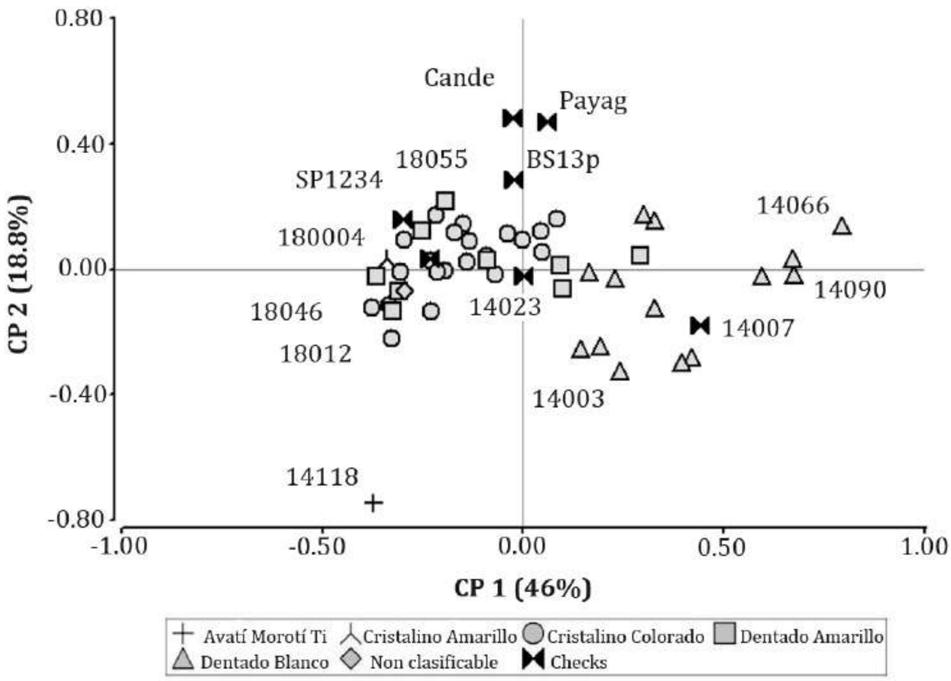

Figure 5. Principal Component Analysis plot of individuals belonging to trials 1 and 2 and consensus configuration of common checks, after applying the proposed methodology.

Figura 5. Representación en Componentes Principales de los individuos de los ensayos 1 y 2 y configuración consenso de los testigos comunes, luego de la aplicación de la propuesta.

Table 2 shows the correlation coefficients between matrices of distance among individuals obtained from the final configurations of the different strategies. The correlation coefficients indicate a high degree of agreement among them. The highest correlation coefficient value was observed between the presented methodological proposal and the strategy of model effects elimination. This was a very promising and encouraging result, since it may improve the way in which germplasm collections are characterized. So far, this goal has been performed by the elimination of model effects, a univariate methodological approach that requires a laborious process. This is due to the model being highly unbalanced, thus hindering the estimation of the effects. In this context the new proposal stands as a useful tool for data analysis in germplasm characterization and evaluation process, providing good results with easy implementation.

Table 2. Correlation coefficients between matrices of distance between individuals obtained from the configurations of the different strategies.

Tabla 2. Coeficientes de correlación entre matrices de distancia entre individuos de las configuraciones provenientes de cada estrategia.

	Concatenation	Elimination of effects	Reference trial	Proposal
Concatenation	1			
Elimination of effects	0.8788	1		
Reference trial	0.7724	0.8491	1	
Proposal	0.8518	0.9518	0.8202	1

The common checks were clustered into significant groups serving as gravity center for accessions that are similar to them. Figure 5 (page 42) shows that non-synthetic common checks were positioned close to the rest of the accessions belonging to their race. Common checks are required to be stable (without genotype-by-assay interaction) and to show a distinct behavior, so that they can serve as geometric reference for those accessions belonging only to a given assay

Conclusions

The objective of this study was to develop a multivariate methodology based on Generalized Procrustes Analysis (GPA) to define similar accession groups in the context of the characterization assays. The GPA is applied to a group of checks, common to all assays. The accessions that are only present in a given assay are considered as supplementary elements. Common checks must fulfill certain assumptions, *i.e.*, they are required not to interact with the evaluation assays and so to be stable across assays. The efficiency of the proposal was illustrated with seven common checks. After verifying the above-mentioned assumptions, the proposed technique was applied to obtain a factorial plane where all evaluated accessions are projected. This configuration was compared with those obtained from three strategies: concatenation of trials, estimation and elimination of the model effects and a reference assay. The correlations between matrices of distance among individuals reveal that the presented proposal provides similar results to those given by the currently used methodology. This traditional methodology is based on the estimation and elimination of effects, and has several disadvantages: a high degree of unbalance and many empty cells that complicate the estimates, in addition of being a univariate approach. These results support that the developed proposal is useful for identifying sets of accessions similar to those involved in germplasm evaluation trials, considering the multivariate structure of the data set.

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Garlic (*Allium sativum* L.) inhibitory effect on platelet activity induced by different agonists

Efecto inhibitorio del ajo (*Allium sativum* L.) sobre la actividad plaquetaria inducida por diferentes agonistas

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Abstract

Platelets are essential elements of human blood. In addition to their normal role, platelets are involved in causing myocardial infarction, stroke and other thrombotic disorders. Platelet activation *in vivo*, probably involves a combination of agonists. Garlic has beneficial effects due to its ability to inhibit platelet aggregation and thromboxane formation. The aim of this work was to evaluate the ability of garlic extracts to inhibit platelet aggregation induced by different agonists and their mixtures in different donors. Significant differences were found in platelet aggregation in response to each agonist ($P \leq 0.05$). The highest antiaggregatory effect was observed with arachidonic acid and the lowest effect with collagen-arachidonic acid mixture. Interaction effects between donor and agonist (or mixtures) were detected. The study showed the potential of aqueous garlic extracts to prevent platelet aggregation induced by different agonist.

Keywords
Allium sativum L. • Perla INTA cultivar • coagulation • antiaggregatory effect

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Resumen

Las plaquetas son componentes esenciales de la sangre humana. Además de su rol normal, las plaquetas pueden estar involucradas en procesos que causan infarto de miocardio, apoplejía y otros trastornos trombóticos. La activación plaquetaria *in vivo*, involucra una combinación de agonistas. El ajo posee efectos beneficiosos debido a su capacidad para inhibir la agregación plaquetaria y la formación de tromboxano. El objetivo fue evaluar la capacidad de extractos acuosos de ajo de inhibir la agregación plaquetaria inducida por diferentes agonistas y sus mezclas en diferentes donantes. Se encontraron diferencias significativas en la agregación plaquetaria en respuesta a cada agonista ($P \leq 0{,}05$). El mayor efecto antiplaquetario se observó con el ácido araquidónico y el menor con la mezcla de colágeno y ácido araquidónico. Se evidenció efecto de interacción entre donante y agonista (o mezclas). El estudio muestra el potencial de los extractos acuosos de ajo para prevenir la agregación plaquetaria inducida por diferentes agonistas.

Palabras clave
Allium sativum L. • Perla INTA cultivar • coagulación • efecto antiagregatorio

Introduction

Platelets are key elements of human blood. They play a central role in the process of thrombus formation as well as an important role in atherogenesis and the progression of atherosclerotic lesions (38). When platelets come in contact with damaged or disrupted endothelium, they are activated, changing their shape and releasing their granules content (33). A great number of agents, including adenosine diphosphate, epinephrine, collagen, thrombin, arachidonic acid, antigen-antibody complexes, serotonin and vasopresin, can induce platelet aggregation (11, 13). Collagen (C) is an extracellular matrix component, which directly binds to receptors that mediate platelet adhesion inducing activation and aggregation. Two receptors are involved in platelet response to collagen; integrin $\alpha_2\beta_1$ acts adhering platelets to collagen, allowing platelets to interact with the lower affinity receptor glycoprotein VI, which is mainly responsible for platelet activation (37). Adenosine diphosphate (APD), the first known low molecular weight platelet aggregating agent, is a weak platelet agonist. However, ADP plays an important role in platelet function given that, when released from the platelet dense granules, it amplifies the platelet responses induced by others agonists (18). Arachidonic acid (AA) is considered a key component in haemostasis and thrombosis, since the release of AA can trigger platelet aggregation and prostaglandin synthesis. AA in reaction with cyclooxigenase is converted to thromboxane A_2, a potent platelet agonist (9). Regarding epinephrine (E), the mechanism by which it induces platelet aggregation is unknown. In some tissues, such as the respiratory epithelium, the activation of the A2AR increases transcellular sodium and chloride cotransport (42).

Several studies have approached platelet aggregation in response to *in vitro* stimulation of these agonists (9, 22, 37, 42). The fact that platelet aggregation *in vivo* involves a combination of agonists, is well known. Consequently, to evaluate various agonists simultaneously according to their way of action and their potential synergistic interaction, becomes useful (23). Platelet aggregation is performed to identify abnormal platelet function, to quantify platelet response, and to monitor platelet inhibition through therapeutic drug (29). As an alternative source of natural antithrombotic drugs, the pharmaceutical industry is currently considering garlic to develop better and safer drugs with lower and less side effects. However, the mechanisms by which aqueous garlic extract elicit their antiplatelet effect are not fully known. Garlic (*Allium sativum* L.) has been used for its antioxidant properties that prevent heart disease including atherosclerosis. Also reduces both plasma cholesterol level and blood pressure (12) and has been recognized as an antiplatelet agent that may contribute to the prevention of cardiovascular disease (34). Raw garlic and some of its preparation like aqueous extracts, garlic oil, aged garlic extract and garlic powder inhibit human platelet aggregation *in vitro* (17) and *in vivo* (4, 20, 40). Many of these physiological effects exerted by garlic are attributed to volatile sulfur-containing flavor compounds, particularly to thiosulfinates, which are responsible for its pungent aroma (24). Allicin (diallylthiosulfinate) is the most abundant compound, representing about 70% of the overall thiosulfinates formed upon crushing garlic cloves (26). It is produced by the interaction of the nonprotein amino acid alliin (+S-allyl-L-cysteine sulfoxide), with the enzyme alliinase (alliin lyase, EC 4.4.1.4) (24). Allicin and other thiosulfinates provide nearly all the antiplatelet activity of raw garlic homogenates in whole blood aggregometry (27). Variability in antiplatelet activity exerted by garlic cultivars belonging to different argentine ecophysiological groups using collagen as agonist has previously been described by our group (15). Perla INTA has the highest organosulfur levels, pungency and *in vitro* antiplatelet activity, being the most suitable garlic cultivar to obtain pharmacological products (15). *In vitro* platelet aggregation can be assayed with an electrical impedance aggregometer using different agonist and several donors. Results may exhibit large variances both among and within platelet donors. Consequently, it is desirable to use an experimental design with several agonists and donor's in order to make their results comparable.

Therefore, this study was conducted to determine the *in vitro* antiplatelet activity of aqueous extract of Perla INTA cultivar induced by different agonists and their mixtures.

Materials and methods

Chemicals

All chemicals used were of analytical grade. Collagen (1mg mL^{-1}) was purchased from Chrono-log Corp, adenosine diphosphate, arachidonic acid and epinephrine were obtained from Helena Laboratory (Beaumont, TX, USA). Stock solutions of each agonist were prepared in deionized water and mixed gently until completely dissolved at the following concentrations: 10 μM ADP, 0,5 mM AA, epinephrine 50 μM. These solutions were stored at -20 °C, except for the collagen solution, which was stored at 4 °C.

Preparation of aqueous garlic extracts

Fresh garlic bulbs of Perla INTA cultivar were chosen from the germplasm collection of INTA La Consulta, Mendoza, Argentina. The bulbs were peeled and blended for 1 min in deionized water (1:10 w/v). The aqueous garlic extract was collected, filtered and kept at room temperature for 15 min to allow enzymatic hydrolysis of the flavor precursors. After that, the extracts were kept frozen at –80 °C.

Aqueous garlic extracts characterization

The characterization of Perla INTA aqueous garlic extract was carried out according to their flavor precursor levels, thiosulfinates, allicin content, and pungency as previously described González *et al*. (2009).

Platelet aggregation

Platelet aggregation was measured *in vitro* using a whole blood electrical impedance aggregometer (10). Blood was collected from nine non-smoker healthy donors (5 women, 4 men), between 26-45 years old, who had no any previous disease and had not taken aspirin or any medication, and all had given informed consent. They also had abstained from eating *Alliums* or other known platelet-inhibitory food or beverages in the previous 2 weeks, for at least 7 days prior to venipuncture. A complete blood count, biochemical analysis and the clotting time was carried out following the Ivy method. Clotting times less than 9.5 minutes were considered normal.

Blood samples were collected by venipuncture (Hematology Service, Central Hospital, Mendoza, Argentina) in tubes containing sodium citrate anticoagulant (3.8%, 1 vol of anticoagulant per 9 vol of blood). Afterward,blood samples were diluted in equal volume of TRIS buffered saline (TBS, pH 7.4) and vortexed. The diluted blood was maintained at room temperature during the experiment and used within 2 h of donation.

Aliquots of 1mL of blood/TBS were incubated for 3 min at 37 °C, then a dose of aqueous garlic extract was added and platelet aggregation was induced by the addition of different agonists or their mixtures. Changes in the impedance was recorded over 6 min. The change in impedance is proportional to the amount of platelet aggregation. All treatments were evaluated with each donor's blood four times. *In vitro* antiplatelet activity (AAI) was expressed as percentage of inhibition of platelet aggregation, compared to control samples prepared in the same way but without adding aqueous garlic extract. The control is considered as 100 % the aggregation or 0% the inhibition for each agonist.

Table 1 shows agonists concentrations prepared as described Bordia *et al.* (1996); Soloviev *et al.* (1999); Rahman and Billington, (2000). Based on previous results a concentration of 7 mg of aqueous garlic extract per mL of blood was chosen for platelet aggregations reactions (15). This concentration is equivalent to a daily consumption of 1 clove (1.5 g of garlic for a person weighing 70 kg) (43).

Table 1. Concentration of stock solutions of each agonist and their mixtures.

Tabla 1. Concentración de las soluciones stock de cada agonista y sus mezclas.

a. Concentration per mL of whole blood.

b. Concentración por mL de sangre completa.

Agonist	Concentration[a]
Collagen	1 µg/mL
Arachidonic acid	0.5 mM
Adenosine diphosphate	10 µM
Epinephrine	50 µM
Mixtures	
Collagen + Arachidonic acid	1 µg/mL + 0.5 mM
Collagen + Adenosine diphosphate	1 µg/mL + 10 µM
Collagen + Epinephrine	1 µg/mL + 50 µM
Adenosine diphosphate + Epinephrine	10 µM + 50 µM

Statistical analysis

Values were expressed as means ± SEM. Data were analyzed by analysis of variance (ANOVA) to test the significant differences. Means were compared using Tukey's test. The results were considered significant at $P \leq 0.05$ unless specified otherwise. Statistical analysis was performed using the software STATISTICA for Windows 6.0. Means of each treatment were compared by least significant difference (LSD) test; $P \leq 0.05$ were considered to be significant.

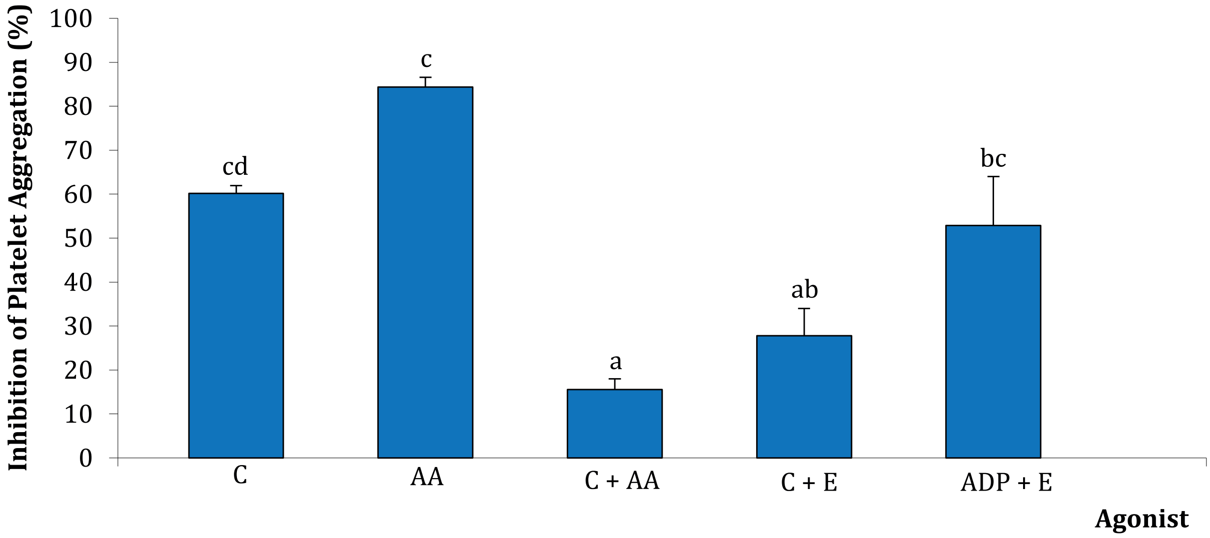
Results

None of the donors included in the assay showed a value of impedance higher than 5 Ω in platelet aggregation induced by ADP. Consequently, this agonist did not cause a significant aggregation in the respective samples. A similar behavior was observed with epinephrine.

Inhibition percentage in platelet aggregation showed significant differences (figure 1, page 50) among different agonists as well as among their combinations. The inhibitory effect on platelet aggregation induced by collagen ranged between 22.5-100%. After adding collagen before aggregation onset, aggregation slopes showed a delay of about 60 s or more, then followed by a steady increase in aggregation. This delay was greater than control samples when garlic extract was added (data no shown).

Antiplatelet aggregation, when AA was used, ranged between 75.7-100%. The aggregation slopes showed a monophasic aggregation response to AA.

On the other hand, antiplatelet effects exerted by Perla INTA aqueous extract were studied by using different agonist mixtures. When a collagen–AA mixture was used as platelet agonist, antiplatelet activity ranged between 3.84-26%. In addition, *in vitro* Perla INTA aqueous extract suppressed platelet aggregation induced by collagen-epinephrine and ADP-epinephrine induced human platelet in a wide range, 10.23-48.48% and 20-80%, respectively. Significant synergistic interaction was observed with collagen in combination with epinephrine, AA and ADP, and ADP in combination with epinephrine when added simultaneously at the selected concentration. Thus, higher impedance values were observed when aggregation was induced by two agonists combination (figure 2, page 50).

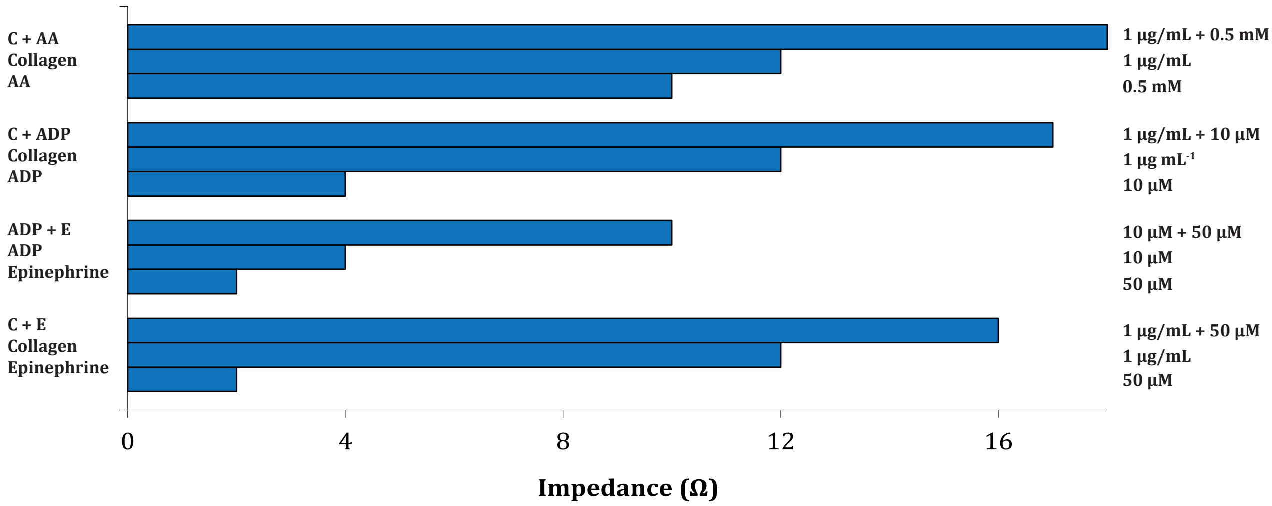


Bars represents means ± SD (n=9). Different letters indicate significantly different according to Tukey test (P ≤ 0.05). ADP: Adenosine diphosphate, E: Epinephrine, C: Collagen, AA: Arachidonic acid.

Barras representan valores promedio ± SD (n=9). Letras distintas indican diferencias significativas según test Tukey HSD a P ≤ 0,05. ADP: Adenosina difosfato, E: Epinefrina, C: Colágeno, AA: Ácido araquidónico.

Figure 1. *In vitro* inhibition of platelet aggregation exerted by Perla INTA aqueous extracts on platelet aggregation induced by different agonists and their mixtures.

Figura 1. Inhibición de la agregación plaquetaria *in vitro* ejercida por extractos acuosos de Perla INTA sobre la agregación inducida por diferentes agonistas y sus mezclas.



Values at right of bars indicate the concentrations of agonists used. ADP: Adenosine diphosphate, E: Epinephrine, C: Collagen, AA: Araquidonic acid.

Valores a la derecha de cada barra indican las concentraciones de agonistas usadas. ADP: Adenosina difosfato, E: Epinefrina, C: Colágeno, AA: Ácido araquidónico.

Figure 2. Aggregation platelet *in vitro*: synergistic effect exerted by different combinations of agonists.

Figura 2. Agregación plaquetaria *in vitro*: efecto sinérgico ejercido por diferentes combinaciones de agonistas.

In general, the antiaggregatory effect showed by Perla INTA aqueous extract on platelet aggregation induced by paired of agonists were minor than antiaggregatory effects observed when platelet aggregation was induced by each agonist alone (figure 1, page 50).

Taking into account average antiplatelet activity of all agonists and mixtures used, this biological activity varied between 18.51% ± 2.43 and 84.40% ± 2.15. The highest antiaggregatory effect was observed with AA and the lowest effect with collagen-AA mixture. ANOVA analysis revealed an interaction effect between donor and agonist (or mixtures) at $P \leq 0.05$ (table 2). ANOVA analysis of donor, agonist and their interaction resulted in 15.2%, 54.74% and 26.2 % of variation to the AAI in this study, respectively, indicating that agonist was the main factor determining AAI, and that donor and donor and agonist interaction, played a minor role. There were not significant differences in platelet aggregation inhibition response between women and men.

Table 2. ANOVA table of *in vitro* antiplatelet activity variation due to donor, agonist, and their interaction.

Tabla 2. Tabla de ANOVA de la actividad antiplaquetaria *in vitro* por donador, agonista e interacción.

Source	d.f	Sum of square		F-value	P>F
Donor	2	6929.9	(15.2%)		
Agonist	4	24945	(54.74%)	59.651	0.0000
Donor x agonist	8	11951.8	(26.2%)	107.36	0.0000
Error	30	1742.6	(3.84%)	25.720	0.0000
Total	44	45559.3	(100 %)		

The chemical profile of the main organosulfur compound present in Perla INTA aqueous garlic extract is shown in table 3. Alliin was the most abundant ACSO, representing more than 70% of the total content of ACSOs, whereas isoallin content was nearly to 23% and methiin content represented less than 10%. Also, this cultivar showed high levels of thiosulfinates, allicin, and pungency.

Table 3. S-alk(en)yl-L-cysteine sulfoxides (ACSOs), thiosulfinates, allicin, and pungency of Perla INTA garlic cultivar**.**

Tabla 3. S-alq(en)yl-L-cisteín sulfóxidos (ACSOs), tiosulfinatos, allicina y pungencia del cultivar Perla INTA.

Variables		Concentration
ACSOs ($mg.g^{-1}fw$)	**Alliin**	21.83 ± 1.33[a]
	Methiin	1.53 ± 0.09
	Isoalliin	6.87 ± 0.32
	Total	30.22 ± 0.58
Total thiosulfinates (mM%g fw)		4.45 ± 0.1
Allicin (mg/g fw)		5.04 ±0.1
Pungency (µMol/g fw)		79.86 ± 1.83

[a] Each value is the mean ± SE (n = 5).

[a] Cada valor representa la media ± DS (n = 5).

Discussion

Under pathological conditions, platelets may become more proadhesive/procoagulant, which leads platelets to adhere to an injured area, and to subsequently release a lot of active constituents inducing plug formation (16). For that reason, antiplatelet therapy is used as a strategy to prevent vascular thrombosis and vascular disorders. Several researchers have shown that garlic and their sub products have *in vivo* (1, 2, 8, 44) and *in vitro* (7, 28, 35) antiplatelet activity. These studies have involved the use of collagen, ADP, AA or epinephrine as agonists, but not their combinations. I*n vivo*, more than one agonist and different combinations are involved in platelet activation. Therefore, the study of the antiplatelet effect of aqueous garlic extracts over platelet activity induced by different agonists and their combinations was considered of great contribution to the understanding of the factors involved in this activity. Two types of aggregation can be distinguished *in vitro*: primary aggregation that is reversible and occurs without the release reaction and secondary aggregation that is irreversible and is associated with the release reaction (36). The reversibility of platelet aggregation induced by each dose of agonists included in this study was assessed from the shape of aggregation tracings. A monophasic curve indicating irreversible aggregation was observed with all the agonist and the combinations evaluated. We found that when platelet aggregation was induced by ADP or epinephrine, the aggregation induced was lower than 40% and 30% respectively. Similar results have been reported (6, 14, 25). Mammalian platelets vary widely in their responses to epinephrine, in part due to they can act via excitatory α receptors and inhibitory β receptors (41). Some people have reduced platelet responsiveness to epinephrine due to the lack of these receptors (39). This fact might explain the results obtained in our work. On the other hand, when these agonists were used in combination, a synergistic effect was observed. This was evidenced by higher responses than the expected for ADP or epinephrine alone. As it has been previously reported by other authors (19, 36).

Regarding collagen, we found that a concentration of 1 μg mL^{-1} induces of 100% of platelet aggregation *in vitro*. In addition, we observed that the concentration of Perla INTA aqueous garlic extract used inhibited the platelet aggregation induced by this agonist. Aqueous garlic extracts contain mainly allicin (26). Allicin easily go through cellular membranes and can react with different sulfhydryl (SH)-containing proteins blocking their free sulfhydryl residues (30). We propose that the antiplatelet effect of Perla INTA aqueous garlic extract is due, at least partially, to the inhibition of collagen binding to specific receptors on platelet surface. Consequently, platelet adhesion and platelet activation could be inhibited. This results are in agreement with Manaster *et al* (2009), who demonstrated that allicin inhibit agonist-induced washed platelet activation via inhibition of platelet signaling. According to Baghalian *et al.* (2005) the minimum allicin content to ensure pharmaceutical and economic viability of garlic should be 4.5 mg g^{-1} fw. The higher level of allicin present in this cultivar could be taken as an opportunity to elaborate pharmacological products based on its health-benefit quality.

A monophasic curve in response to a concentration of 0.5 μM of AA was obtained. Both aqueous and organic garlic extracts have been found to inhibit several steps of the AA-cascade in platelets (3). Moreover, garlic extracts inhibit incorporation of labeled AA into platelet phospolipids and inhibit AA-metabolizing enzymes in platelets (15). Ali (1995) showed that raw garlic inhibited cyclooxygenase activity non-competitively and irreversibly. Our results confirm these observations. The highest antiaggregatory effects exerted by Perla INTA aqueous extract were observed when AA was used as agonist.

Platelet aggregation *in vivo* probably involves a combination of agonists, being collagen more important at the beginning, thrombin becomes important later on, and with other agonists in different mixtures throughout (19). Platelets exhibit diverse responses, including shape change, aggregation, and secretion in response to a variety of agonists. These agonists differ in their intrinsic ability to produce the effects on physiological responses by platelet (14, 21). The degree of inhibition depends on the potential activity of each platelet agonist used in the present work. Perla INTA aqueous extract strongly inhibited platelet aggregation induced by a strong agonist like collagen and also by AA. However, the antiaggregatory effect was lower when the aggregation was induced by collagen together with AA. These results demonstrate the synergistic interaction exerted for the combination of agonists used. These results are in agreement with those reported by Razi *et al* (2005) and Huang and Detwiler (1981), who showed the potentiated response to platelet aggregation in combination of collagen and AA, and epinephrine. The synergism between the different platelet agonists increases platelet aggregations. Full platelet aggregation can also be induced by the simultaneous addition of subthreshold levels of platelet stimuli, which fail to induce platelet aggregation on their own merit (21).

Other important aspect that was evaluated was the influence of donor, agonist and their interaction. Although, the analysis revealed an interaction effect between donor and agonist, the type of agonist was the main factor determining of *in vitro* antiplatelet activity. These results were consistent with previous reports that showed that the response of platelets to agonists has a large inter-individual variability within the population (31, 32).

Conclusion

This study reveals the potential antiaggregatory effect of Perla INTA aqueous garlic extract, when this activity is induced by different agonists and their mixtures. Several mechanisms are involved in this inhibitory effect, and they include the modification of platelet membrane, a direct inhibitory effect on the AA metabolism and lipoxygenase enzyme and inhibition of calcium mobilization. The modification of platelet membrane inhibits the signaling pathway and consequently the platelet activation and platelet adhesion. When enzymes involved in several steps of AA-cascade are inhibited, endoperoxides intermediates are not converted to prostanglandins and thromboxane B_2 which are potent platelet aggregators. Furthermore, a synergistic effect was evidenced. This aspect is important since the therapeutic effect of antiplatelet herbal drugs can be affected. However, the biochemical basis of these synergistic responses needs further exploration.

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Peanut (*Arachis hypogaea* L.) yield under irrigation levels in off-season cultivation

Rendimiento de maní (*Arachis hypogaea* L.) bajo niveles de riego en cultivos fuera de temporada

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Abstract

Water deficit is considered the most critical environmental factor for peanut production in Brazil, as it constitutes one of the major constraints to the expansion of its cultivation in the suitable crop zones of the country. Determining crop water demand is fundamental to increasing yield with lower water consumption. The present study aimed to evaluate the effects of full and deficit irrigation levels (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% and L5 = 100% replenishment of crop evapotranspiration) on the development, growth and yield of peanut crop sown in two times, February and March. Treatments were distributed in a split-plot randomized complete block design, with four replicates, using a line-source sprinkler system. Irrigation depths from 65 to 314 mm were applied with the levels L1 to L5 during the first and second cropping cycles. Full irrigation with sowing in March was more advantageous due to yield increase of up to 30% compared to sowing in February, but crop cycle was 25 days longer. Water stress caused by deficit irrigation reduced plant height, seed mass and pod yield, while full irrigation (L5) led to yields from 4,141 to 5,102 kg ha^{-1} for February and March, approximately three times higher than those obtained with the lowest irrigation level (L1).

Keywords
Arachis hypogaea L. • deficit irrigation • water stress

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Resumen

El déficit de agua se considera el factor ambiental más crítico para la producción de maní en Brasil, ya que constituye una de las principales limitaciones para su expansión en las zonas adecuadas para su cultivo en el país. Determinar la demanda de agua de los cultivos es fundamental para aumentar el rendimiento con un menor consumo de agua. El presente estudio tuvo como objetivo evaluar los efectos de los niveles de riego deficitarios y completos (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% y L5 = 100% de reposición de la evapotranspiración del cultivo) en el desarrollo, crecimiento y rendimiento de la cosecha de maní sembrado en dos veces, febrero y marzo. Los tratamientos se distribuyeron en un diseño de bloques completos aleatorios de parcelas divididas, con cuatro repeticiones y utilizando un sistema de aspersores de fuente de línea. Las láminas de riego aplicadas fueron entre 65 a 314 mm (L1 a L5) en ambos ciclos de cultivo. El nivel de riego total con siembra en marzo fue más ventajoso debido al aumento del rendimiento de hasta un 30% en comparación con la siembra en febrero, pero el ciclo de cultivo fue 25 días más largo. El estrés hídrico causado por el riego deficitario redujo la altura de la planta, la masa de semillas y el rendimiento de vainas. El cultivo de maní con riego completo (L5) tuvo rendimientos de 4,141 a 5,102 kg ha^{-1} (febrero y marzo respectivamente) siendo aproximadamente tres veces más que los obtenidos con el nivel de riego deficitario más bajo (L1).

Palabras claves
Arachis hypogaea L. • riego deficitario • estrés hídrico

Introduction

Peanut (*Arachis hypogaea* L.) farming is the fourth largest oil crop in global production with an estimated yield of 45.45 10^6 Mg in the 2017/2018 cropping season (35). In Brazil, peanut is currently cultivated in 138,500 ha, with a production of 0.52 10^6 Mg and average yield of 3.71 Mg ha^{-1}, which corresponds to a 13% expansion of area and a 3% increase in its production compared to the previous season (12). São Paulo is the largest producing state, with 95% of the national production, where the peanut crop is planted mainly in succession to sugarcane in the renewal of sugarcane fields. In Brazil, peanut is cultivated in two periods: in the first one, during the rainy season, the crop is sown between October and November, and in the second one, during the dry season (off-season), it is sown between February and March. In the São Paulo state, about 95% of the cultivated area is limited to the first crop season (12).

The off-season is a strategy for producers who intend to increase economic return with the crop and who carry out activities other than sugarcane production. Peanut cultivation in the dry season requires improved crop management (27), such as the use of irrigation to guarantee high yields and, consequently, higher profitability, since dry spells are very frequent during this period of the year and can drastically reduce the productive potential of the crop (12).

Since 70% of the peanut growing area fall under arid and semi-arid regions, water deficit during the cultivation period is considered the major constraint to peanut yield worldwide because it frequently affects crop growth and development of this crop (22, 30). Water is considered one of the main resources required by plants for growth and is a limiting factor for crop development and agricultural yield (11). An annual estimate of global losses in peanut yield caused by drought equivalent to US$ 520 million (19).

Besides the negative impact on yield, water deficit stands out among the major constraint to the expansion of peanut cultivation to the suitable crop zones in Brazil. Water deficit causes reductions of 25 to 30% in peanut yield in the country (28, 31).

In the literature there are limited research on the effects of water deficit on peanut, most of which were carried out with cultivars of erect growth habit, which are cultivated in a small area in Brazil. Therefore, it is essential to study alternatives to minimize the impacts of water deficit on peanut cultivation in the producing regions, since it is a factor that significantly limits its yield (18, 34). Among the alternatives to mitigate the negative effects of water deficit on peanut yield, it is necessary to conduct studies aiming to evaluate cultivation practices that contribute to the increase of yield and production stability, such as irrigation management, sowing dates and selection of new cultivars with better adaptive capacity to marginal environments.

Therefore, this study aimed to evaluate the effects of full and deficit irrigation levels and sowing dates on the yield, growth and development of peanut cultivated in the off-season, in order to define adequate strategies to increase yield.

Material and Methods

The experiment was set up in the field in the traditional region of peanut production in the municipality of Jaboticabal-SP, under Aw climate (humid subtropical), according to Köppen's classification (3). The soil of the area is classified as *Latossolo Vermelho Eutroférrico típico* (Oxisol), with a very clayey texture, moderate A horizon, kaolinitic, on gently undulating and undulating relief (14). Its physical and chemical characteristics are presented in tables 1 and 2, respectively.

Table 1. Physical characteristics of the soil of the experimental area. Jaboticabal, SP, Brazil.

Tabla 1. Características físicas del suelo del área experimental. Jaboticabal, SP, Brasil.

Depth (cm)	Sd* (g cm^{-3})	FC* (cm^3 cm^{-3})	PWP* (cm^3 cm^{-3})	Sand (g kg^{-1})	Clay (g kg^{-1})	Silt (g kg^{-1})
0–20	1.45	0.45	0.33	310	470	220
20–40	1.49	0.41	0.30	270	520	200

*Sd: soil bulk density; FC: field capacity; PWP: permanent wilting point.

*Sd: densidad del suelo; FC: capacidad de campo; PWP: punto de marchitez permanente.

Table 2. Chemical characteristics of the soil of the experimental area. Jaboticabal, SP, Brazil.

Tabla 2. Características químicas del suelo del área experimental. Jaboticabal, SP, Brasil.

Depth (cm)	pH $CaCl_2$	OM (g dm^{-3})	P_{resin}	S	H+Al	Al	K	Ca	Mg	SB	CCE	V%
			(mg dm^{-3})		($mmol_c$ dm^{-3})							
0–20	5.6	40	67	5	21	1	3.4	36	13	52.7	73.9	71
20–40	5.8	40	68	5	20	1	3.2	36	11	50.3	70.4	71

Hydrogen potential (pH), in $CaCl_2$; Organic Matter (OM); Phosphorus Resin (Presi); Sodium (S); Potential Acidity (H + Al); Exchangeable Aluminum (Al); Potassium (K); Calcium (Ca); Magnesium (Mg); Sum of Bases (SB); Capacity Cation Exchange (CCE); Base Saturation (V%).

Potencial de hidrógeno (pH), en $CaCl_2$; Materia Orgánica (OM); Resina de fósforo (Presi); Sodio (S); Acidez potencial (H + Al); Aluminio intercambiable (Al); Potasio (K); Calcio (Ca); Magnesio (Mg); Suma de bases (SB); Capacidad de intercambio catiónico (CCE); Saturación de bases (V%).

Treatments consisted of 5 depth of irrigation water applied during the growing season (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% and L5 - 100%), corresponding to fractions of replenishment of crop evapotranspiration (ETc), associated with two sowing dates (February - S1 and March – S2), according to figure 1 (page 58).

The experiment was carried out in a split-plot randomized complete block design, with sowing date in the plot and irrigation levels in the subplot, with four replicates. It was set up in an area of 216 m^2, with 20 plots for each sowing date, totaling 40 plots. Each plot consisted of 4 crop rows with length of 2.4 m and evaluations were carried out in the middle portion of the two central rows. The two external rows, as well as 0.5 m on each end of the central rows, were considered as borders and were not used for evaluations.

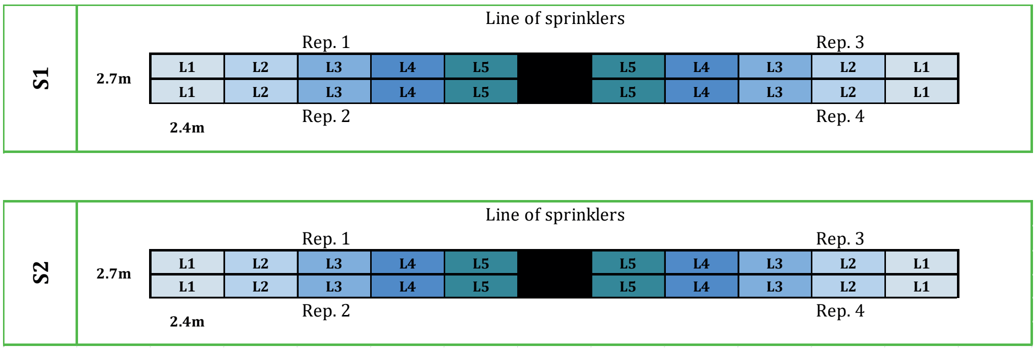

Figure 1. Scheme of the experimental area, with line of sprinklers and experimental units with treatments of irrigation depths (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% and L5 - 100% replenishment of crop evapotranspiration) and sowing dates (S1 - February and S2 - March).

Figura 1. Esquema del área experimental, con línea de aspersores y unidades experimentales con tratamientos de láminas de riego (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% y L5 - 100% de reposición de evapotranspiración del cultivo) y fechas de siembra (S1 - febrero y S2 - marzo).

The adequate temperature range for peanut growth is considered by some authors to be between 10 °C and 33 °C (4, 29). In the present experiment, the temperature values remained within the acceptable range, except for the minimum temperature, which reached values below the lower limit in mid-May and late June (figure 2A, page 59). In mid-May, the crop sown in February was at the full seed stage (R6), while the crop sown in March was at the early pod formation stage (R3). At the end of June, the crops sown in S1 and S2 were at the harvest stage (R8) and full seed stage (R6), respectively. The average temperatures in the first cropping cycle were higher than those of the second one (22.8 and 21.9°C, respectively), as shown in figure 2A (page 59). When the averages are compared considering the first 90 days of each cropping cycle, the difference of temperature was even greater for the first one (24.1°C), compared to the second one (22.8°C).

ETc was calculated as the product between crop coefficient (Kc) and reference evapotranspiration (ETo), with Kc values interpolated during the phenological cycle (*i.e.* $Kc_{initial}$ = 0.4, Kc_{mid} =1.15 and Kc_{final} = 0.6) and ETo calculated by the Penman-Monteith method (2), using daily meteorological data from the weather station of FCAV-UNESP, located close to the experimental area.

During the experimental period, the rainfall and number of days with rainfall were lower than the average of the last 40 years. This water deficit condition allowed evaluating the effects of the treatments imposed on the crop, as it will be discussed below. In the first cropping cycle, there was no need for water supplementation during the vegetative stage (V1 to V6), because of the rain occurred (figure 2B, page 59). In the second cropping cycle, water replacement was necessary at 20 days after sowing (DAS). There were rains in February and March, followed by a long dry period until the end of July, which coincided with most of the cycle for the crop sown in March. Irrigation was applied every week from March, according to the quantities recommended in each treatment. For the crop sown in February, the highest values of ETc (4 to 5 mm day^{-1}) occurred from March 10 to March 31 (figure 2C, page 59), coinciding with high temperatures in the period (figure 2A, page 59). For the crop sown in March, the highest values of ETc were between 3 and 4 mm day^{-1} for the period from April 20 to May 20 (figure 2C, page 59).

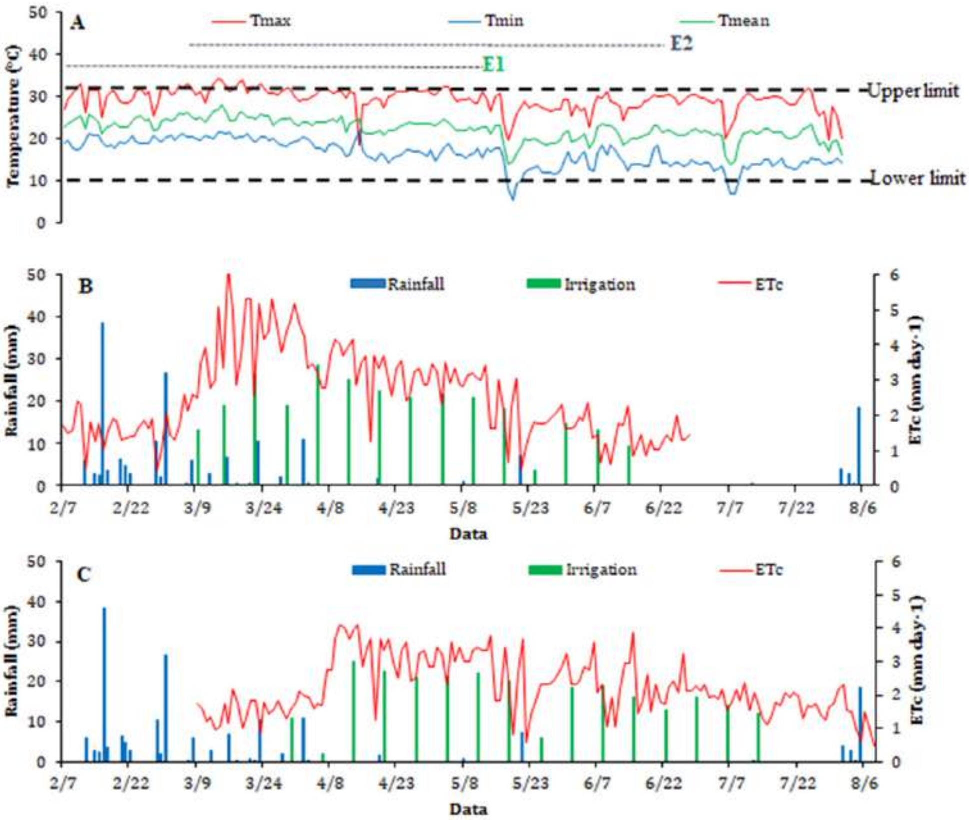

Figure 2. Maximum (Tmax), mean (Tmed) and minimum (Tmin) temperatures (A) and rainfall, crop evapotranspiration (ETc) and irrigation applied in the treatment with full irrigation (L5) for sowing in February (B) and March (C).

Figura 2. Temperaturas máximas (Tmax), medias (Tmed) y mínimas (Tmin) (A) y lluvia, evapotranspiración del cultivo (ETc) y riegos aplicados en el tratamiento con riego completo (L5) para siembra en febrero (B) y marzo (C).

The accumulated values of rainfall were 157 and 51 mm and of ETc were 323 and 299 mm, for the crops sown in February and March, respectively (figure 3, page 60). Most of the rainfall in the first cropping cycle occurred in February (about 100 mm), coinciding with the initial stages of the crop (V1 to V6). The irrigation depths were similar and ranged from 66 to 314 mm in the first cropping cycle and from 65 to 310 mm in the second one. Consequently, the total depths (rainfall + irrigation) received in the treatments ranged from 223 to 470 mm in the first cropping cycle and from 116 to 360 mm in the second cropping cycle (figure 3, page 60). This difference was due to the rainfall, since the irrigation depths applied were similar for both sowing seasons. The maximum total depths received in this experiment were lower than those reported in other studies, in which the peanut crop requires at least 500 mm during the crop cycle (20). This difference is due to the lower water demand of the crop in the autumn-winter period, as conducted in this experiment.

Irrigation was applied weekly in quantities corresponding to the fractions of the ETc accumulated in the period in each treatment, considering an 85% application efficiency. Water depths in each treatment were applied using a line-source sprinkler system (17), which allows obtaining a decreasing gradient of water depth perpendicular to the irrigation line, corresponding to the distribution factor (figure 4, page 60), as established in the treatments (figure 1, page 58). The experiment used Senninger® 3023-2 sprinklers and M 08Qx05 ¾" nozzles, spaced every 6 m in the central row and operated with 350 kPa pressure. The distribution factors of the sprinklers were determined in a field test using collectors spaced by 1 m up to the limit distance of water application by the sprinklers, perpendicularly to the irrigation line, with 2 replicates.

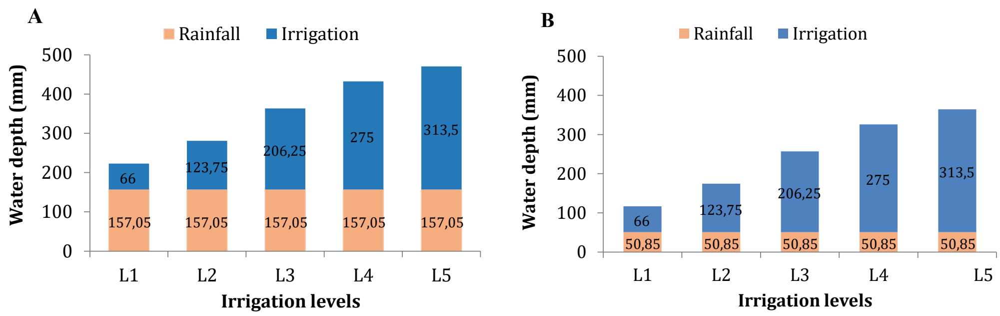

Figure 3. Seasonal water depths (irrigation + rainfall) as a function of irrigation levels (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% and L5 - 100% replenishment of crop evapotranspiration) and sowing dates (February (A) and March (B)).

Figura 3. Láminas de agua estacionales (riego + lluvia) en función de los niveles de riego (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% y L5 - 100% de reposición de la evapotranspiración del cultivo) y las fechas de siembra (febrero (A) y marzo (B)).

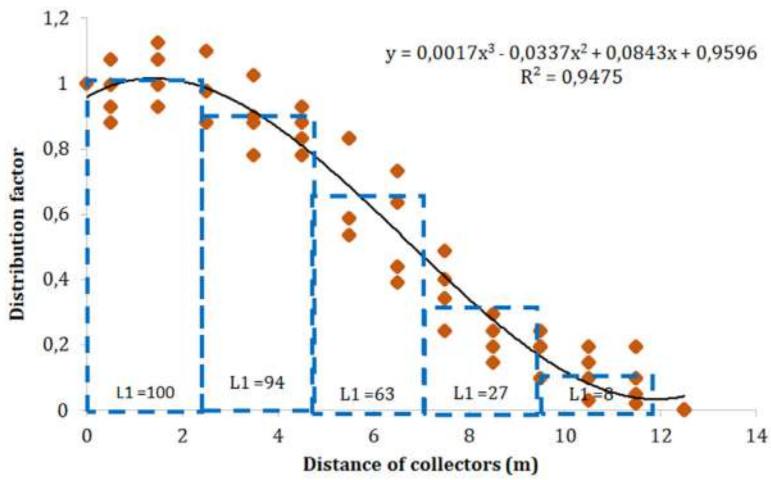

Figure 4. Distribution factor of the irrigation depths applied by the line-source sprinkler system.

Figura 4. Factor de distribución de las láminas de riego aplicadas por el sistema de aspersores de fuente de línea.

The sowing dates were February 6 and March 6, 2018. The cultivar IAC 505, which has a spreading growth habit, cycle between 130 and 140 days, moderate resistance to leaf diseases and relative drought tolerant (16). The crop was sown at spacing of 0.9 m between rows and density of 15 seeds m^{-1}. At sowing, fertilization was applied using the 0-20-20 (N, P, K) formulation at dose of 300 kg ha^{-1}. Seeds were previously treated with the fungicide Thiram (Vitavax®-Thiram 200 SC, 250 mL of c.p. 100 kg^{-1}) to avoid the incidence of pathogens. In order to maintain the phytosanitary control of the experiment, weekly fungicide applications were carried out with the following active ingredients: Pyraclostrobin + Epoxiconazole (Opera®) and Cyproconazole (Alto® 100), at doses of 109.8 g a.i. ha^{-1}, 720 g a.i. ha^{-1} and 25 g a.i. ha^{-1}, respectively. The insecticide Thiamethoxam (Engeo Pleno™ S) was also used at dose of 115 g a.i. ha^{-1} and weeds were controlled by manual weeding to avoid interference with the crop.

The following agronomic characteristics of the crop were evaluated from 20 days after sowing (DAS) until harvest: a) plant height - main stem height was measured in five plants of each experimental unit; b) Canopy cover fraction- measured by pictures taken from the experimental plots and analyzed by the Canopeo® software, (26); c) Intercepted photosynthetically active radiation fraction (IRF) - calculated as the ratio of Photosynthetically active radiation (PAR) measured above and below the crop canopy by means of a bar with a line-quantum sensor (AccuPAR radiometer; Decagon Devices, Inc., Pullman, WA), with measurements taken between 11 am and 2 pm, by placing the bar above the canopy and below the green leaves, making four measurements per plot; d) pod yield - plants in the evaluated area of each plot were threshed at the end of the cycle to separate the pods and then determine their mass; e) unit seed mass was determined using a sample from the five plants of the plot, according to the Rules for Seed Analysis (9); f) degree-days (DD, °C) - computed as the sum of positive values calculated by equation 1:

$$DD = \left(\frac{Tmax - Tmin}{2} \right) - Tb \quad [1]$$

where:

Tmax and Tmin are maximum and minimum daily temperature, respectively (°C)

Tb is base temperature (12 °C);

g) irrigation water productivity (IWP, kg m^{-3}) - calculated by equation 2 as the pod yield produced per unit of applied irrigation water (1).

$$IWP = \frac{Y_i}{10\, D_i} \quad [2]$$

where:

Y_i is the yield (kg ha^{-1}) of the crop attained with seasonal irrigation depth D_i (mm).

The results were subjected to analysis of variance (ANOVA) by F test and the means were compared by Tukey test at 5% probability level, using Agrostat software (7).

Results

The phenological cycle, from sowing to physiological maturity (S to R7) ended at 117 and 142 DAS for the crops sown in February and March, respectively, totaling 1,288 and 1,282 DD (degree-days) (figure 5). In both seasons, the vegetative stage (VE-R1) had a similar duration (35 days and 431 and 447 DD). The duration of the reproductive stage (R1-R7) was 75 and 100 days with 857 and 835 DD for the first and second sowing dates, respectively (figure 5).

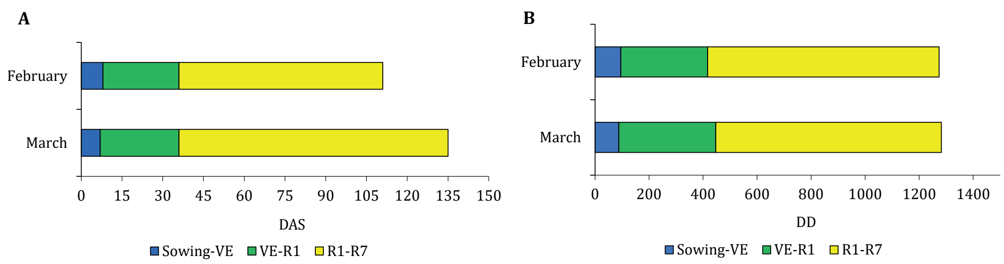

Figure 5. Phenological cycle of the cultivar IAC 505 in days after sowing (DAS) (A) and degree-days (DD) (B), for sowing in February and March.

Figura 5. Ciclo fenológico del cultivar IAC 505 en días después de la siembra (DAS) (A) y grados-días (DD) (B), para siembra en febrero y marzo.

Plant height was similar for all treatments until flowering (R1), however, for the reproductive stage, plants receiving higher irrigation application had increased growth rates, reaching 30 cm for treatment L1, compared to 18 cm for L5, for both, S1 and S2 planting dates (figure 6, page 62).

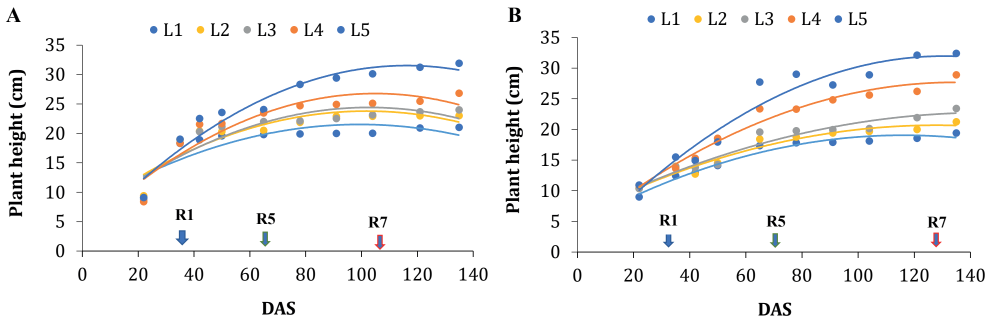

Figure 6. Plant height as a function of the level of irrigation water applied (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% and L5 = 100% replenishment of crop evapotranspiration), for peanut sown in February (A) and March (B). R1, R5 and R7 are flowering, pod formation and physiological maturity stages, respectively.

Figura 6. Altura de la planta función del nivel de agua de riego aplicado (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% y L5 = 100% de reposición de la evapotranspiración del cultivo), para maní sembrado en febrero (A) y marzo (B)). R1, R5 y R7 son etapas de floración, formación de vainas y madurez fisiológica, respectivamente.

Canopy cover fraction had similar behavior to plant height, with the effects of irrigation levels becoming evident from flowering, followed by maximum canopy cover in mid grain filling period, corresponding to 70 to 80% for L1 and L2, as compared to 40 to 50% for L4 and L5 (figure 7). Slower senescence was observed in the treatments with higher irrigation depths. The aerial view taken at maximum canopy cover (65 DAS) also show the treatment´s effect by relating the gradual increase in canopy cover and intercepted radiation with increase in irrigation depth (figure 8, page 63).

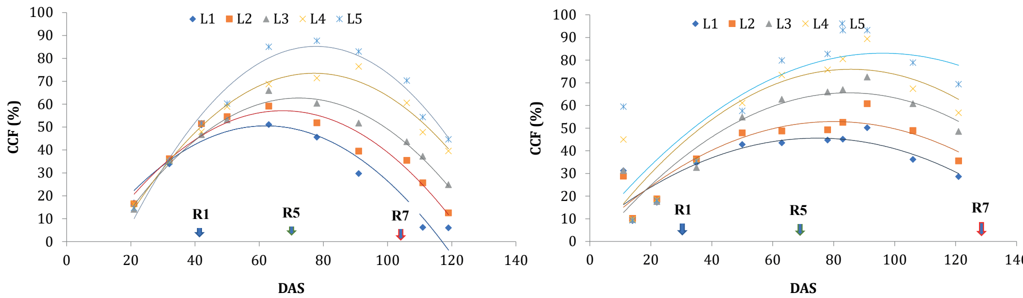

Figure 7. Canopy cover fraction as a function of the level of irrigation applied (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% and L5 = 100% replenishment of crop evapotranspiration), for peanut sown in February (A) and March (B). R1, R5 and R7 are flowering, pod formation and physiological maturity stages, respectively.

Figura 7. Fracción de la cubierta del dosel en función del nivel de riego aplicado (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% y L5 = 100% de reposición de la evapotranspiración del cultivo), para el maní sembrado en Febrero (A) y marzo (B). R1, R5 y R7 son etapas de floración, formación de vainas y madurez fisiológica, respectivamente.

Unit seed mass increased linearly with the increase in the water depth received in both cropping cycles, ranging from 0.60 to 0.80 g (figure 9A, page 63). Similarly, pod yield also increased linearly with the increase in water depth, from about 1,500 kg ha^{-1}, in both cropping cycles, to 4,141 kg ha^{-1} in the first one and to 5,102 kg ha^{-1} in the second one (figure 9B, page 63).

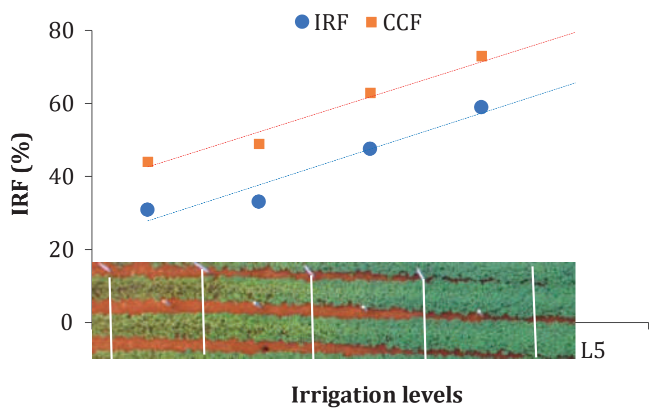

Figure 8. Intercepted photosynthetically active radiation fraction (IRF), canopy cover fraction (CCF) and aerial view of plots at 65 days after sowing for the irrigation levels applied (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% and L5 = 100% replenishment of crop evapotranspiration) for peanut sown in February.

Figura 8. Fracción de radiación interceptada (IRF), fracción de cobertura del dosel (CCF) y vista aérea de las parcelas a los 65 días después de la siembra para los niveles de riego aplicados (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94 % y L5 = 100% de reposición de la evapotranspiración del cultivo) para el maní sembrado en febrero.

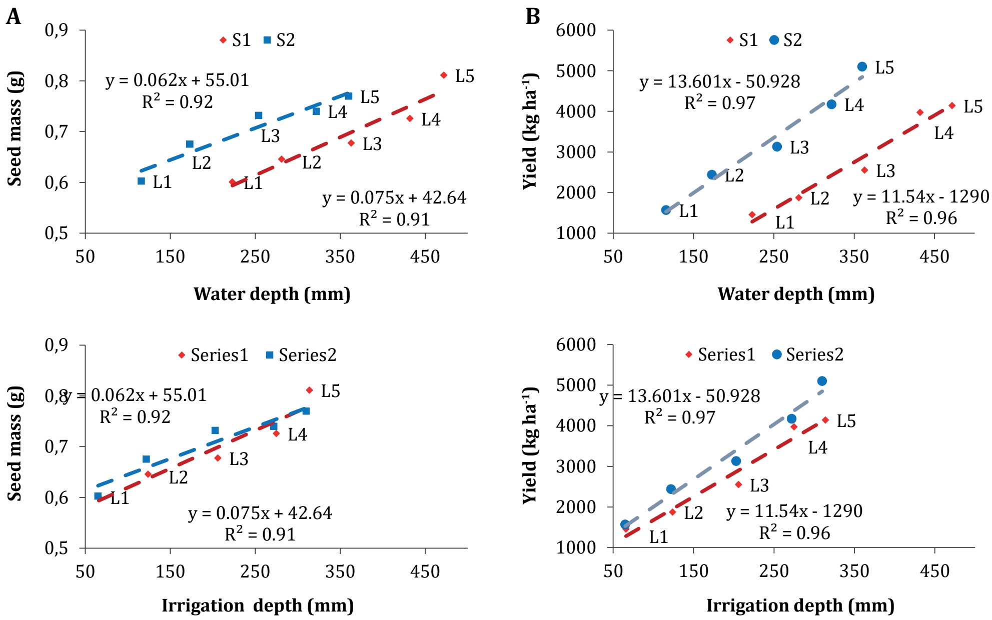

Figure 9. Unit seed mass (A) and pod yield (B) for peanut sown in February (S1) and March (S2), as a function of the seasonal water depth (rainfall + irrigation) and irrigation water depth, according to the level of irrigation (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% and L5 - 100% replenishment of crop evapotranspiration).

Figura 9. Massa de semillas (A) y rendimiento de vaina (B) para el maní sembrado en febrero (S1) y marzo (S2), en función de la profundidad del agua estacional (lluvia + riego) y la profundidad de agua riego, según el nivel de riego (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% y L5 - 100% de reposición de la evapotranspiración del cultivo).

The analysis of variance for pod yield showed significant effects ($p \geq 0.01$) of sowing date, irrigation levels and the interaction between these factors (table 3, page 64). Pod yield was higher in the second cropping cycle, compared to the first one, at the irrigation levels

L2 (30%), L3 (23%) and L5 (23%), and similar at the other levels. For the same sowing date, reducing irrigation application caused significant reductions on yield at all irrigation levels, except for L4 and L5 in the first cropping cycle, indicating similarity between the water depths of 94% and 100% ETc, which correspond to 275 and 314 mm, respectively. Such 12% saving in the applied water depth represents lower cost of production without losses in the yield of this cultivar. However, this effect did not occur in the second cropping cycle, since the application of water depths of the same magnitude as those applied in the first one for the treatments L4 and L5 (272 and 310 mm, respectively) resulted in a 22% reduction of yield.

Table 3. Analysis of variance and comparing means for pod yield (kg ha^{-1}) as a function of irrigation level (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% and L5 = 100% replenishment of crop evapotranspiration) and sowing date.

Tabla 3. Análisis de varianza y medias de comparación para el rendimiento de vainas (kg ha^{-1}) en función del nivel de riego (L1 = 8%, L2 = 27%, L3 = 63%, L4 = 94% y L5 = 100% de reposición de evapotranspiración del cultivo) y fechas de siembra.

Sowing\Irrigation[1]	L1	L2	L3	L4	L5	Mean
February	1.454Da	1.873Cb	2.550Bb	3.971Aa	4.141Ab	2.798
March	1.568Ea	2.436Da	3.129Ca	4.172Ba	5.102Aa	3.281
Mean	1.511	2.154	2.839	4.072	4.622	3.031
ANOVA[2]	F					
Sowing (S)	39.86**					
Irrigation (L)	334.08**					
S x L	5.70**					
CV% (S)	7.96					
CV% (L)	6.60					

[1]Uppercase letters compare irrigation levels per sowing and lowercase letters compare sowing dates in each level of irrigation; [2]** significant at p< 0.01.

[1]letras mayúsculas comparan niveles de riego por siembra y letras minúsculas comparan la fecha de siembra en cada nivel de riego; [2]** significativo en p <0,01.

The amount of irrigation required to produce the same mass of pods was larger in the first crop period, indicating lower productivity of the irrigation water compared to the second crop period (figure 10).

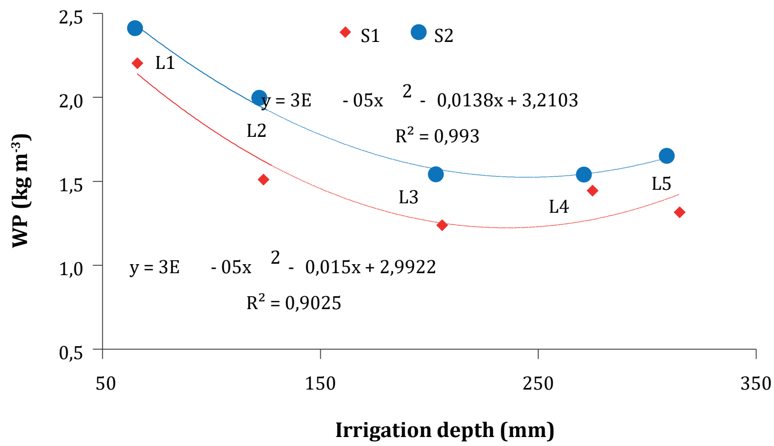

Figure 10. Water productivity (WP) for sowing in February (S1) and March (S2), as a function of the seasonal irrigation depth, according to the level of irrigation (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% and L5 - 100% replenishment of crop evapotranspiration).

Figura 10. Productividad del agua (WP) para siembra en febrero (S1) y marzo (S2), en función de la profundidad riego estacional, según el nivel de riego (L1 - 8%, L2 - 27%, L3 - 63%, L4 - 94% y L5 - 100% de reposición de la evapotranspiración del cultivo).

Discussion

Peanut crop is considered as a day-neutral plant with respect to photoperiodism, in which day length does not affect flowering, whereas phenological stage duration is dependent on-air temperature (15). The crop cycles in this experiment were close to the cycle duration for the cultivar IAC 505, which is 130 to 140 days (16). Because of similar temperature in both planting dates (figure 2A, page 59), vegetative stage duration had no difference (figure 5A, page 61). Reproductive stage was 25 days shorter in the crop sown in February, as compared to March. This effect might be due to the higher temperature during the first crop cycle (figure 2A, page 59), as given by the sum of DD (figure 5B, page 61), while in the cycle of the crop sown in March, the days with temperatures lower than the base temperature resulted in lower thermal sum, delaying the physiological stages.

The irrigation levels did not affect plant height and canopy cover in the vegetative stage, because of the low crop water demand in the first stages, since plant size (figure 6, page 62) and canopy cover (figure 7, page 62) were small. Therefore, the low water depths applied, in addition to rainfall, were enough to meet crop water demand and not caused delay in the stage VE to R1. During the reproductive stage, the varying irrigation depths applied in the treatments provided sufficient water supply for the treatments L4 and L5 an increasing gradual water deficit for the treatments L1 to L3, due to lower replenishment of crop consumptive use. Consequently, lower growth rates in height and canopy cover were observed in the treatments with water deficit, which can be attributed to the reduction of turgor, which in turn directly affects plant growth. Those results corroborate Larcher (2006) and Suleiman *et al.* (2013).

The close correlation between canopy cover fraction and intercepted radiation by the canopy (figure 8, page 63) makes evident that the higher values measured for canopy cover fraction during the crop cycle correspond to increase in biomass, as irrigation application increased from L1 to L5. In fact, abiotic stress factors, such as water stress, can cause changes in plant growth and metabolism since the beginning of the development cycle, leading to reductions in the capture of solar radiation and, consequently, lower growth rate and reduction in crop yield (10, 25).

The increments on yield obtained between the highest and lowest levels of water replacement (L5 and L1) were around 3 times for both sowing dates (figure 9B, page 63). The difference in irrigation water productivity (figure 10, page 64) was due to the lower yields of the first cropping cycle (table 3, page 64 and figure 9B, page 63), since applied seasonal irrigation depths were similar (figure 3, page 60).

Higher pod yield in the second cropping cycle may be due to the duration of the reproductive stage (R1-R7), which was 25 days longer than that of the first cropping cycle (figure 5, page 61), because of the lower crop development rate under milder temperature. Studies on plant development demonstrate that the increase in temperature affects grain yield because of the reduction in the duration of the phenological stages (32), especially the reproductive stage (8), resulting in smaller seeds and lower yields (13).

The result of reduction in yield due to water stress agrees with Kheira *et al.* (2009) and Azevedo *et al.* (2017), who concluded that water suppression at any stage of the peanut cycle causes a reduction in its yield. According to Nakagawa and Rosolem (2011), peanut sensitivity to water stress is lower in the period from seedling emergence to early formation of the floral organs and expressively increases during the flowering and fruiting stages, which occurred in the treatments with deficit irrigation for both sowing dates. These results also corroborate those observed by Barbieri *et al.* (2017), who found differences in the yields of the cultivars IAC Tatu (erect) and Runner IAC 886 (dwarf) under water replacement levels.

The yields obtained at the higher irrigation levels (L4 and L5) were similar or superior to the yield obtained in the rainy season in the farms of the region of the experimental area (12), which was 3,798 kg ha^{-1}. For the dry season, the estimated yield for the region of Jaboticabal was 1,541 kg ha^{-1}, which is equivalent to the values observed at the lowest level of water replenishment (L1).

Although promising, the results of this study are not sufficient to recommend the practice of irrigation in peanut cultivation on a large scale. It is still necessary conduct studies over longer periods and in other producing regions to evaluate the productive potential and economic viability of irrigated cultivation.

Conclusions

In the absence of water deficit, peanut yield was 30% higher for sowing in March than for sowing in February. Water deficit reduced plant height, leaf area, mass of seeds and pod yield and increase crop cycle. Adequate water supply promoted by full irrigation (L5) led to yields from 4.141 to 5.102 kg ha^{-1}, for sowing in February and March, respectively, which were approximately three times higher than those obtained with the lowest level of irrigation (L1).

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Agronomic performance of maize (*Zea mays* L.) genotypes under *Azospirillum brasilense* application and mineral fertilization

Rendimiento agronómico de genotipos de maíz (*Zea mays* L.) bajo la aplicación de *Azospirillum brasilense* y fertilización mineral

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Abstract

Maize has a high nitrogen (N) demand; as a result, more sustainable alternatives are needed to reduce demand for mineral fertilizers. The hypothesis is that the agronomic performance of maize genotypes diverges in their potential when subjected to different supplies of N. This study aimed to evaluate and characterize the agronomic performance of maize genotypes submitted to topdressing nitrogen fertilization and inoculation with *Azospirillum brasilense* and select the best genotypes within each management. An experiment was conducted in the second season of 2017, in Jaboticabal-SP (Brazil), using 48 maize genotypes in a randomized block design. Treatments consisted of: 1) application of 140 kg ha^{-1} nitrogen, using urea as mineral fertilizer; 2) *A. brasilense* inoculation via soil, at a rate of 600 mL ha^{-1}, as biological fertilization. Grain yield and agronomic traits were evaluated. The data were subjected to analysis of variance (F-Test), means were compared by Scott-Knott test at 5% probability, and multivariate statistical analysis was performed by principal component analysis. The maize genotypes showed contrasting agronomic performance in relation to the nitrogen supplies evaluated. Inoculation with *A. brasilense* via soil has a significant effect on increasing maize grain yield, making it a viable and more sustainable alternative in the supply of N. The choice of the genotype is an essential factor for the successful use of mineral nitrogen fertilization or *A. brasiliense* inoculation.

Keywords

diazotrophic bacteria • grain yield • biological nitrogen fixation • nitrogen

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Resumen

El maíz tiene una alta demanda de nitrógeno (N), como resultado, se necesitan alternativas más sostenibles para reducir la demanda de fertilizantes minerales. La hipótesis es que el comportamiento agronómico de los genotipos de maíz diverge en su potencial cuando se someten a diferentes suministrados de N. Este estudio tuvo como objetivo evaluar y caracterizar el rendimiento agronómico de los genotipos de maíz sometidos a fertilización nitrogenada e inoculación con *Azospirillum brasilense* y seleccionar los mejores genotipos dentro de cada manejo. Se realizó un experimento en la segunda temporada de 2017, en Jaboticabal-SP (Brasil), utilizando 48 genotipos de maíz en un diseño de bloques al azar. Los tratamientos consistieron en: 1) aplicación de 140 kg ha^{-1} de nitrógeno, utilizando urea como fertilizante mineral; 2) Inoculación de *A. brasilense* vía suelo, a razón de 600 mL ha-[1], como fertilización biológica. Se evaluaron el rendimiento de granos y los rasgos agronómicos. Los datos se sometieron a análisis de varianza (prueba F), y las medias se compararon mediante la prueba de Scott-Knott con una probabilidad del 5%, y el análisis estadístico multivariado se realizó mediante análisis de componentes principales. Los genotipos de maíz mostraron un comportamiento agronómico contrastante en relación con los suministrados de N evaluados. La inoculación con *A. brasilense* vía suelo tiene un efecto significativo en incrementar el rendimiento de granos de maíz, convirtiéndolo en una alternativa viable y más sustentable en el suministrado de N. La elección del genotipo es un factor esencial para el uso exitoso de la fertilización con nitrógeno mineral o la inoculación con *A. brasiliense*.

Palabras claves
bacterias diazotróficas • producción de granos • fijación biológica de nitrógeno • nitrógeno

Introduction

Proper management of nitrogen fertilization is essential for high grain yield in cereal crops such as maize. At harvest, nitrogen deficiency can reduce grain yield by 14 to 80% (8, 28). Maize is socio-economically important as it is the most grown crop species worldwide (21, 23).

Among the soil nutrient fertilizers available, nitrogen (N) is the most expensive fertilization cost and can reach about 30% of total production cost (10). However, when applied to the soil, it can cause environmental damages since a is usually lost by leaching and volatilization (19). In addition, nitrogen fertilizer manufacturing consumes much oil, which is a non-renewable energy source. Therefore, new alternatives need to be sought to streamline use of nitrogen fertilizers (5, 13), as one of the major agricultural challenges for the coming years is to produce sustainable food and optimize existing resources (17).

Studies have indicated plant breeding and *Azospirillum* genus bacteria inoculation as alternatives to partially supply maize nitrogen demands. These bacteria are highly capable of performing biological nitrogen fixation (BNF) (25), which can reduce use of nitrogen fertilizers.

These bacteria also play a role in plant hormone production such as auxins, cytokines, and gibberellins, which stimulate plant shoot and root growth (26), and hence plant light interception and dry mass yield (22). Such stimulus also increases plant water and nutrient uptakes by increasing root system branching (20), thereby improving corn grain yield (4, 15).

The results of *Azospirillum brasilense* inoculation depend on biotic and abiotic factors such as genotype, soil microbial community, and climate variations (14). Genotypes may have different physiological performances in terms of N uptake (9). Thus, identifying and selecting maize genotypes responsive to inoculation and nitrogen fertilizations is a forthcoming approach to improve crops and reduce nitrogen fertilizer use and respective environmental contamination (18).

The hypothesis for the present study is that the agronomic performance of maize genotypes diverges in their potential when subjected to nitrogen topdressing fertilization and inoculation with *A. brasilense*. This study aims to evaluate and characterize the agronomic performance of second-season maize genotypes as a function of topdressing nitrogen fertilization and *A. brasilense* inoculation via soil and select the best genotypes within each management.

Material and methods

An experiment was conducted in the second season of 2017, at the *Fazenda de Ensino, Pesquisa e Extensão* (FEPE) of the São Paulo State University, School of Agricultural and Veterinarian Sciences, Jaboticabal-SP (Brazil). The study area is at an average altitude of 615 meters, near the coordinates of 21° 14' 05'' S latitude and 48° 17´ 09'' W longitude. According to Köppen's classification, the climate is classified as Aw, which stands for humid tropical with rainy season in summer and dry winter. The soil was classified as eutrophic Red Latosol (Oxisol) (6). The region has average precipitation of 1.425 mm.

Soil chemical attributes and particle size were determined in the 0.00–0.20 and prior to common maize sowing. The results were pH ($CaCl_2$) 5.45; organic matter (g dm^{-3}) 24.37; N 0.18%; P (mg dm^{-3}) 8.26; K ($mmol_c$ dm^{-3}) 3.08; Ca ($mmol_c$ dm^{-3}) 38.73; Mg ($mmol_c$ dm^{-3}) 16.98; S (mg dm^{-3}) 7.07; B (mg dm^{-3}) 0.21; Fe (mg dm^{-3}) 25; H+AL (mmolc dm^{-3}) 20.13; cation exchange capacity ($mmol_c$ dm^{-3}) 78.93; base saturation 74.17%; clay 540 g kg^{-1}; silt 230 g kg^{-1}; sand 230 g kg^{-1}.

Forty-eight maize (*Zea mays* L.) genotypes were used in the experiment. These correspond to 46 synthetic maize populations from random crossbreeding, which were developed by Phoenix Agricultural Ltda, as well as two commercial cultivars AL Bandeirantes (Check A) and hybrid DKB 390 VT PRO2 (Check B).

Sowing was performed on February 17, 2017. Plots were delineated in randomized blocks, arranged in strips, devised by a plot seeder. Sowing fertilization was performed using 350 kg ha^{-1} of the 8-28-16 formulation. Each plot consisted of four 5-m long rows spaced 0.50 m between rows and 0.33 m between plants, with a population of 60,000 plants ha^{-1}. As a useful area for evaluation and harvesting, only the two central lines were used.

All the genotypes were supplied with nitrogen by: 1) biological fixation with *A. brasilense* inoculation in the soil; and 2) chemical fertilization with urea as source. Both topdressing and inoculation were performed on March 23, 2017, when plants were at V_4 stage.

We used the commercial inoculant Qualyfix Gramíneas® (*A. brasilense*, strain AbV5 and AbV6, at concentration of 5 x 10^8 cells mL^{-1}), which was applied at the manufacturer's recommended rate (600 mL ha^{-1} for soil spraying). The inoculant was applied to the soil with the aid of a costal sprayer at 10 cm from maize row. Nitrogen topdressing was performed at a rate of 140 kg ha^{-1} N, using urea as source. It was applied in continuous fillet at 10 cm from crop row. After fertilization and *A. brasilense* application, irrigation was performed by a sprinkler system. Weed and pest controls were performed according to recommendations for maize cropping (7).

The following variables were evaluated: number of vegetative days until male (MF) and female (FF) flowerings, plant height (PH), ear height (EH), lodging and breaking rates (L + B), stunting rate (STU), *Fusarium* spp. (FUS) and grain yield (GY).

We also evaluated number of plants tilted more than 45° from the vertical or lodged on the soil at harvest time. This result was added to the breaking parameter, which is the number of plants with broken stem below the main ear. The data were transformed to $\sqrt{x+1}$ for data normality, and later converted to percentage values.

Before threshing, five maize ear samples were collected randomly from each plot to be evaluated for *Fusarium* spp. (FUS) symptoms. The number of ears presenting FUS symptom was counted and transformed to $\sqrt{x+1}$ for data normality and then converted to percentage.

Grain yield (GY) was obtained by threshing ears harvested within the useful area of each plot, weighing the grains and correcting to 13% moisture, and then converting the data to tons per hectare (t ha^{-1}).

The stuting rate (STU) was analyzed according to percentage of *Daubulus maidis* incidence in each plot, obtained by equation 1.

$$\text{Incidence (\%)} = \frac{\text{NPS X 100}}{\text{NTP}} \quad [1]$$

where:

NPS = number of plants showing symptoms of generalized redness or yellowing and whitish streaks caused by *Dalbulus maidis*

NTP = total number of plants in the plot

The data were transformed to $\sqrt{x+1}$, aiming at normality, and later converting them to percentage.

During the experiment, the averages of temperature maximum and minimum and rainfall were 29.1 °C, 16,6 °C and 358 mm, respectively (figure 1). Meteorological data were provided by the agroclimatological station at FCAV/UNESP (College of Agricultural and Veterinary Sciences – University of São Paulo State). Water was provided via sprinkler irrigation when there were longer periods with no rain. Harvesting was performed manually on 07/03/2017, at 136 days after emergence (DAE).

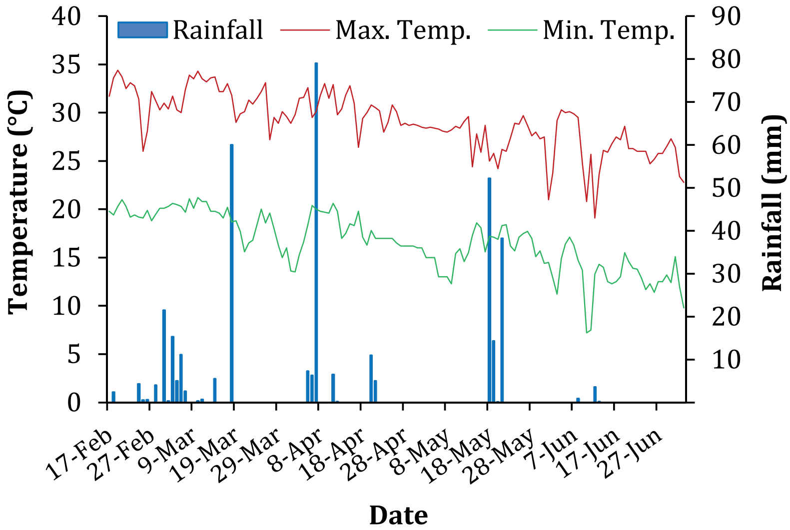

Figure 1. Rainfall (mm) and minimum and maximum temperatures (°C) from February 17 to July 03, 2017.

Figura 1. Precipitaciones (mm) y temperaturas mínimas y máximas (°C) del 17 de febrero al 03 de julio de 2017.

Data from each variable was recorded separately, and genotypes and the two N supplies were considered as fixed factors. A factorial variance analysis (2 nitrogen supplies x 48 genotypes) was performed, considering all genotypes and agronomic traits. Means were compared by the Scott-Knott test at 5% probability, using AgroEstat® software (2).

After standardization, all data were submitted to principal component analysis (PCA). The number of components was chosen according to Kaiser's criterion (11), wherein eigenvalues > 1.00 are selected. The results of principal component analysis (PC) were presented in biplot graphs, with genotypes plotted in the X-axis and matrices in the Y-axis, being represented by points. A scatterplot of the principal component analysis was used to have a clear view of the performance of each genotype in each variable. Genotypes were identified by a -2 to 2 ellipse in PC1 and PC2, wherein genotypes within the ellipse have no specific properties, while those located outside the ellipse are characterized as promising for each N supply. The analyses were performed using the Statistica 7.0® software (24).

Results and Discussion

The results of the analysis of variance (table 1, page 72) showed that N source had a significant effect on grain yield (GY) and on days for female flowering (FF), when exchanging the N supply via inoculation with *A. brasilense* for topdressing, Regarding the source of genotype variation, all parameters differed significantly, except for male flowering (MF) and lodging + breaking rates (L + B), These results corroborates the hypothesis that the agronomic performance of maize genotypes diverges in their potential when subjected to nitrogen topdressing fertilization and inoculation with *A. brasilense*.

The interaction GEN x NSS (genotype x N supply source) showed a significant difference only for *Fusarium* spp., therefore, genotypes and N supply vary differently for incidence of this disease (table 1, page 72). Similar results were observed by Khan and Doohan (2009), who found decreases between 12 and 21% in the disease incidence for inoculated plants.

These authors pointed out that favorable conditions for *Fusarium* spp. development may have been due to an increase in fertilization rates, besides being directly related to leaf disease intensity.

In both N supply systems, the genotypes presented overall GY means of 2.14 t ha^{-1} (table 1), among which Check A and Check B stood out with averages of 3.23 and 3.55 t ha^{-1}, respectively (table 1). These averages may reflect the low L + B rates, STU and FUS of these genotypes. In this same context, genotype 22 had the lowest yield, with an average of 1.21 t ha^{-1}, which may be linked to other to other factors such as the low PH and high staging value presented by this genotype, in addition to the above average L + B rates (table 2, page 73-74).

Table 1. Simple factorial variance analysis of 48 maize genotypes in response to topdressing nitrogen fertilization and *Azospirillum brasilense* inoculation.

Tabla 1. Análisis de varianza factorial simple de 48 genotipos de maíz en respuesta a la fertilización nitrogenada y la inoculación de *Azospirillum brasilense*.

SV		F Test							
	DF	GY	MF	FF	PH	EH	L + B	STU	FUS
NSS	1	7.57**	0.88^{ns}	65.33*	0.01^{ns}	0.01^{ns}	0.19^{ns}	0.58^{ns}	0.01^{ns}
GEN	47	0.82**	6.10^{ns}	12.63**	0.03**	0.01**	45.18^{ns}	1.47**	0.18**
GEN*NSS	47	0.13^{ns}	4.06^{ns}	3.68^{ns}	0.01^{ns}	0.01^{ns}	6.66^{ns}	0.21^{ns}	0.15*
Mean	-	2.14	63.42	62.81	1.67	0.90	16.87	26.63	12.39
CV	-	24.29	3.27	3.59	5.58	8.66	29.29	18.33	11.69

* Significant at 5% probability and ** Significant at 1% probability by the F-test: SV: source of variation; DF: degrees of freedom; NSS: nitrogen supply source; GEN: genotype; GEN * NSS: interaction between genotype and nitrogen supply; CV: coefficient of variation (%); ns: not significant; GY: grain yield (t ha^{-1}); MF: male flowering (days); FF: female flowering (days); PH: plant height (m); EH: ear height (m); L + B: lodging and breaking of plants (%); STU: stunting (%); FUS: *Fusarium* spp. (%)

* Significativo al 5% de probabilidad y ** Significativo al 1% de probabilidad según lo teste-F: FV: fuente de variación; GL: grados de libertad; FSN: fuente de suministro de nitrógeno; GEN: genotipo; GEN * FSN: interacción entre genotipo y suministro de nitrógeno; CV: coeficiente de variación (%); ns: no significativo; GY: rendimiento de granos (t ha-1); PH: altura de la planta (m); EH: altura de espiga (m); MF: floración masculina (días); FF: floración femenina (días); L: vuelco de plantas (%); B: quebradura de tallos (%); STU: Cinta roja (%); FUS: *Fusarium* spp. (%).

In comparing the performance of genotypes with respect to the two N supply sources, only genotypes 27 and 34 showed significant differences in GY. These genotypes presented higher GY by *A. brasilense* application, with increments of 1.08 and 1.19 t ha^{-1}, respectively, in relation of mineral fertilizantion (table 2, page 73-74). These results are in agreement with those found by Müller *et al.* (2016), who obtained increases of up to 28% in grain yield after inoculation of *A. brasilense* in relation to genotypes with topdressing nitrogen fertilization.

Superior grain yield was also reported by Araújo *et al.* (2014) after inoculating maize with the same diazotrophic bacteria. When topdressed (table 2, page 73-74), 31.25% of the genotypes were superior to the others for GY. The same was observed in *A. brasilense* inoculation, in which 45.83% of the genotypes differed from the others regarding their response to inoculation. For both N supply sources, each genotype showed significant GY differences, therefore, this trait is greatly influenced either by chemical or biological sources.

Genotypes showed significant differences in PH and EH when comparing both N supplies. Genotype 18 increased plant height and ear insertion height by 0.20 m when inoculated with *A. brasilense*, that is, it was responsive to diazotrophic bacteria application (table 2, page 73-74). Favorable effects provided by bacteria may have occurred due to changes in the root system of inoculated maize plants, leading to improvements in plant growth, water absorption, and nutrient intake (3), highlighting the height gains.

Genotype 32 presented the same increase (0.20 m) in plant and EH when urea was supplied as topdressing (table 2, page 73-74); therefore, this genotype was more responsive to urea fertilization in terms of plant height. This result corroborates Vogel *et al.* (2013), who observed that nitrogen-deficient maize plants are more capable of synthesizing carbohydrates during photosynthesis, thus growing more.

Table 2. Means of agronomic traits of 48 maize genotypes in response to topdressing nitrogen fertilization (N) and inoculation with *Azospirillum brasilense* (AZOS).

Tabla 2. Medias de los rasgos agronómicos de 48 genotipos de maíz en respuesta a la fertilización nitrogenada (N) y la inoculación con *Azospirillum brasilense* (AZOS).

G	GY		MF		FF		PH		EH		L + B		STU		FUS	
	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS
1	2.56 a	2.52 a	60.00 Bb	65.00 A	60.00 Bc	65.00 A	1.64 b	1.70 a	0.82 b	0.85 b	18.68	13.34	18.82 b	20.06 b	7.55	8.99 b
2	1.71 b	1.72 b	64.50 a	65.50	65.00 b	66.00	1.67 b	1.65 b	0.92 b	0.90 b	16.17	22.84	24.35 a	24.04 a	14.52	10.10 b
3	1.96 b	2.78 a	62.00 b	63.50	62.50 c	63.50	1.64 b	1.57 b	0.90 b	0.83 b	16.35	15.26	26.70 a	8.53 b	12.70	10.62 a
4	1.46 b	1.87 b	64.50 a	66.00	64.50 b	65.50	1.45 b	1.37 b	0.86 b	0.76 b	19.02	16.72	15.13 b	7.63 b	6.43	4.06 b
5	1.93 b	2.55 a	62.00 b	63.50	61.00 c	64.00	1.62 b	1.53 b	0.84 b	0.81 b	6.08	11.90	10.25 b	14.88 b	10.25	12.50 a
Check A	3.11 a	3.34 a	65.00 a	64.50	63.50 b	65.00	1.80 a	1.94 a	0.99 a	1.12 a	10.27	8.33	12.65 b	9.26 b	14.14	7.41 b
7	1.61 b	1.68 b	64.50 a	64.50	64.00 b	63.50	1.59 b	1.53 b	0.84 b	0.86 b	12.96	19.91	31.48 a	37.27 a	7.41	12.04 a
8	2.13 a	2.92 a	61.00 b	61.50	59.50 c	61.00	1.63 b	1.69 a	0.89 b	0.94 b	14.04	6.79	24.69 a	13.81 b	8.74	8.69 b
Check B	3.34 a	3.76 a	62.50 b	63.00	61.50 c	62.50	1.84 a	1.81 a	1.03 a	1.07 a	1.85	6.52	0.00 b	0.00 b	5.88	4.03 b
10	2.56 a	2.58 a	60.00 b	62.50	60.00 c	61.00	1.73 a	1.67 b	0.86 b	0.85 b	9.17	8.17	15.83 b	19.55 b	9.58	10.10 b
11	1.63 b	2.03 b	62.50 b	64.50	64.00 b	65.50	1.53 b	1.49 b	0.83 b	0.73 b	10.51	2.00	10.77 b	16.42 b	7.18	14.33 a
12	2.79 a	2.66 a	61.00 b	65.00	60.00 c	64.00	1.81 a	1.81 a	1.02 a	0.92 b	18.41	9.47	8.29 b	11.11 b	6.67	7.55 b
13	1.86 b	1.93 b	63.00 b	62.00	61.50 c	61.00	1.74 a	1.74 a	0.94 a	0.92 b	15.44	13.92	7.97 b	26.28 a	12.13	11.45 b
14	2.36 a	2.62 a	61.50 b	64.00	59.00 c	62.00	1.67 b	1.64 b	0.87 b	0.83 b	17.96	20.94	21.77 a	10.47 b	11.06	14.96 a
15	1.73 b	2.43 a	65.50 a	65.00	63.00 b	64.00	1.65 b	1.66 b	0.97 a	0.90 b	12.88	12.06	21.36 a	28.90 a	13.48	12.84 a
16	2.19 a	2.49 a	63.50 b	62.50	59.50 c	62.50	1.83 a	1.74 a	0.95 a	0.91 b	19.48	22.58	31.80 a	36.06 a	14.43	10.76 b
17	1.55 b	1.57 b	62.50 b	63.50	59.50 c	61.50	1.80 a	1.89 a	0.96 a	1.03 a	19.69	20.83	34.07 a	33.33 a	16.76 B	4.17 Ab
18	1.95 b	2.35 b	66.00 a	63.00	61.50 c	62.00	1.56 Bb	1.76 Aa	0.82 Bb	0.99 Aa	16.79	18.59	28.81 a	26.42 a	17.16 B	5.63 Ab
19	1.62 b	2.01 b	64.00 a	63.00	60.50 c	64.50	1.69 a	1.60 b	0.90 b	0.82 b	10.09	11.07	35.64 a	24.31 a	10.09	11.07 b
20	1.86 b	1.92 b	63.00 b	62.50	61.00 c	62.50	1.76 a	1.71 a	0.86 b	0.87 b	15.62	18.15	33.08 a	36.31 a	13.77	15.48 a
21	1.37 b	2.24 b	67.00 a	63.00	62.50 c	63.50	1.63 b	1.53 b	0.91 b	0.83 b	24.83	27.74	42.39 a	35.64 a	12.29	19.41 a
22	0.87 b	1.56 b	67.50 a	65.00	69.50 a	66.00	1.55 b	1.63 b	0.92 b	1.00 a	26.32	27.39	46.89 a	39.74 a	13.66	8.52 b
23	1.27 b	2.02 b	66.00 a	63.50	64.50 b	64.50	1.58 b	1.59 b	0.87 b	0.97 a	12.90	14.91	49.59 a	32.87 a	15.17	13.70 a
24	2.16 a	2.65 a	63.00 b	62.00	65.00 b	64.50	1.76 a	1.81 a	0.94 a	0.98 a	18.10	13.70	26.19 b	12.41 b	13.81	14.26 a
25	1.15 b	2.04 b	66.50 a	64.00	70.00 Aa	65.00 B	1.71 a	1.68 a	0.96 a	0.92 b	25.43	24.04	48.08 a	40.96 a	16.88	9.42 b
26	1.80 b	2.17 b	61.50 b	63.00	60.50 c	65.00	1.65 b	1.71 a	0.89 b	0.92 b	31.00	32.44	43.88 a	40.64 a	12.25	22.95 a

Means followed by the same uppercase letters in the row and lowercase letters in the column do not differ from each other by the Scott-Knott test at 5% probability; GY: grain yield ($t\ ha^{-1}$); MF: male flowering (days); FF: female flowering (days); PH: plant height (m); EH: ear height (m); L + B: lodging and breaking of plants (%); STU: stunting (%); FUS: *Fusarium* spp. (%)

Las medias seguidas de las mismas letras mayúsculas en la fila y las letras minúsculas en la columna no difieren entre sí según la prueba de Scott-Knott al 5% de probabilidad; GY: rendimiento de granos (t ha-1); PH: altura de la planta (m); EH: altura de espiga (m); MF: floración masculina (días); FF: floración femenina (días); L: vuelco de plantas (%); B: quebradura de tallos (%); STU: Cinta roja (%); FUS: *Fusarium* spp. (%).

Table 2 (cont.). Means of agronomic traits of 48 maize genotypes in response to topdressing nitrogen fertilization (N) and inoculation with *Azospirillum brasilense* (AZOS).

Tabla 2 (cont.). Medias de los rasgos agronómicos de 48 genotipos de maíz en respuesta a la fertilización nitrogenada (N) y la inoculación con *Azospirillum brasilense* (AZOS).

G	GY		MF		FF		PH		EH		L + B		STU		FUS	
	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS	N	AZOS
27	2.03 Bb	3.11 Aa	61.50 b	63.00	60.00 c	61.50	1.69 a	1.66 b	0.84 b	0.87 b	27.38	29.17	42.26 a	40.18 a	14.88	20.24 a
28	1.99 b	2.44 a	62.50 b	63.50	61.00 c	62.50	1.62 b	1.60 b	0.87 b	0.95 a	42.36	41.71	19.46 b	45.32 a	5.30	15.64 a
29	1.89 b	2.09 b	62.00 b	61.50	60.50 c	62.00	1.73 a	1.61 b	0.93 b	0.92 b	25.76	26.88	32.95 a	35.38 a	15.15	8.89 b
30	2.35 a	2.61 a	63.00 b	63.50	61.50 c	63.50	1.74 a	1.69 a	0.89 b	0.96 a	17.69	17.56	22.13 b	18.75 b	15.51 B	6.55 Ab
31	2.30 a	2.78 a	65.00 a	64.00	62.50 c	64.00	1.69 a	1.62 b	0.84 b	0.83 b	26.28	25.18	34.98 a	27.54 a	10.97	12.68 a
32	1.91 b	2.66 a	64.50 a	63.50	64.00 b	64.50	1.78 Aa	1.58 Bb	0.92 Ab	0.74 Bb	11.52	13.34	29.35 a	26.68 a	15.36	20.06 a
33	2.32 a	2.51 a	64.50 a	65.50	61.50 c	64.00	1.69 a	1.59 b	0.88 b	0.90 b	17.11	16.71	34.37 a	30.29 a	15.41	13.57 a
34	1.60 Bb	2.79 Aa	63.00 b	61.50	59.50 c	61.50	1.77 a	1.63 b	0.95 a	0.90 b	23.60	23.91	31.29 a	18.55 b	10.31	10.82 b
35	2.17 a	2.98 a	62.50 b	61.00	62.00 c	62.00	1.58 b	1.59 b	0.80 b	0.83 b	15.97	12.37	15.41 b	12.79 b	13.59	16.50 a
36	2.00 b	2.23 b	65.50 a	62.50	64.50 b	65.00	1.65 b	1.59 b	0.85 b	0.91 b	14.29	16.82	19.64 a	14.19 b	7.14	10.62 b
37	1.86 b	2.16 b	65.00 a	63.50	63.00 b	63.50	1.74 a	1.56 b	0.98 a	0.87 b	22.22	17.39	32.46 a	37.63 a	13.60	12.29 a
38	1.83 b	1.96 b	59.00 Bb	63.50 A	59.00 c	63.50	1.67 b	1.56 b	0.87 b	0.86 b	12.00	13.25	16.00 b	19.02 b	12.00	18.80 a
39	1.97 b	2.05 b	64.50 a	62.50	65.00 b	64.00	1.79 a	1.76 a	1.06 a	0.96 a	14.00	17.50	38.71 a	19.64 b	11.88	19.64 a
40	2.06 b	1.75 b	63.50 b	64.50	62.00 c	64.50	1.63 b	1.67 b	0.82 b	0.86 b	6.55	6.07	29.64 a	20.31 a	17.09	9.97 b
41	2.26 a	2.60 a	65.50 a	64.00	62.00 c	62.50	1.67 b	1.72 a	0.89 b	0.90 b	13.38	10.76	21.38 b	23.90 a	22.50	19.52 a
42	1.58 b	1.89 b	65.00 a	63.00	63.50 b	64.00	1.66 b	1.69 a	0.82 b	0.85 b	15.79	13.04	33.82 a	28.04 a	15.39	20.87 a
43	1.60 b	2.34 b	66.50 a	63.00	66.00 b	63.50	1.52 b	1.64 b	0.79 b	0.89 b	17.92	16.35	40.60 a	27.69 a	12.66	13.27 a
44	3.15 a	2.59 a	62.50 b	63.00	60.00 c	60.50	1.70 a	1.72 a	0.88 b	0.94 b	6.34	6.17	25.54 a	45.25 a	10.60	12.33 a
45	1.92 b	2.23 b	61.50 b	62.00	59.50 c	63.00	1.64 b	1.67 b	0.84 b	0.87 b	13.14	14.58	34.29 a	29.17 a	5.57	14.58 a
46	1.79 b	2.33 b	64.50 a	62.00	62.00 c	61.00	1.72 a	1.65 b	0.89 b	0.90 b	9.38	10.00	23.96 a	27.50 a	13.54	12.50 b
47	1.45 b	2.13 b	63.00 b	62.00	61.00 c	63.00	1.61 b	1.60 b	0.82 b	0.87 b	16.87	17.49	34.63 a	37.77 a	14.32	18.14 a
48	1.12 b	1.62 b	63.00 b	63.50	64.00 b	64.50	1.47 b	1.58 b	0.81 b	0.86 b	23.82	19.86	44.66 a	36.07 a	12.68	9.57 b
M	1.94 B	2.34 A	63.49	63.35	62.23 B	63.40 A	1.67	1.66	0.89	0.90	16.95	16.79	27.66	25.60	12.28	12.50

Means followed by the same uppercase letters in the row and lowercase letters in the column do not differ from each other by the Scott-Knott test at 5% probability; GY: grain yield (t ha^{-1}); MF: male flowering (days); FF: female flowering (days); PH: plant height (m); EH: ear height (m); L + B: lodging and breaking of plants (%); STU: stunting (%); FUS: *Fusarium* spp. (%)

Las medias seguidas de las mismas letras mayúsculas en la fila y las letras minúsculas en la columna no difieren entre sí según la prueba de Scott-Knott al 5% de probabilidad; GY: rendimiento de granos (t ha-1); PH: altura de la planta (m); EH: altura de espiga (m); MF: floración masculina (días); FF: floración femenina (días); L: vuelco de plantas (%); B: quebradura de tallos (%); STU: Cinta roja (%); FUS: *Fusarium* spp. (%).

Both *A. brasilense* inoculated and urea topdressed plants obtained similar increase in PH and EH, being thus responsive to both N supply sources. These responses can reduce final production cost and environmental impact.

Regarding the incidence of L + B, all genotypes behaved similarly with no significant differences between inoculation and topdressing. When comparing the 48 genotypes for each N supply, 68.75 and 62.50% of the genotypes were more responsive to topdressing and inoculation, respectively (table 2, page 73-74). Moreover, no disease infestation was observed in genotype Check B in both environments, Therefore, this genotype may be tolerant to vector transmission (leafhopper - *Dalbulus maidis*), thus benefiting plant development and health in these plots, as well as improving productivity.

By comparing *Fusarium* spp. incidence in both N supply sources, significant differences were observed in genotypes 17, 18, and 30, which showed higher tolerance when inoculated with *A. brasilense*. This benefit may be due to a better responsiveness to plant inoculation, possibly due to an improved root system with higher nutrient absorption capacity, which is also reflected in healthier and better nourished plants. However, when topdressed, genotypes showed no significant differences, therefore, all plants behaved similarly. On the other hand, when N is supplied by biological means, a significant difference was shown on 54.16% of the genotypes, thus highlighting a possible response of inoculation with the bacteria.

By analysis of principal components (PC), genotype distribution was evaluated for each N supply source, using a biplot graph method (figure 2).

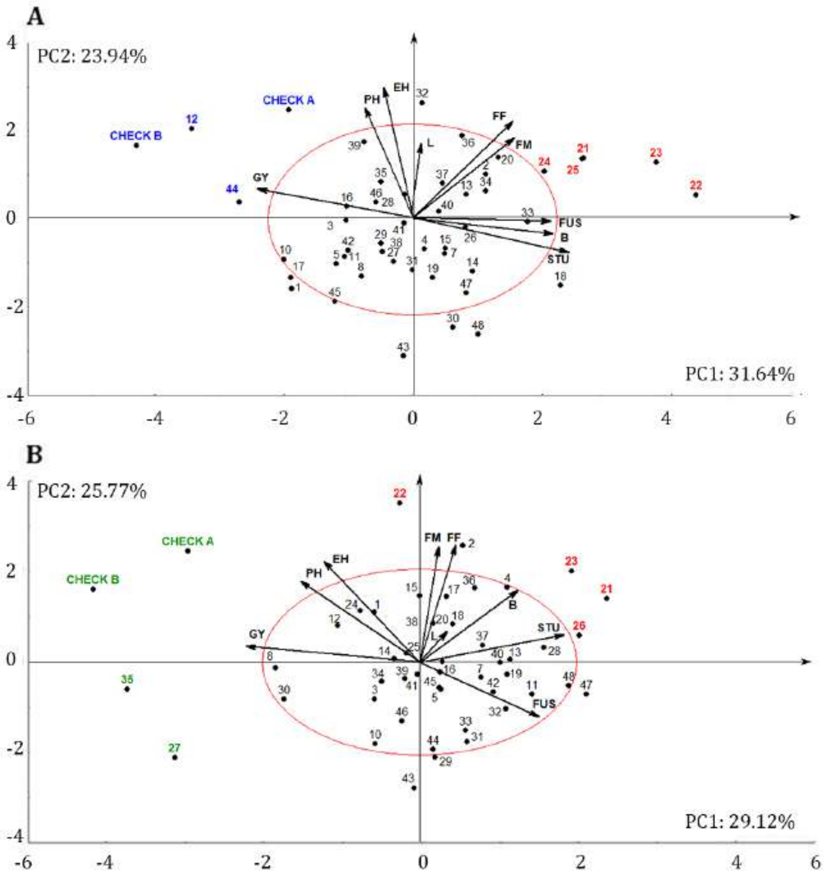

Figure 2. Biplot dispersion graph of 48 maize genotypes supplied with nitrogen topdressing (A) and inoculated with *A. brasilense* via soil (B). GY: grain yield (t ha^{-1}); PH: plant height (m); EH: ear height (m); MF: male flowering (days); FF: female flowering (days); L: plant lodging (%); B: plant breakage (%); STU: stunting (%); FUS: *Fusarium* spp. (%).

Figura 2. Gráfico de dispersión biplot de 48 genotipos de maíz suministrados con nitrógeno urea (A) y inoculacion con *A. brasilense* aplicado al suelo (B). GY: rendimiento de granos (t ha^{-1}); PH: altura de la planta (m); EH: altura de espiga (m); MF: floración masculina (días); FF: floración femenina (días); L: vuelco de plantas (%); B: quebradura de tallos (%); STU: Cinta roja (%); FUS: *Fusarium* spp. (%).

When fertilized with urea topdressing, the first two PCs explained 55.58% of the original data variability (31.64% and 23.94%, respectively) (figure 2A, page 75). As for the biological supply with *A. brasilense* inoculation, 54.89% of the original data variability was explained by the first two PCs (29.12% and 25.77% respectively) (figure 2B, page 75).

For nitrogen fertilization in topdressing (figure 2A, page 75), the genotypes Check A, Check B, 12, and 44 were discriminated by the parameters grain yield, plant height, and ear height. Check B was the most productive genotype, with high values of plant height and ear height, and the lowest values of incidence of lodging + breaking, stunting, and *Fusarium* spp.

Genotype 44 had the second-highest yield but high values for STU and FUS, which indicates problems in plant development and grain quality. In turn, genotypes 43 and 48 showed high values of male and female flowerings, and stunting but low values for GY, PH, and EH. Because of such characteristics, these genotypes should be discarded from breeding programs. The same was observed in genotypes 1, 18, and 30; yet, they had grain yields considered above the general average and with high values for L + B, STU, and FUS as well. Genotype 32 was discriminated by PH and EH, presenting a significant difference between the sources of N supply, in addition to values above the general average for grain yield and *Fusarium* spp.

On the positive side of the X-axis (figure 2A, page 75), it can be noted that genotypes 21, 22, 23, 24, and 25 were discriminated by lower GY, which may be explained by high percentage of L + B, stunting and FUS, which directly affect grain yield, besides presenting the longest flowering cycles.

For inoculation with *A. brasilense* (figure 2B, page 75), it can be noted on the negative side of the X-axis that the genotypes Check A, Check B, 27, and 35 were characterized by the variables GY, PH, and EH, corroborating results shown in table 2 (page 73-74). Check B was the most productive and with above-average values for PH and EH, as well as lower rates of L + B, STU and FUS.

Genotype Check A presented the second highest GY, the highest values for PH and EH, besides good plant health. Genotype 35 had the fourth highest yield but low PH and EH, with above average values for incidence of FUS. Despite the high GY, Genotype 27 also had higher and undesirable indices for lodging, breaking, stunting, and FUS Genotype 43 showed lower value for GY, PH and EH but high rates for L + B, STU and FUS. when compared to the genotypes located on the upper-left side of the graph (figure 2, page 75).

Genotype 2 presented low grain yield in relation to the above genotypes, presenting late flowering and above-average values for L + B. The same was seen in genotype 47, which also showed above-average values for incidence of STU and FUS. Genotype 22 presented high EH and low presence of FUS. However, it presented high values for L + B and STU but later flowering and the lowest grain yield (figure 2B, page 75).

On the positive side of the X-axis (figure 2, page 75), it can be noted that the genotypes 21, 23, and 26 were in the opposite direction to the others, which indicates low GY and high L + B values, as well as high incidence of STU and FUS, which directly affect plant development and yield. Therefore, these genotypes may have presented the lowest responses to *A. brasilense* inoculation.

The hypothesis tested in the present study was confirmed, as the maize genotypes showed different agronomic performance when submitted to topdressing mineral fertilization and inoculation with *A. brasilense* via soil. It was observed that genotypes 27, 34 and 35 showed superior agronomic performance when inoculated with *A. brasilense*, while genotypes 12 and 44 obtained higher agronomic performance under nitrogen mineral fertilization.

Conclusions

The maize genotypes showed contrasting agronomic performance in relation to the nitrogen supplies evaluated. Inoculation with *A. brasilense* via soil has a significant effect on increasing maize grain yield, making it a viable and more sustainable alternative in the supply of N. However, the responses of agronomic attributes vary with the genotype. The choice of the genotype is an essential factor for the successful use of mineral nitrogen fertilization or *A. brasiliense* inoculation.

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Forage production and leaf proportion of lucerne (*Medicago sativa* L.) in subtropical environments: fall dormancy, cutting frequency and canopy effects

Producción de forraje y proporción de hojas de alfalfa (*Medicago sativa* L.) en ambientes subtropicales: efectos del grado de latencia, la frecuencia de corte y la canopia

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Abstract

In subtropical and humid environments, winter-active cultivars of lucerne usually produce more forage with lower leaf proportion (leaf to stem ratio) than winter-dormant ones. The aim of present research was to analyze i) forage production of cultivars contrasting in fall dormancy under contrasting cutting frequencies, and (ii) the origin of differences between cultivars in the leaf proportion trait. In each of two subtropical locations of Argentina, an experiment including three lucerne cultivars (FD4= winter-dormant, FD6=semi winter-dormant, FD9=winter-active) and three cutting frequencies ('high': defoliated when intercept 0.50 of incident radiation, 'intermediate': when intercept 0.95 of incident radiation, 'low': when intercept 0.95 of incident radiation plus 150 growing degree days) was established. A significant cultivar*cutting frequency interaction was detected. In treatments where the cutting interval was longer (*e.g.* 'high' *vs.* 'low' cutting frequency) the more winter-active cultivars were more productive than the more winter-dormant ones (FD9>FD6>FD4), mainly due to a higher stem production and without major differences in leaf production. In turn, in treatments where the cutting interval was shorter, the cultivars showed similar forage production (FD9=FD6=FD4). Compared at similar canopy height, the differences between cultivars in leaf proportion were practically irrelevant. We confirm that (i) in subtropical and humid environments, the differences in forage production between cultivars contrasting in their fall dormancy depend on the cutting frequency, and that (ii) leaf proportion in aerial biomass of lucerne pastures is governed mainly by plant morphology, especially canopy height.

Keywords
lucerne • fall dormancy • cutting frequency • forage production • leaf to stem ratio

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Resumen

En ambientes subtropicales húmedos los cultivares de alfalfa sin latencia invernal suelen mostrar mayor producción de forraje y menor proporción de hoja que los cultivares con latencia invernal. El objetivo del trabajo fue analizar i) la producción de forraje de cultivares de alfalfa de distinto grado de latencia invernal bajo diferentes frecuencias de corte, y ii) el origen de las diferencias observadas entre cultivares en la proporción de hoja. En dos localidades subtropicales de Argentina, se estableció un experimento que incluía tres cultivares de alfalfa (FD4= con latencia invernal, FD6= con latencia invernal intermedia, FD9= sin latencia invernal) y tres frecuencias de corte ('alta': defoliados cuando se interceptó el 50% de la radiación incidente, 'intermedia': cuando se interceptó el 95% de la radiación incidente, 'baja': cuando se acumularon 150 grados días de crecimiento a partir del momento en que se llegó al 95% de intercepción de la radiación incidente). Se detectó una interacción significativa entre frecuencia de corte y cultivar. En los tratamientos con largos intervalos entre cortes (*e.g.* 'alta' frecuencia *vs.* 'baja' frecuencia), los cultivares con menor latencia invernal produjeron más forraje (FD9>FD6>FD4), debido principalmente a una mayor producción de tallos y sin mayores diferencias en la producción de hojas. A su vez, las diferencias entre cultivares desaparecieron (FD9=FD6=FD4) en los tratamientos con menores intervalos entre cortes. Comparados a similar altura de canopia, las diferencias entre cultivares en la variable proporción de hoja fueron irrelevantes. Este trabajo confirma que (i) en ambientes subtropicales, las diferencias en producción de forraje entre cultivares depende de la frecuencia de corte, y que (ii) la proporción de hoja depende principalmente de la morfología de las plantas, especialmente de la altura de la canopia.

Palabras clave

alfalfa • latencia invernal • frecuencia de corte • producción de forraje • relación hoja:tallo

Introduction

Lucerne cultivars are grouped according to fall dormancy (or winter activity) based on shoot elongation during autumn (19, 22). In temperate latitudes, lucerne cultivars contrasting in fall dormancy showed similar annual forage production, regardless of the cutting frequency (9, 19, 21, 23). In subtropical locations with humid and mild winters, winter-active cultivars produce more forage than winter-dormant ones (12, 13, 15), but such differences tend to disappear as the cutting interval decreases (6).

In turn, most of the literature indicates that winter-dormant cultivars had higher leaf proportion in their aerial biomass than winter-active ones (8, 14, 24). Leaves had better nutritive quality than stems (5) and positive relationships between leaf proportion and animal performance, were reported (4). Therefore, in lucerne cultivars, a typical trade-off between forage production (higher in 'winter-active' cultivars) and nutritive forage quality (lower in 'winter-active' cultivars) is frequently observed (8, 14, 24).

It was demonstrated that, as plants got bigger, biomass allocation in support tissues (stems) increases relatively more than in metabolic tissues (leaves) (11). A recent study in a temperate location showed that the relationship between yield and quality was independent of genotype and changes in leaf/stem ratio were associated with changes in canopy biomass and canopy height (21). These findings suggest that the origin of the above-mentioned differences between cultivars in leaf proportion may be not intrinsic (*i.e.* they were not due to a cultivar effect). We hypothesized that in lucerne, the variability in leaf proportion could be simply explained by the variability in aerial biomass or in canopy height.

The aim of the present research was to analyze i) forage production of cultivars contrasting in fall dormancy under contrasting cutting frequencies, and (ii) the origin of differences between cultivars in the leaf proportion trait.

Material and methods

Experimental conditions, design and measurements

Two similar trials were carried out in two locations of the Humid Pampas of Argentina: Paraná (31°44' S, 60°31' W, Exp. 1, irrigated) and Marcos Juarez (32°42' S, 62°06' W, Exp. 2, rainfed). The climate is sub-tropical humid (16). Monthly mean temperature ranges from 10.7°C in July to 24.6°C in January. Average annual rainfalls are 1069 and 900 mm for Paraná and Marcos Juarez, respectively. Treatments were established on Aquic Argiudoll (Paraná) and Typic Argiudoll (Marcos Juarez) soils (20). In both locations, soils showed no physical restriction to root growth and adequate levels of P availability (> 20mg/kg P Bray) and organic matter content (~3.0-3.2%).

Cultivars were sown, at a seeding rate of 24 kg/ha, with 15 cm row spacing on 20th April 2010 (Paraná) and on 24th September 2009 (Marcos Juarez) in plots of 1.5 x 6.0 m size in a randomized complete block with three replicates. At sowing, calcium triple superphosphate was surface broadcasted at a rate of 40 kg/ha of P to provide non-limiting P availability. Plots were hand-weeded approximately every 20 days; and chemical control of insects was done as necessary. Irrigation at Paraná was provided by drip-irrigation (driplines spaced 0.60 m apart bearing 1 L/h emitters every 0.30 m) every time soil water content was near 30 mm below the drained upper limit (*i.e.* 210 mm for 1 m soil depth) estimated using evapotranspiration and rainfall as described by Allen *et al*. (1998).

In each plot, a factorial combination of cultivar and cutting frequency was established. Cultivars contrasting in fall dormancy were: a winter-dormant cultivar (FD4), a semi-dormant cultivar (FD6), and a winter-active cultivar (FD9). Each cultivar was defoliated at 'high', 'intermediate' and 'low' frequency. The cutting frequency was based in radiation capture and thermal time accumulation. Plots of 'high' and 'intermediate' cutting frequency were defoliated each time they intercepted 0.50 ('high' frequency) and 0.95 ('intermediate' frequency) of incident radiation. In turn, plots of 'low' cutting frequency had to spend 150 growing degree days (GDD) once 0.95 of incident radiation, was intercepted. In consequence, the interval between cuttings was not fixed to a number of days.

Climate during the experimental period was measured daily at 1.5 m height with a portable meteorological station (LI-1200S, Li-Cor Inc., Lincoln, NE). Main data are shown in figure 1 (page 82). Briefly, mean day temperature was similar to historical records and between locations. In both locations, rainfall was lower than historical records (data not shown) and in Marcos Juarez (Exp. 2, rainfed) a strong water deficit was observed from June 2010 to late January 2011 (figure 1d, page 82).

In Paraná (Exp. 1), treatments started on 16/11/2010 and ended, for 'high', 'intermediate' and 'low' cutting frequencies on the 27/12/2011 (407 days), 29/12/2011 (409 days) and 28/12/2011 (408 days), respectively. In Marcos Juarez (Exp. 2), they all started on 15/3/2010 and ended on 17/3/2011 (368 days), 28/3/2011 (379 days) and 1/4/2011 (383 days) also for 'high', 'intermediate' and 'low' cutting frequencies, respectively.

Radiation interception was measured, on sunny days, at midday every 3-5 days using a tube solarimeter type TSL (Delta-T Devices Ltd., Burwell, Cambridge, UK). In each plot, one above-canopy measurement (Ia) was followed by three below-canopy measurements (Ib). The proportion of radiation intercepted by the canopy was estimated by the following equation: 1 – Ib/Ia. Growing degree days (GDD) were calculated as the sum of daily mean temperatures above the base temperature of 5°C (3, 10).

Prior to cutting, canopy height was measured in fifteen representative sites of each plot. Forage dry matter (kg DM/ha) was estimated by harvesting all strips of 5 m length and 1 m wide (5 m^2 in the center of the plot) with a motor mower (cutting height: 5 cm above ground level). Fresh forage was weighted at field. After that, two sub-samples (~ 200 g) were extracted in order to determine moisture content and components (leaves and stems). Leaves (blades, petiole, stipules and 3 cm of stem tip) and stems were hand separated. All samples were dried at 60°C for 72 h.

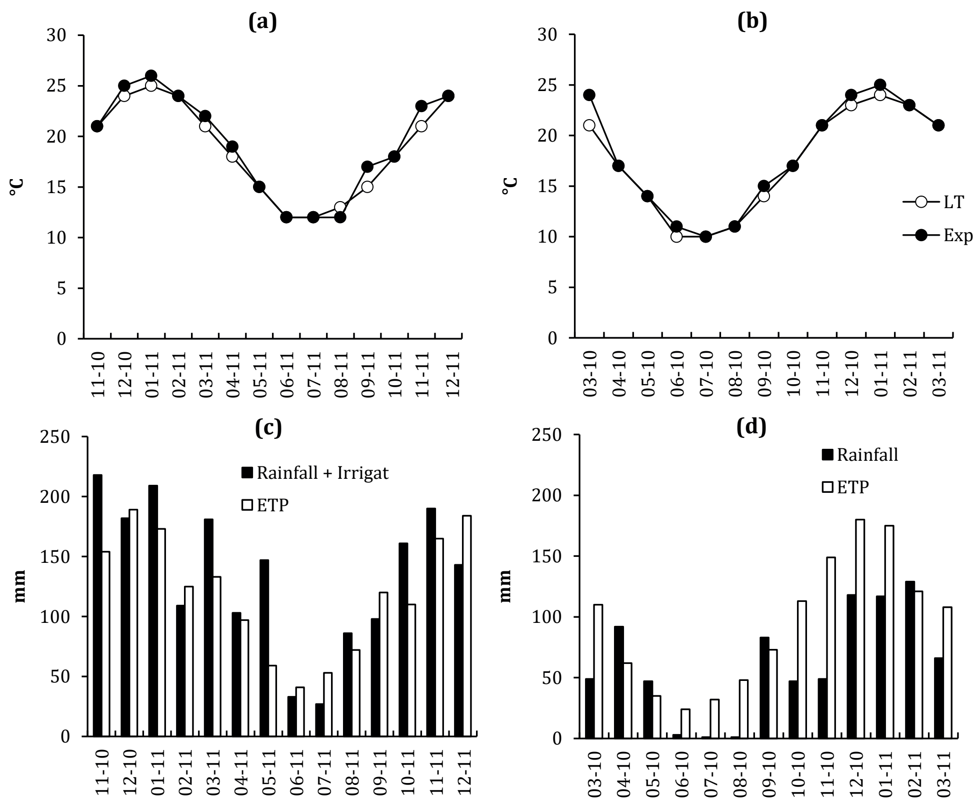

Figure 1. Mean air temperature (°C), rainfall (mm), irrigation (mm) and evapotranspiration (ETP, mm) observed in the experimental period (Exp) and for the long term (LT) in Paraná (a, c) and Marcos Juarez (b, d). 2010-2011.

Figura 1. Temperatura media del aire (°C), precipitaciones (mm), irrigación (mm) y evapotranspiración (ETP, mm) durante el período experimental (Exp) y los datos históricos (LT) en Paraná (a, c) y Marcos Juarez (b, d). 2010-2011.

Statistical analysis

Both experiments were analyzed separately. All data was checked for normality and homogeneity of variances. For total forage and total leaf production (forage mass and leaf mass produced in approximately one year) analysis of variance (ANOVA) was used. When significant effects resulted from ANOVA (P < 0.05), Fisher's least significant difference was used to identify treatment differences. In order to distinguish intrinsic (cultivar differences) from size-mediated effects, we plotted, for each defoliation treatment, leaf proportion *versus* canopy size traits (aerial biomass and canopy height). After that, data within similar size range, were selected. Range of aerial biomass and canopy height in data selected for Paraná (Exp. 1) were: 'high' frequency= 10-25 cm and 600-1700 kg DM/ha; 'intermediate' frequency= 20-45 cm and 1600-4900 kg DM/ha; 'low' frequency= 25-65 cm 2000-5700 kg DM/ha. Range of aerial biomass and canopy height in data selected for Marcos Juarez (Exp. 2) were: 'high' frequency= 10-25 cm and 400-1600 kg DM/ha; 'intermediate' frequency= 10-50 cm and 900-4000 kg DM/ha; 'low' frequency= 15-55 cm 1000-4200 kg DM/ha. With this dataset, a statistical comparison (t-test) was made (2, 17) within each defoliation frequency treatment, using only data sharing the same size trajectory. Regression functions were fitted between explanatory (aerial biomass, canopy height and thermal time between cuttings) and dependent (leaf proportion) variables using Genstat version 18 (VSN, International Ltd).

Results and discussion

In both experiments, significant cultivar*cutting frequency interactions were detected. In irrigated conditions (Exp. 1: Paraná), forage production was similar ($p > 0.05$) between cultivars at 'high' cutting frequency but differences were detected at lower cutting frequencies (figure 2a, page 84). At 'intermediate' cutting frequency, the winter-active and semi-dormant cultivars (FD6 and FD9) produced more forage ($p < 0.05$) than the winter-dormant cultivar (FD4). At 'low' cutting frequency, FD9 was more productive, FD4 was less productive and FD6 was intermediate but not significantly different from FD4 and FD9 (figure 2a, page 84). Under rainfed conditions (Exp. 2: Marcos Juarez), the cultivars showed similar forage production at 'high' and 'intermediate' cutting frequencies, but at 'low' cutting frequency, FD9 and FD6 resulted significantly more productive than FD4 (figure 2b, page 84).

Results of the present research were similar to that observed in subtropical latitudes (6) but differed from those observed at temperate latitudes where interaction between cultivar and cutting frequency was not detected (9, 14, 19, 21, 23). Thus, in subtropical latitudes, cultivar choice must consider the cutting frequency of the productive system. However, leaf yield was quite similar between cultivars (figure 2c, d, page 84). In fact, significant differences between cultivars were detected only at 'low' cutting frequency under rainfed conditions of Marcos Juarez (figure 2d, page 84). Such results imply that the higher forage productivity of more winter-active cultivars (FD6 and FD9) observed at 'intermediate' and 'low' cutting frequencies, was mainly due to a higher production of stems (figure 2e, f, page 84), the less nutritive fraction of aerial biomass (5).

In turn, differences in forage production between cutting frequencies (figure 2a, b, page 84) were explained by differences in both leaf and stem production (figure 2c, d, e, f, page 84). However, these differences were always of greater magnitude in stem than in leaf production. In other words, such results also imply that the higher forage productivity observed in the low cutting frequency managements ('low' and 'intermediate'), were mainly explained by differences in stem biomass.

Stem biomass is the less nutritive fraction of aerial biomass (5). Thus, breeding programs focused on traits related to fiber stem digestibility will be highly valuable for systems using winter-active cultivars and/or systems prone to using long intervals between cuttings (7) (*e.g.* conserved forage industry). Of course, this could also be important for grazing systems, since stem biomass accumulation has a negative impact on animal performance (4). Certainly, the choice of a cultivar also depends on other characteristics such as persistence and pest/disease tolerance.

Table 1 (page 85) shows the comparison between cultivars in leaf proportion of harvested aerial biomass (kg DM/ha), among data sharing a range of similar aerial biomass and canopy height at the moment of cutting (17). At high defoliation frequency, FD4 was leafier than FD6 (Paraná and Marcos Juarez) and FD9 (only in Marcos Juarez). At the intermediate defoliation frequency, FD4 was leafier than FD9 (Paraná and Marcos Juarez) and FD6 (only in Marcos Juarez). All cultivars showed the same leaf proportion at low defoliation frequency. FD6 and FD9 showed similar leaf proportion irrespective of the defoliation frequency treatment (table 1, page 85). It is important to note that most differences between cultivars disappeared when leaf proportion was compared at a similar canopy height (table 1, page 85). In fact, at similar canopy height, FD4 was leafier than FD9 and FD6 only at intermediate defoliation frequency (Paraná) and at high defoliation frequency (Marcos Juarez), respectively.

An inverse relationship between forage production (higher in winter-active cultivars) and leaf proportion (lower in winter-active cultivars) is frequently reported (8, 14, 24). Our results indicate that the variability in leaf proportion was mainly explained by variability in both, canopy biomass and canopy height (figure 3a, b, c, d, page 86). Similar findings were also reported in a temperate and humid environment (21). Thus, it is possible to speculate that differences in leaf proportion between cultivars contrasting in fall dormancy are not intrinsic (*i.e.* the differences in leaf proportion are not due to a cultivar effect). One of the most important implications of previous findings is that changes in leaf proportion obtained in isolated plants during breeding programs are hard to be sustained or observed under pasture conditions. In fact, leaf/stem partition in plants growing in dense canopies follows changes in canopy biomass and canopy height, which in turn are driven by the competition among plants by light (11).

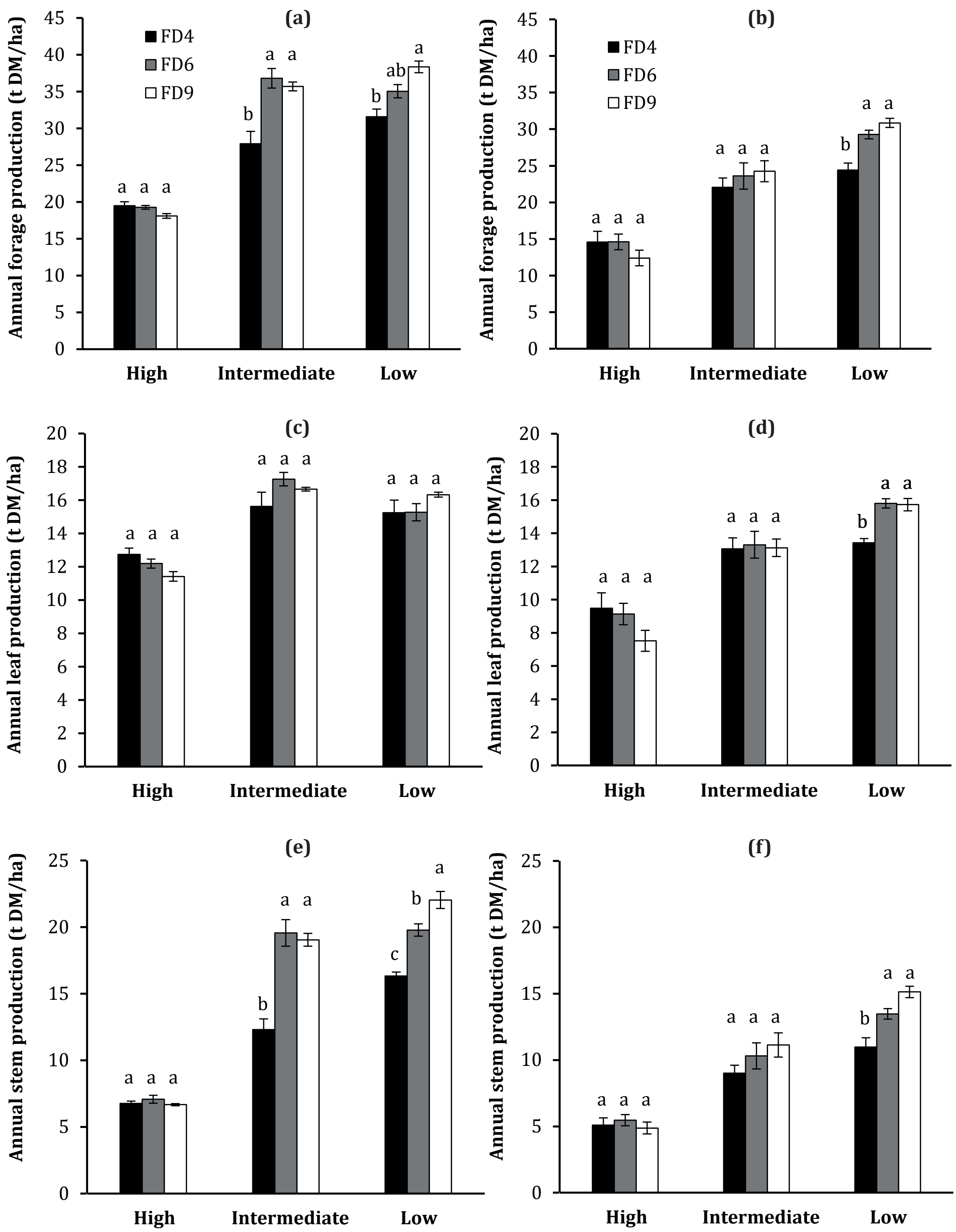

Figure 2. Annual forage (a, b), leaf (c, d) and stem (e, f) production (t DM/ha) for lucerne cultivars differing in fall dormancy (FD4, FD6 and FD9) and subjected to different cutting frequencies (High, Intermediate and Low). Paraná (a, c, e) and Marcos Juarez (b, d, f). Different letters indicate differences between cultivars (P < 0.05), within each cutting frequency. Bars are the standard error of each mean. 2010-2011.

Figura 2. Producción anual (t DM/ha) de forraje (a, b), de hojas (c, d) y de tallos (e, f) para cultivares que difieren en su latencia invernal (FD4, FD6, FD9) sometidos a diferentes frecuencias de corte (High, Intermediate y Low). Paraná (a, c, e) y Marcos Juarez (b, d, f). Letras diferentes denotan diferencias entre cultivares (P < 0,05), dentro de cada frecuencia de corte. Las barras representan el error estándar de la media. 2010-2011.

		Paraná		Marcos Juarez	
		Similar biomass	Similar height	Similar biomass	Similar height
Defoliation	Cultivars	Leaf prop	Leaf prop	Leaf prop	Leaf prop
High	FD4 vs. FD6	0.67 vs. 0.63*	0.66 vs. 0.64	0.66 vs. 0.62*	0.64 vs. 0.62*
	FD4 vs. FD9	0.67 vs. 0.64	0.66 vs. 0.64	0.66 vs. 0.61*	0.64 vs. 0.63
	FD6 vs. FD9	0.63 vs. 0.64	0.64 vs. 0.64	0.62 vs. 0.61	0.62 vs. 0.63
Intermediate	FD4 v. FD6	0.56 vs. 0.48*	0.56 vs. 0.53	0.60 vs. 0.57	0.58 vs. 0.58
	FD4 vs. FD9	0.56 vs. 0.47*	0.56 vs. 0.50*	0.60 vs. 0.56*	0.58 vs. 0.56
	FD6 vs. FD9	0.48 vs. 0.47	0.53 vs. 0.50	0.57 vs. 0.56	0.58 vs. 0.56
Low	FD4 v. FD6	0.50 vs. 0.45	0.48 vs. 0.44	0.56 vs. 0.57	0.56 vs. 0.57
	FD4 vs. FD9	0.50 vs. 0.44	0.48 vs. 0.44	0.56 vs. 0.53	0.56 vs. 0.53
	FD6 vs. FD9	0.45 vs. 0.44	0.44 vs. 0.44	0.57 vs. 0.53	0.57 vs. 0.53

Table 1. Leaf proportion for cultivars differing in fall dormancy (FD4, FD6 and FD9) and subjected to different cutting frequencies (High, Intermediate and Low) in Paraná and Marcos Juarez. Statistical comparison (t-test) was made using data sharing the same size (*i.e.* similar range of aerial biomass or similar range of canopy height). *Denote differences between cultivars (P < 0.05). 2010-2011.

Tabla 1. Proporción de hoja para cultivares de distinta latencia inverna (FD4, FD6 y FD9) sometidos a diferentes frecuencias de corte (High, Intermediate y Low) en Paraná y Marcos Juarez. La comparación estadística (test-t) se realizó usando solo datos que compartían un rango similar de tamaño (*i.e.* similar rango de biomasa aérea y de altura de canopia). *Denota diferencias entre cultivares (P < 0,05). 2010-2011.

Our work shows that variability on leaf proportion was better explained (higher R^2) by changes in canopy height than by changes in aerial biomass (figure 3a, b, c, d, page 86). In addition, variability in leaf proportion was poorly correlated with thermal time (figure 3e, f, page 86). Previous findings confirm that the observed differences in leaf proportion (leaf to stem ratio) between cultivars and defoliation frequencies were simply different points on a common allometric trajectory. Therefore, in lucerne pastures, biomass allocation among leaves and stems (*i.e*. the nutritive value of lucerne) can be modeled and predicted, for very contrasting lucerne genotypes and under a huge range of defoliation managements (3) using biomass based relations (18) or, even better, using plant height (21).

Temperature is the main driver of phenology and development (10, 11). The poor correlation observed between thermal time and leaf proportion (figure 3e, f, page 86) indicates that traditional criteria based on phenological development (*i.e*. flowering, growing degree-days, node number in stem) seems inappropriate for precise and quality-based defoliation management guidelines, for lucerne pastures (11, 21). Furthermore, the well-known differences in the aerial biomass/canopy height ratio of lucerne cultivars contrasting in fall dormancy (21, 23) are also an issue to be considered.

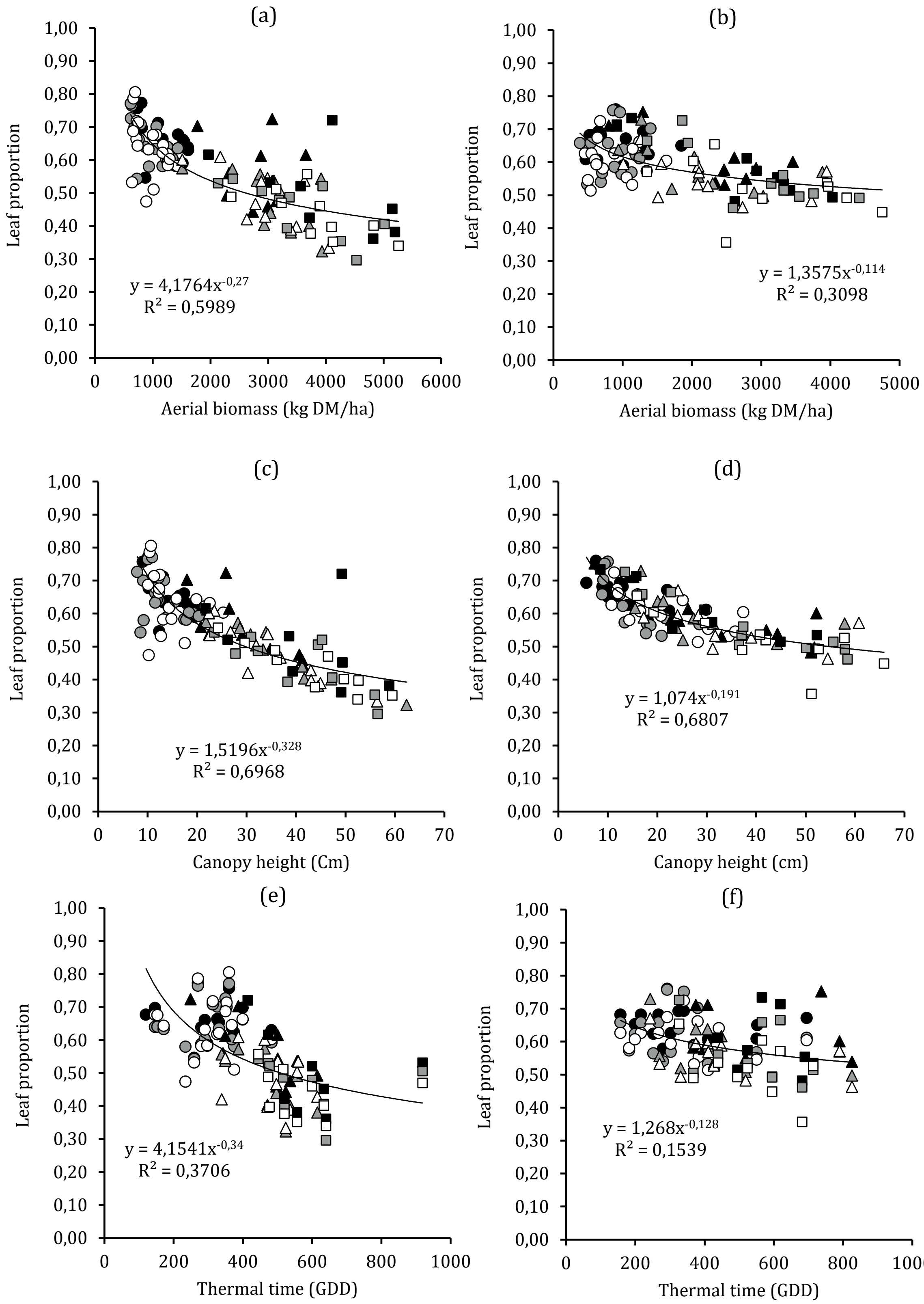

Figure 3. Leaf proportion *versus* aerial biomass (a, b), canopy height (c, d) and thermal time (e, f) for cultivars differing in fall dormancy (FD4: black symbols, FD6: grey symbols, FD9: white symbols) subjected to different cutting frequencies (High: circles, Intermediate: triangles, Low: quadrats) in Paraná (a, c, e) and Marcos Juarez (b, d, f). 2010-2011.

Figura 3. Relación de la proporción de hoja con la biomasa aérea (a, b), la altura de la canopia (c, d) y la suma térmica (e, f) para cultivares que difieren en latencia invernal (FD4: símbolos negros, FD6: símbolos grises, FD9: símbolos blancos) sometidos a diferentes frecuencias de corte (High: círculos, Intermediate: triángulos, Low: cuadrados) en Paraná (a, c, e) y Marcos Juarez (b, d, f). 2010-2011.

Conclusions

This work confirms that in subtropical and humid environments, differences in forage production between cultivars contrasting in winter activity depend on the cutting frequency. Moreover, the results obtained demonstrate that the variability in leaf proportion was mainly explained by the variability in canopy height.

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Root-shoot growth and time to transplant of different lettuce (*Lactuca sativa* L.), genotypes during nursery

Relación raíces/parte aérea y momento de trasplante para diferentes genotipos de lechuga (*Lactuca sativa* L.), durante la etapa de propagación

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Abstract

Although vegetable nursery growers decide on plug sizes based on the types, production time and schedule of the crops to grow, they usually choose individual small cell sizes because these allow short plant-raising periods and reduced costs. However, larger plugs produce a finished plant after transplant in a shorter period of time than smaller plugs. Nursery growers end the propagation period when roots take up the plug cell and plantlets can be removed from the plug tray without damage. Thus, this study aimed to investigate the effect of three plug-cell sizes and a single BAP application (100 mg L^{-1}) on the shoot and root growth and time to transplant of different lettuce genotypes. Nursery decreased as plug cell volume increased and with the single BAP spray. A novelty result was that the transplant dates were assigned when the marginal root dry weight accumulation decreased, *i.e.,* based on objective rather than on subjective observations. The significant leaf area and dry weight accumulation found could be explained by growth parameters such as the rate of leaf appearance, the relative rate of leaf area expansion, the relative growth rate, the net assimilation rate and the partition coefficient from root: shoot allometries.

Keywords
synthetic cytokinin • photo-assimilate partitioning • plug tray • root restriction • vegetables

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Resumen

Los propagadores hortícolas deciden el tamaño de la celda basados en la especie y la dinámica de crecimiento, de esta manera eligen los tamaños más pequeños bajo el supuesto de disminuir el período de terminación del esqueje y reducir los costos de producción. Los propagadores sugieren trasplantar cuando las raíces ocupan toda la celda y las plántulas pueden ser extraídas de la celda sin daños visibles; cuanto mayor es el tamaño de la celda, el tiempo hasta trasplante disminuye. El objetivo de este estudio ha sido investigar el efecto de tres tamaños de celda y el asperjado con BAP (100 mg L^{-1}) sobre la acumulación de biomasa aérea y radical y el momento de trasplante para diferentes genotipos de lechuga. Nuestros resultados muestran que el ciclo de propagación disminuyó a medida que se incrementó el volumen de la celda y en las plántulas asperjadas con BAP. El resultado novedoso de este trabajo es que el momento de trasplante fue determinado objetivamente cuando la acumulación marginal de peso seco radical comenzaba a disminuir. Los cambios en la expansión de área foliar y acumulación de peso seco pudieron ser explicados a partir del uso de diferentes estimadores del crecimiento.

Palabras clave

Citocininas • partición de foto asimilados • tamaño de celda • restricción radical • hortalizas

Introduction

In recent years, plant growers have adopted the plug system for seedling propagation based on the assumption that this system may allow minimizing labor costs, increasing crop uniformity, and reducing cropping duration. Plug production is characterized by the use of specialized plug trays, in which the higher the number of cells per tray, the smaller the cells. Although decisions on plug sizes are based on the type, production time and schedule of the crops to grow, vegetable plug growers have mainly adopted containers of reduced size based on the hypothesis that these can increase nursery yield. This choice allows increasing plant density, but has the disadvantage that it restricts root growth, followed by considerable changes in plant growth and physiology (1). In addition, although seedling trays with individual small cell sizes allow short plant-raising periods and reduced costs, larger plugs produce a finished pot plant after transplant in a shorter period of time than smaller plugs (3).

Nursery root restriction would lead to a decrease in both root growth and cytokinin synthesis or translocation. When the container base impedes vertical root growth, the availability of cytokinin synthesized in the root apex and reallocated to shoots would be reduced (12). In this context, data from our laboratory have shown that the exogenous application of the cytokinin 6-benzylaminopurine (BAP) at the pre-transplant stage to plants grown in small cell volumes may override the shoot growth limitation due to root restriction and that the plant response to BAP is related to both the dose and application time (13). Although information on the effect of physical root restriction as a result of a limited plug cell volume on plant growth is available (12), the physiology of the roots growing under impeded plug cell tray conditions during nursery is still vaguely understood.

Based on the above, the aim of this work was to study the growth pattern during nursery of four genotypes of lettuce (*Lactuca sativa* L.), the most commonly consumed fresh leafy vegetable and one of the main crops grown under greenhouse conditions (22), in the presence of different degrees of root restriction by the use of different plug cell volumes and a single BAP spray as an attempt to explain the potentially different plantlet sizes at the transplant stage. The hypothesis tested indicate that a single BAP application can override plug cell restriction through an increase in leaf area, shoot biomass accumulation and photo-assimilate partitioning to shoots.

Materials and Methods

The experiment was carried out in a greenhouse at the Facultad de Agronomía de la Universidad de Buenos Aires, Argentina (34°35'59''S, 58°22'23''W) from June 5th to August 28th 2018 (experiment 1) and repeated four times: i) between July 3rd and September 25th 2018 (experiment 2), ii) between August 7th and October 9th 2018 (experiment 3), iii) between October 2nd and December 3rd 2018 (experiment 4), iv) and between November 8th and December 23rd 2018 (experiment 5).

Four lettuce seed genotypes 'Lores' (Vilmorin, France), 'Sandrine' (Clause-Tézier, Spain), 'Taina' (Cohorsil, Honduras) and 'Luana' (Sakata, Japan)] were germinated and grown in 50-, 128- and 288-plug cell plastic trays (55.70, 17.37 and 6.18 cm^3 $cell^{-1}$ respectively) filled with Klasmann 411® medium (Canadian *Sphagnum* peat moss-perlite-vermiculite 70/20/10 v/v/v). Seedlings were sprayed with BAP (SIGMA EC 214-927-5) (Sigma-Aldrich Co., St. Louis, MO, USA) with two treatments, C: control: 0 mg L^{-1} and BAP: 100 mg L^{-1} when the first pair of true leaves developed. BAP was previously diluted in alcohol 80%. In this way, 24 combinations were generated that made up 24 experimental units.

Plants were irrigated with high-quality tap water (pH: 6.64 and electrical conductivity of 0.486 dS m^{-1}) by using intermittent overhead mist and weekly fertigation (1N:0.5P:1K:0.5Ca v/v/v/v) (Stage 2: 50 mg L^{-1} N; Stages 3-4: 100 mg L^{-1} N). The volume per pot varied according to the container's volume.

Half hourly averages of the air temperature were measured using a HOBO H08-001-02 data logger (Onset Computer Corporation, MA, USA) protected from direct radiation by aluminum foil shades. Global solar radiation was recorded with an additional PAR HOBO data logger. The mean air temperature during the experiment was 19.5 $\pm$ 2.0 °C, while the mean photosynthetic active radiation ranged between 17.33 and 21.81 mol photons m^{-2} day^{-1}.

Plants for destructive measurements (10 per treatment and block) were harvested at emergence and at 7-day intervals. Roots were washed fresh weights (FW) were recorded. Dry weights (DW) were obtained after drying to constant weight at 80°C for 96 hours, root, stem and leaf dry weights (DW) were recorded. The number of leaves was recorded as well and each leaf area was determined using the ImageJ® (Image Processing and Analysis in Java) software. We assumed that plants reached the transplant stage when the marginal root DW through time decreased.

The rate of leaf appearance (RLA), the relative growth rate (RGR), the rate of leaf area expansion (RLAE), the mean net assimilation rate (NAR) and the allometric coefficients between roots and shoots were calculated according to Di Benedetto and Tognetti (2016).

Since there were no significant differences between the experiments, data shown are limited to those of experiment 3, which was conducted in a time of the year in which the four lettuce genotypes are usually propagated.

The experimental design was a randomized design in three blocks of 10 observations of each combination of levels of the evaluated effects (plug cell volume × BAP application × lettuce genotype). Data were subjected to analysis of variance (ANOVA). The STATISTICA 8 (StatSoft) software was used and the assumptions of ANOVA were checked. Least significant differences (LSD) values were calculated. Means were separated by Tukey's tests ($P \leq 0.05$). Slopes from straight-line regressions of RLA, RLAE, RGR, NAR and allometric values were tested using the SMATR package.

Results

In control plants, nursery cultivation, estimated through the time between seed germination and the transplant stage, was directly related to the plug cell volume: as the plug cell volume increased, the nursery period of all the lettuce genotypes tested decreased. The nursery period was also decreased by the BAP spray at the pre-transplant stage (figure 1, page 92).

Data are the mean of the five experiments performed (n = 15). The vertical line indicates least significant differences (LSD).

Los datos son promedio de las cinco repeticiones del experimento (n = 15). Las líneas verticales indican la mínima diferencia significativa (MDF).

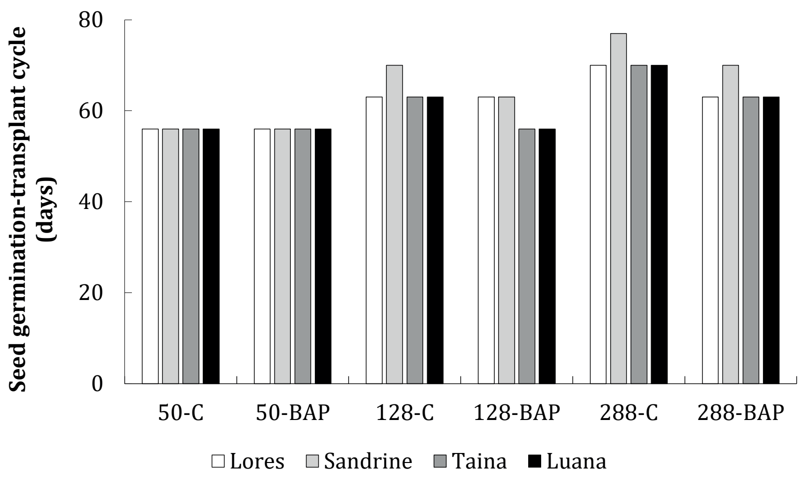

Figure 1. Effects of three plug cell volume (50-, 128- and 288-cell trays^{-1}) and a pre-transplant BAP spray (100 mg L^{-1}) (C: control plants) at the time from seed germination and transplant stage of four lettuce genotypes.

Figura 1. Efecto de tres tamaños de celda (50-, 128- y 288-celdas bandeja^{-1}) y el asperjado con BAP en pre-trasplante (C, control: 0 mg L^{-1} y BAP: 100 mg L^{-1}) sobre el ciclo de propagación para cuatro genotipos de lechuga.

At the end of experiment, total leaf areas and shoot FW were highest in control plants grown in 50-plug cell trays and decreased as the plug cell volume decreased. The single BAP spray decreased leaf areas but increased shoot FW in all plug cell volumes tested. The lettuce genotypes showed only quantitative differences to this general response pattern (table 1, page 93).

During the experiment, all lettuce genotypes showed higher RLA in 50-plug cell trays than in smaller plug cell volumes. The single BAP spray always increased RLA values respect to the controls. However, only minor differences in RLAE between treatments were found and no effect of BAP was observed (table 1, page 93).

RGR, which was determined as an estimator of DW accumulation, showed quantitative differences between lettuce genotypes, but, in most plants, the highest RGR values were found in plants grown in 50-plug cell trays and BAP-sprayed plants. We also observed significant differences in NAR, which supports the results observed in lettuce plants from the highest plug cell volumes and BAP-sprayed ones. When RGR was disaggregated into shoots (data not shown) and roots, the RGR of the latter (RGR_{Roots}) was highest in the plants grown in the largest plug cell volume. When the plants were sprayed with BAP, some lettuce genotypes showed a tendency towards a decreased root DW accumulation (table 1, page 93).

The increased β coefficients observed in the root-shoot allometries showed a higher photo-assimilate partition to roots as the plug cell volume decreased. In contrast, the BAP spray increased photo-assimilate partition to shoots (decreased β coefficients) (table 1, page 93).

Discussion

To investigate the impact of plug size on below-ground (root growth and resource capture) and above-ground (shoot weight, leaf area) traits, different combinations of shoot and root growth were created using plug cells of different sizes and a single BAP application (100 mg L^{-1}).

Time to transplant is usually a trait with no objective protocols; nursery growers define the end of the propagation period when roots take up the plug cell and plantlets can be removed from the plug tray without damage (6). However, the novelty of the results shown in figure 1 is that they were obtained from data collected when marginal root DW through time decreased (data not shown).

Table 1. Effects of three plug cell volume (50-, 128- and 288-cell trays^{-1}) and a pre--transplant BAP spray (100 mg L^{-1}) on the growth parameters of four lettuce genotypes.

Tabla 1. Efecto de tres tamaños de celda (50-, 128- y 288-celdas bandeja^{-1}) y el asperjado con BAP en pre-trasplante (100 mg L^{-1}) sobre los estimadores de crecimiento para cuatro genotipos de lechuga.

	Leaf area (cm^2 plant^{-1})	Shoot FW (g plant^{-1})	RLA (leaves day^{-1})	RLAE (cm^2 cm^{-2} day^{-1})	RGR (g g^{-1} day^{-1})	NAR (g cm^{-2} day^{-1}) x 10^{-5})	RGR_{Roots} (g g^{-1} day^{-1})	β coefficient
Lores								
50-C	181.01aC	4.202bC	0.200bB	0.075aA	0.113aA	257.34bB	0.114aA	1.054dC
50-BAP	149.47bB	4.815aC	0.218aB	0.072aA	0.118aA	295.63aD	0.108bA	0.905eB
128-C	101.98cB	2.769dA	0.141dB	0.062bA	0.097bA	163.12eB	0.093cA	1.121bC
128-BAP	71.40dB	3.063cA	0.182cB	0.065bA	0.100bA	216.98cB	0.092cA	1.047dA
288-C	23.96eB	0.613fC	0.130eB	0.040cA	0.065dB	173.90eA	0.072dA	1.673aB
288-BAP	22.94eA	0.869eB	0.148dB	0.038cA	0.079cA	203.61dA	0.071dA	1.093cB
Sandrine								
50-C	214.92aA	5.462bB	0.229bA	0.079aA	0.104bB	242.21cC	0.100bB	1.173cB
50-BAP	115.80bC	5.753aB	0.257aA	0.071bA	0.112aA	342.00aB	0.110aA	0.975fA
128-C	108.60bB	2.364dD	0.163dA	0.066cA	0.098cA	223.48dA	0.082cB	1.277bB
128-BAP	68.79cB	2.877cB	0.195cA	0.062cA	0.099cA	328.42bA	0.087cA	1.049eA
288-C	30.58dA	0.737fA	0.152eA	0.045dA	0.075dA	181.29fA	0.074dA	1.337aC
288-BAP	23.71dA	1.014eA	0.164dA	0.040dA	0.078dA	206.85eA	0.070dA	1.088dB
Taina								
50-C	200.50aB	5.778bA	0.121bD	0.074aA	0.101bB	262.43bB	0.096bB	1.253bA
50-BAP	172.41bA	5.974aA	0.139aD	0.073aA	0.112aA	316.40aC	0.113aA	0.817dC
128-C	96.78cC	2.704dA	0.104cC	0.063bA	0.090cA	175.11dB	0.079dB	1.265bB
128-BAP	77.63dA	2.740cC	0.127bC	0.059bA	0.096bA	215.53cC	0.088cA	0.822dB
288-C	20.65eB	0.590fD	0.086dD	0.041cA	0.056dB	120.60eC	0.071dB	1.346aC
288-BAP	16.85eB	0.636eD	0.102cD	0.037cA	0.062eA	214.91cA	0.054eA	1.209cA
Luana								
50-C	216.86aA	4.190bC	0.139bC	0.077aA	0.101aB	346.26bA	0.105bB	1.251cA
50-BAP	99.81bD	4.486aD	0.155aC	0.066bB	0.104aA	400.05aA	0.118aA	0.989eA
128-C	84.99cD	2.523dC	0.113dC	0.061bA	0.102aA	176.23dB	0.090cA	1.338bA
128-BAP	78.83dA	2.725cC	0.127cC	0.061bA	0.104aA	220.02cB	0.097bA	1.045dA
288-C	24.02eB	0.684fB	0.104eC	0.040cA	0.062bB	160.45eB	0.074dA	1.833aA
288-BAP	20.14eA	0.760eC	0.114dC	0.038dA	0.063bA	174.15dB	0.070dA	1.064dC

Different capital letters indicate significant differences ($P < 0.05$) for the different lettuce genotypes for the same treatment. FW: fresh weight; RLA: rate of leaf appearance; RLAE: relative rate of leaf area expansion; RGR: relative growth rate; NAR: net assimilation rate.

Letras minúsculas diferentes indican diferencias significativas ($P < 0,05$) entre tratamientos para cada genotipo de lechuga. Letras mayúsculas diferentes indican diferencias significativas ($P < 0,05$) entre genotipos para el mismo tratamiento. FW: peso fresco; RLA: tasa de aparición de hojas; RLAE: tasa relativa of expansión de área foliar; RGR: tasa de crecimiento relativo; NAR: tasa de asimilación neta.

A limited plug cell volume restricts the vertical root growth when the root apical meristem reaches the bottom of the cell or the pot (12). Thus, both primary root growth and root branching decrease and, presumably, the concentration of cytokinin, the main endogenous hormone synthesized by root apical meristems, would decrease as well. At the transplant stage, lettuce plants grown in plug trays may show a well-developed root system with white roots and without damage, but with a root girdling growth around the cell (3). Although a new root system may develop through the lateral roots that arise adventitiously, the effects of the root restriction remain for the following weeks (12). In the present study, as the plug cell volume increased, the time from sowing to transplant decreased. Similarly, the BAP spray decreased the nursery period about two weeks in lettuce plants grown in 128- and 288-plug cell trays and showed no changes in those grown in 50-cell plug cell trays.

Since we found no significant qualitative differences between experiments, data from table 1 (page 93), which include most growth parameters used to explain the results shown in figure 1 (page 92), are limited to the results of experiment 3, which was conducted in a period during which the four lettuce genotypes are usually propagated.

Transplant growers need to produce plants that are visually appealing and of acceptable quality for their customers. Aesthetically, the main trait related to plant quality for commercial acceptance of vegetable transplants is total leaf area. Since leaves are the plant organs responsible for light interception, in physiological terms, this implies the need to expand the leaf area at the highest growth rate, which includes both leaf number and individual leaf size. Here, at the transplant stage, plantlets showed higher total leaf area and shoot FW in 50-cell plugs and decreased as the plug cell volume decreased. When plants were sprayed with BAP, seedlings increased their shoot FW related to controls but showed a decreased total leaf area (table 1, page 93). These results are in agreement with previous reports (4, 21).

Leaf area development can be characterized by means of two growth parameters: (i) RLA, which is an estimator of leaf initiation and plastochron length, and (ii) RLAE, which allows quantifying leaf expansion. Changes in total leaf area are mainly related to the meristematic shoot apex capacity to initiate and expand leaf primordia. Table 1 (page 93) shows that, in control plants, as the plug cell volume increased, RLA and RLAE increased. The BAP spray increased RLA in higher proportions than RLAE.

Davière and Achard (2017) have shown that the plastochron length of transgenic plants with reduced cytokinin levels may be altered, which explains the ability of a single BAP spray to override root restrictions. Similarly, we have previously shown that the effects of cytokinin on leaf growth rates include both a decrease in the plastochron length and an increase in leaf expansion, accompanied by an increase in the size of the shoot apex meristem through the synthesis of high-molecular weight substances essential for cell growth (9). These two processes are mediated by the down-regulation of the KNOTTED and WUSCHEL genes (18) associated with a high-cytokinin/low-gibberellin ratio.

The primary shoot apical meristem, which is responsible for generating all the aboveground organs, is controlled by hormones, including auxin, cytokinin, and gibberellin, which act both independently and in combination (10). The main function of endogenous cytokinin is to control the cell cycle and shoot apical meristem growth (2). An increase in RLA would indicate a shorter plastochron, which would in turn require an increase in the shoot apical meristem and non-limiting photo-assimilate supply to hold vegetative plant growth. The latter needs an increase in the photosynthetic capacity and/or a change in photo-assimilate partitioning. The higher total leaf area observed in control than in BAP-sprayed plants would be explained by a limited photo-assimilate availability required to expand the high leaf number previously initiated.

Transplants grown in large plug-cell trays are usually taller and have greater leaf areas and shoot-root DW than those grown in small plug-cell trays. Data from control plants are in agreement with this general view. However, BAP-sprayed plants, which had higher final yield (table 1, page 93), showed lower leaf areas as a result of a higher leaf number but lower individual leaf area (data not shown), which forces us to change the concept of transplant quality. Although the leaf area determines the plant capacity of light interception, the relative growth rate (RGR) may vary mainly as a result of the 'physiological component' NAR (net assimilation rate). Our results showed that the higher plug cell volume, the higher the RGR and NAR of control plants. On the other hand, cytokinin are also known to enhance carbon fixations per unit leaf area. The exogenous application of cytokinin has been shown to stimulate the transcription of a wide number of chloroplast genes, which code for the large subunit of Rubisco (24). In previous studies, we also found that direct addition of cytokinin increases leaf thickness (11), and that this may further enhance carbon assimilation per unit leaf area. At the early stages of leaf development, treatment with exogenous BAP accelerates the division of mesophyll cells, whereas at the later stages of development, BAP treatment activates the expansion of growing cells and of those that have just accomplished their growth (17). The results here found in BAP-sprayed plants are in agreement with these previous reports (table 1, page 93).

Poorter *et al.* (2012) pointed out that the reduced growth in small pots is caused mainly by a reduction in photosynthesis per unit leaf area. In addition, In addition, Li *et al.* (2016) has been indicated that, in general, NAR is the best general predictor of the variation in RGR, in agreement with previous reports from our laboratory in spinach (14, 15) and lettuce (21). Root restrictions often depress the photosynthetic capacity (23).

For optimal development of the plant as a whole, root and shoot biomass have to be balanced. Photo-assimilates from leaves are used for root and stem growth. A plausible control mechanism for organ growth is the regulation of relative allocation of assimilates among the plant organs. Although the interspecific variation between biomass allocations to above-ground versus below-ground organs is broadly coordinated, these relationships can be largely modified by plant phenotypic adjustments to variable environmental conditions. Changes in the allometric slopes reflect variations in carbon partitioning (19), and carbohydrate partitioning between competing sites is fundamental to plant growth, especially because plants are capable of modifying their resource allocation to favor the growth of their growing parts. The RGR_{Roots} found in the present study (table 1, page 93) would be a direct response to a limited volume for root growth.

On the other hand, sink strength and source activity can be altered by endogenous hormones and environmental factors. Among endogenous hormones, auxin and cytokinin have major roles in source nutrient remobilization and sink development (25). Plant tissues and organs rich in cytokinins are known to attract assimilate translocation and increase the sink capacity of leaves treated with cytokinins such as benzyl adenine. Changes in the allometric slopes (β coefficient) reflect variations in carbon partitioning in response to the interactions between biotic and abiotic environments. In agreement with the results shown in table 1 (page 93), data from our lab in control *Impatiens walleriana* plants (6) showed a higher photo-assimilate partitioning towards roots as the plug-cell volume or growing medium quality decreased. In that study, we also showed that a single 100 mg L^{-1} BAP spray increased the photo-assimilate partitioning towards shoots. In addition, when we analyzed the stem-leaves allometries, we found an increase in the partitioning towards leaves in control plants and an increase in the partitioning towards stems in BAP-treated ones. Although the smaller the cell volume, the higher the percentage of dry matter allocated to roots, the increase in photo-assimilate partitioning towards the shoot apical meristem has been associated with a positive feedback during the first 30 days after an exogenous BAP spray (7).

The genotypes evaluated in the present study were two butter heads and two crisp head lettuce genotypes with different environment requirements. The butter head Lores is seeded and grown during spring, summer and autumn, while the butter head Sandrine is suggested for growing during autumn and winter. On the other hand, the crisp head Taina is particularly suited for warm season production, while the crisp head Luana is sown during autumn and winter. Our results indicated, with minor quantitative differences, that the four lettuce genotypes evaluated showed the same response pattern to both limited plug cell volumes and BAP application.

Conclusions

Present results suggest that the growth response of lettuce seedlings during nursery yields can be optimizing with a correct plug cell volume chosen and BAP use as a result of the higher plantlet and the lower growth time related to costs cultivation, which can validate the proposed hypothesis. Time from transplant was achieved when the marginal root DW through time decreased. This novelty approach give a better objective methodology to define time to transplant although this commercial use is now far. On the other hand, our results showed that plug purchasers would be change their visual criteria when they buy BAP-treated lettuce for increased field yields.

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Effect of LED lights on the antioxidant properties of baby spinach leaves (*Spinacia oleracea* L.)

Efecto de las luces LED sobre las propiedades antioxidantes de hojas de espinaca tipo "baby" (*Spinacia oleracea* L.)

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Abstract

The present study employed white (W), blue (B: 468 nm), red (R: 629 nm) and green (G: 524 nm) monochromatic LED lights for 26 days, from 11:00 to 18:00 h (7 h per day), with an average photosynthetic photon flux density (PPFD) of 26.0 m^{-2} s^{-1} on baby spinach leaves (*Spinacia oleracea* L.), cvs. Falcon F1 and Viroflay, grown in a hydroponic system. Regardless of the cultivar, the fresh and dry weights were positively influenced when the plants were irradiated by R-light in comparison to W-light. Independent of the cultivar, the leaves treated with B-light reached a significantly higher phenolic compound concentration and antioxidant capacity than plants irradiated with W-light. In addition, the green light increased total phenolic compound concentration. According to the results, the use of LED lights is a promising technique for the production of antioxidant compound-enriched leafy vegetables.

Keywords
LED light • biomass • spectrum • antioxidant capacity • hydroponic system • spinach

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Resumen

El presente estudio empleó luces LED monocromáticas blanca, azul (468 nm), roja (629 nm) y verde (524 nm) durante 26 días, desde las 11:00 hasta las 18:00 h (7 horas por día), con una densidad media de flujo fotosintético de 26,00 μmol m^{-2} s^{-1} sobre dos cultivares de espinaca (*Spinacia oleracea* L.) de hoja baby (Falcon F1 y Viroflay) cultivadas en un sistema hidropónico. Respecto del cultivar, los pesos fresco y seco fueron influenciados positivamente cuando las plantas fueron irradiadas con luz roja en comparación con las irradiadas con luz blanca. Independientemente del cultivar, las hojas tratadas con luz azul alcanzaron una concentración de compuestos fenólicos y capacidad antioxidante superiores a la de las plantas irradiadas con luz blanca. Además, la luz verde incrementó la concentración de compuestos fenólicos. De acuerdo con los resultados, el uso de luces LED es una técnica prometedora para la producción de hortalizas de hoja enriquecidas en compuestos antioxidantes.

Palabras clave

luces LED • biomasa • espectros • capacidad antioxidante • sistema hidropónico • espinaca

Introduction

Spinach (*Spinacia oleracea* L.) is a leafy vegetable belonging to the family *Amaranthaceae*. This species is recognized for its high concentration of iron, calcium, potassium and vitamins such as A, B and C. Moreover, it has several phenolic compounds that promote good health beyond their basic nutrition, which are also attributed to its antioxidant characteristics. These compounds help avoid diseases and cell damage caused by reactive oxygen species (ROS) produced in the respiration process (1, 3, 15).

To increase the quality of leafy vegetables, light supplements are being studied. Nowadays, light-emitting diode (LED) lights are being used, given their advantage of a long lifespan, low energy cost and low temperature production. Its operation is based on the adjustment to different light spectrum, which can also be adjusted for different light quality, mainly red (R), green (G) and blue (B)-lights, stimulating germination, promoting vegetative growth and synthesizing antioxidant compounds in plants grown in greenhouses (30). Weston and Barth (1997) Rouphael *et al.* (2012) indicated that the factors that most influenced the antioxidants content of vegetables were temperature and light intensity. These climatic factors can be modified by the use of greenhouses, which offers a productive advantage by reducing the high climatic variability present in an outdoor crop (16).

Colonna *et al.* (2016) reported a propensity to increase the nutritive value and phenolic compounds of different species of leafy vegetables grown in a greenhouse when were harvested at 8:00 h (with a low photosynthetic photon flux density (PPFD): 200 – 400 μmol m^{-2} s^{-1}) in comparison to harvesting at 14:00 h (with a high PPFD: 800 – 1200 μmol m^{-2} s^{-1}).

On the other hand, Samuolienè *et al.* (2012) determined a positive trend of B and G LED light supplementation (30 μmol m^{-2} s^{-1}) for 16 hours per day on vitamin C and tocopherol concentration in lettuce (*Lactuca sativa* L.) cultivated in a greenhouse compared to natural light intensity conditions. Moreover, Son and Oh (2015) showed that the use of R (600 – 700 nm), G (500 – 600 nm) and B (400 – 500 nm) LED lights with a PPFD of 173 μmol m^{-2} s^{-1} for 12 hours per day for 18 days increased the production of biomass and secondary metabolites on lettuce treated with a greater proportion of R and B-lights, respectively. Johka *et al.* (2010) indicated that blue LED improved seedling quality and growth after transplanting red leaf lettuces. Son and Oh (2015) observed that total phenolic concentration, total flavonoid concentration, and antioxidant capacity of lettuces grown under high ratios of blue LED were significantly higher compared with red LED or control conditions. Among the secondary metabolites, Ávalos and Pérez-Urria (2009) indicated that changes in quality and light intensity increased phenolic compounds and the antioxidant capacity of vegetables.

On the other hand, spinach (*Spinacia oleracea* L.) is widely regarded as a functional food due to its diverse nutritional composition, which includes vitamins and minerals, and to its phytochemicals and bioactives that promote health beyond basic nutrition (33). Spinach leaves showed biological activities that contribute to the anti-cancer, anti-obesity, hypoglycemic, and hypolipidemic properties (33). Viroflay baby spinach grown under red photoselective filter reached higher phenolic compound content, foliar area, width, length and yield than control plants (25). In this same study, Viroflay baby leaves cultivated under blue filter showed significantly higher dry weight than those in red and gray filters (25).

The aim of this study was to evaluate light supplementation with B, G and R- lights on the physical aspects and synthesis of antioxidant compounds in two baby spinach leaves (*Spinacia oleracea* L.) cultivars grown in a floating hydroponic system in a plastic greenhouse.

Materials and methods

Plant material and experiment

The experiment and analysis were conducted in the greenhouse and laboratory of the Postharvest Study Center (CEPOC) at the Faculty of Agricultural Sciences, University of Chile, located in La Pintana, Santiago, Chile. The experiment lasted from the end of September to November 2017 in a closed hydroponic floating system located inside a plastic greenhouse.

The plant material used was spinach belonging to two cultivars: Falcon F1, which is a vigorous plant, with a smooth, large, dark green leaf (World South Seeds Ltda., Chile) and Viroflay characterized by a very fast growing, very productive, with dark green semi-smooth leaves (World South Seeds Ltda., Chile).

A germination test was performed according to ISTA standards (20) and the results of which showed 96% for both cultivars. Inert substrate was mixed in a 1:1 proportion with granulated rock wool (Agrolan®, El Volcán S. A., Chile) and expanded perlite A6 (Harbolite Chile Ltda., Chile) and used in the sowing. Irrigation was done with tap water after sowing and when the plants had the first true leaf completely expanded, modified 50% Hoagland II nutritive solution was added (18). The pH of the solution was kept between 5.5 and 5.8 to maximize nutrient absorption by the crop (19, 21). The transplant was done when the plants had 1 to 2 completely expanded true leaves (18 days after sowing) in a floating root closed hydroponic system, making sure that the roots were in contact with the nutrient solution. The hydroponic system was 7.0 x 1.5 m with a maximum capacity of approximately 950 L. The plants were placed on high-density expanded polystyrene plates located in the system, which were previously perforated with a 3 bobbin arrangement, with a density of 63 plants m^{-2}. One day after transplant, the same nutrient solution was added keeping the range of pH between 5.5 to 5.8. The experimental unit (EU) was a quadrant of 0.9 x 0.5 m polystyrene floating plate containing the root system, with 15 plants located in the central area to avoid interaction with other treatments. An empty space of 14 cm without plants was kept between each EU. Treatments were distributed randomly in a margin of 7.0 x 0.5 m of the floating system.

The plants were grown in the greenhouse with natural sunlight. During this period, the natural sunlight was between 11.85 to 13.82 h per day (from sunrise to sunset time).

The treatments were applied with LED tapes (DEMASLED, TEXMCCW, Chile) located in each EU divided into 9 sections. The light spectrum of the LED lights were measured in each EU in 9 equidistant sectors with a smart spectrometer sensor (Lighting Passport, Asensetek, Taiwan). Additionally, the PPFD of each LED light used to enrich the natural light of the greenhouse was measured with the point sensor of a radiometer (DELTA OHM, HD9021, Italy) in the same 9 equidistant sectors per EU as previously described. The natural sunlight was enriched with white (W), blue (B), green (G) or red (R) LED lights for 26 days from transplanting to the harvest. LED light supplementation was performed daily from 11:00 to 18:00 h (to take advantage of the period of maximum photosynthetic activity). The average PPFD was 26 $\mu mol\ m^{-2}\ s^{-1}$ of photosynthetically active radiation (PAR): white (W) 25.85 ± 0.55; blue (B) 25.59 ± 0.60; green (G) 26,60 ± 1.50; and red (R) 25.93 ± 0.60 $\mu mol\ m^{-2}\ s^{-1}$. The light spectrum were 468, 534 and 629 nm for B, G and R- lights, respectively.

The harvest took place when the plants had 5 to 6 fully expanded true leaves (45 days after sowing) and they had a maximum length of 10 cm (commercial size for baby leaves). At this time, each EU was assessed independently. All the leaves in each EU were harvested and packed into low-density polyethylene sealed bags. Five or six leaves per plant from three plants were packed per bag. After harvest, early in the morning, five randomly selected bags from each EU were stored at 5 °C for two hours till afternoon for the physical measurements of the leaves. For the chemical analysis, another eight bags were frozen at -80 °C in an ultra-low temperature freezer (SANYO, MDF – U33V, Japan).

Physical parameters

At harvest, a visual assessment was made of leaf damage per plant. The damage intensity on the leaves and the number of affected leaves per repetition were recorded. The damage intensity was obtained according to the affected leaf area, dividing the leaf into four quadrants. According to the affected quadrants, a damage coefficient was calculated per leaf considering the expression $Damage = (C1 * n) + (C2 * n) + (C3 * n) + (C4 * n)$

Where:

C = the damage coefficient value (Dc) based on the number of affected quadrants

n = the number of damaged leaves per EU (figure 1).

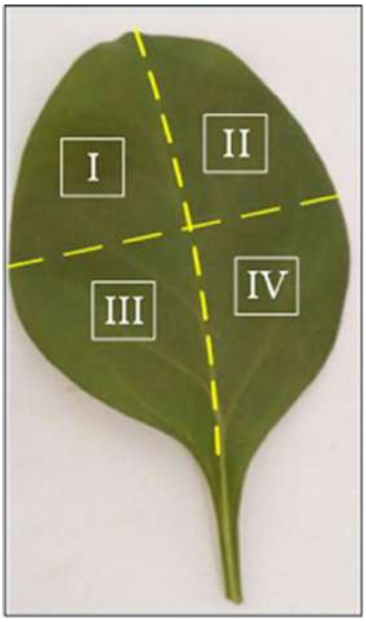


Figure 1. Quadrants used for the damage scale of spinach leaves under light supplementation with LED lights.

Figura 1. Cuadrantes utilizados para la escala de daño de las hojas de espinaca bajo suplementación lumínica con luces LED.

The color was measured in six random leaves selected from three random plants. The measurements were taken in the axial part of the distal sector of the leaf using a tristimulus compact colorimeter (Minolta Chroma meter, CM – 2500d, Japan). The results were expressed as hue, chroma (C) and lightness (L) (25). The longest three leaves were measured from another five random plants per repetition (to limit the length). The measurements were made with a metric rule, where the maximum length and width were represented for the distance obtained from the base to the apex of the leaf above the midrib and the greatest distance perpendicular to the central rib of the leaf, respectively (25). The results were expressed in cm. The fresh weight (FW), dry weight (DW) and dry weight percentage (%DW) were measured with a precision balance (Radwag, AS 100/C/2, Poland) (25). For the DW, the samples were dried in a freeze dryer (ilShinBioBase, FD5508, Korea) until reaching a constant weight. The FW and DW were expressed in g per plant. The %DW was calculated as a quotient between DW and FW, expressed as g of DW in 100 g of FW (25).

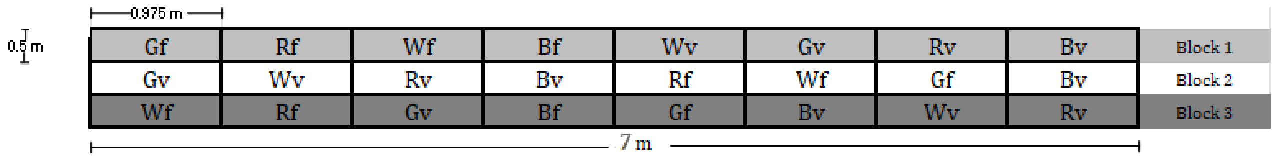
Antioxidant parameters

Initially, 1 g of frozen leaves obtained from a sample of 5 random plants per repetition were extracted with methanol 70% (v/v) following the adapted method proposed by Swain and Hills (1059). The sample was crushed in Ultraturrax (IKA, T18 basic, USA) until a uniform consistency (approximately 30 s) and centrifuged (HERMLE Labortechnik, Z326K, Germany) for 15 min at 6037 gN. The supernatant liquid was filtered through a 0.45 µm PVDF filters and stored at -20 ° C in a horizontal freezer (Electrolux, EC305ZBGW / 220/50, Sweden) for 1 week until the analysis. Total phenolic content (TPC) were measured using the method proposed by Singleton and Rossi (1965), with the Folin-Ciocalteu method. The results were expressed as µg of gallic acid equivalent (GAE) g^{-1} of fresh weight (FW).

The antioxidant capacity was measured using the DPPH (1, 1 – diphenyl 2 – picrylhydrazyl) and FRAP (ferric reducing antioxidant power) measurement protocols. The DPPH assay was performed using the method proposed by Brand-Williams *et al.* (1995), while the FRAP assay followed the method proposed by Benzie and Strain (1996). The results were expressed as μg Trolox equivalent (TE) g^{-1} of fresh weight (FW).

Statistical analysis

A completely random block design was produced with a factorial structure of 4 x 2 with three repetitions, where the first factor was the spectrum of LED light employed in the light supplementation, which had 4 levels: W, B, G and R. The second factor corresponded to the spinach cultivar: Falcon F1 and Viroflay (figure 2).



B: blue, W: white, R: red, G: green, f: Falcon F1, and v: Viroflay.

B: azul, W: blanco, R: rojo, G: verde, f: Falcon F1, v: Viroflay.

Figure 2. Scheme of the experimental design used for light supplementation with blue (468 nm), red (629 nm), green (524 nm) and white LED lights, with a light intensity of 26 μmol m^{-2} s^{-1} of PAR, on two cultivars of spinach for 26 days.

Figura 2. Esquema del diseño experimental usado para la suplementación lumínica con luces LED de espectro azul (468 nm), rojo (629 nm), verde (524 nm) y blanco, con una intensidad lumínica de 26 μmol m^{-2} s^{-1} de PAR, sobre dos cultivares de espinaca durante 26 días.

An analysis of variance (ANOVA) was carried out and Tukey's multiple comparison test was done in order to find differences among treatments. All the analyses were done with a 5% significance level. The statistical analyses were performed with the InfoStat statistics software (9).

Results

Physical parameters

The leaves of two spinach cultivars treated by W, B, G and R- lights showed no visible damage and did not affect the hue, C or L color parameters as described in table 1.

Table 1. Hue, chroma (C) and lightness (L) of the leaves from two spinach cultivars (Falcon F1 and Viroflay) cultivated for baby leaves under white, blue (468 nm), green (524 nm) and red (629 nm) LED light supplementation for 26 days.

Tabla 1. Tono, croma (C) y luminosidad (L) de las hojas de los dos cultivares de espinaca (Falcon F1 y Viroflay) cultivadas para hojas baby bajo suplemento de luces LED blanca, azul (468 nm), verde (524 nm) y roja (629 nm) durante 26 días.

LED light spectrum	Hue		C		L	
	Falcon F1	Viroflay	Falcon F1	Viroflay	Falcon F1	Viroflay
W	109.59±1.78aA	110.01±1.32aA	40.66±3.04aA	38.77±1.99aA	25.14±3.69aA	22.23±1.29aA
B	109.85±1.72aA	109.57±1.40aA	40.17±2.34aA	40.66±3.04aA	24.84±3.05aA	23.38±2.65aA
G	111.34±0.57aA	108.31±0,99aA	37.74±0.38aA	41.86±2.49aA	21.82±0,70aA	23.09±2.73aA
R	109.84±1.75aA	110.09±1.56aA	40.32±2.82aA	39.82±1.46aA	24.79±3.39aA	22.82±1.50aA
Significance						
LED	NS	NS	NS	NS	NS	NS
Cv	NS	NS	NS	NS	NS	NS
LED x Cv	NS	NS	NS	NS	NS	NS

Values indicate means ± standard deviation (S.D.). W: White; B: blue; G: green; R: red. LED: LED light spectrum. Cv: Cultivar. Lowercase letters compared in the column and uppercase letters compared in the rows indicate significant differences between LED light spectrum and cultivars, respectively (p – value < 0.05). NS: not significant (p – value > 0.05).

Los valores indican las medias ± la desviación estándar (S.D.). W: blanco; B: azul; G: verde; R: rojo. LED: Luz LED. Cv: Cultivar. Letras minúsculas comparan en columnas y letras mayúsculas comparan en líneas indicando diferencias significativas entre espectros de luces LED y cultivares, respectivamente (valor p < 0,05). NS: no significativo (valor de p > 0,05).

According to the results obtained, cv. Viroflay had significantly longer and narrower leaves than cv. Falcon F1. Regardless of the cultivar, significant differences were observed in the length of the leaves irradiated with different spectrum of LED light. Thereby, plants irradiated with R-light obtained the longest leaves compared to the other treatments. Falcon F1 leaves grown under R-light had a length of 6.91 cm and those treated with W-light reached 6.33 cm. In the case of Viroflay, the leaves grown under R and W-lights had lengths of 7.68 and 7.03 cm, respectively. Regarding the maximum width, regardless of the cultivar, no significant differences were observed among leaves treated with different LED light spectrum (table 2).

Table 2. Length and width of the leaves from two spinach cultivars (Falcon F1 and Viroflay) cultivated for baby leaves, under white, blue (468 nm), green (524 nm) and red (629 nm) LED light supplementation for 26 days.

Tabla 2. Longitud y anchura de las hojas de espinaca de los dos cultivares (Falcon F1 y Viroflay) de hojas baby, bajo un suplemento de luces blanca, azul (468 nm), verde (524 nm) y roja (629 nm) durante 26 días.

LED light spectrum	Length (cm)		Width (cm)	
	Falcon F1	Viroflay	Falcon F1	Viroflay
W	6.33±0.15aA	7.03±0.15aB	3.55±0.28aB	2.69±0.03aA
B	6.42±0.22aA	6.81±0.58aB	3.44±0.18aB	2.64±0.37aA
G	6.21±0.39aA	7.38±0.34aB	3.35±0.16aB	2.68±0.01aA
R	6.91 ± 0.07bA	7.68 ± 0.31bB	3.54 ± 0.26aB	2.83 ± 0.37aA
Significance				
LED	*	*	NS	NS
Cv	*	*	*	*
LED x Cv	NS	NS	NS	NS

The values show means ± standard deviation (S.D.). W: White; B: blue; G: green; R: red. LED: LED light spectrum. Cv: Cultivar. Lowercase letters compared in the column and uppercase letters compared in the rows and indicate significant differences among LED light spectrum and cultivars, respectively. * p – value < 0.05 NS: not significant (p – value > 0.05).

Los valores muestran las medias ± la desviación estándar (S.D.). W: blanco; B: azul; G: verde; R: rojo. LED: Luz LED. Cv: Cultivar. Letras minúsculas comparan en columnas y letras mayúsculas comparan en líneas indicando diferencias significativas entre espectros de luces LED y cultivares, respectivamente. *valor p < 0,05. NS: no significativo (valor de p > 0,05).

Falcon F1 had significantly higher values of FW, DW and DW% than Viroflay. With respect to the LED light spectrum, regardless of the cultivar, plants irradiated with R-light reached significantly higher values of FW and DW than the other treatments, where FW was 2.59 g (Falcon F1) and 2.24 g (Viroflay) and DW was 0.25 g (Falcon F1) and 0.21 g (Viroflay) respect W-light, what FW was 2.04 g (Falcon F1) and 1.95 g (Viroflay) and DW was 0.19 g (Falcon F1) and 0.18 g (Viroflay). %DW showed no significant differences among the plants irradiated with different LED light spectrum (table 3, page 104).

Antioxidant parameters

A significantly higher total phenolic content (TPC) was found in Viroflay than Falcon F1. Regardless of the LED lights, the highest TPC was obtained in leaves treated by B and G- lights compared to the W treatments (table 4, page 104).

The highest antioxidant capacity was detected in Falcon F1 leaves by both the DPPH and FRAP protocols. In Falcon F1 and Viroflay, 925.8 and 886.1 μg TE g^{-1} FW were observed using the DPPH protocol, respectively. On the other hand, Falcon F1 and Viroflay showed 1092.3 and 989.1 μg TE g^{-1} FW using the FRAP protocol, respectively. Independent of the cultivar, leaves irradiated with B-light showed the highest antioxidant capacity compared to W, G and R- lights using both the DPPH and FRAP protocols (table 4, page 104). The DPPH protocol detected 998.8 and 949.5 μg TE g^{-1} FW in Falcon F1 and Viroflay treated with B-light, respectively. Using the FRAP protocol, the leaves treated with B-light showed 1234.9 and 1115.9 μg TE g^{-1} FW in Falcon F1 and Viroflay, respectively. In Falcon F1 leaves treated with W, G and R-lights, antioxidant capacity yielded 904.7, 879.3 and 920.6 μg TE g^{-1} FW using the DPPH protocol, respectively and 1070.3, 1041.9 and 1022.1 μg TE g^{-1} FW using the FRAP protocol, respectively. On the other hand, in Viroflay leaves antioxidant capacity was 856.3, 846.4 and 892.0 μg TE g^{-1} FW using the DPPH protocol respectively, and 987.2, 916.4 and 936.9 μg TE g^{-1} FW using the FRAP protocol, respectively.

Table 3. Fresh weight (FW), dry weight (DW) and dry weight percentage (%DW) of the leaves from two spinach cultivars (Falcon F1 and Viroflay) cultivated for baby leaves, under white, blue (468 nm), green (524 nm) and red (629 nm) LED light supplementation for 26 days.

Tabla 3. Peso fresco (FW), peso seco (DW) y porcentaje de peso seco (%DW) de las hojas de dos cultivares de espinaca (Falcon F1 y Viroflay) cultivados para hojas baby, bajo suplementos de luces LED blanca, azul (468 nm), verde (524 nm) y roja (629 nm) durante 26 días.

LED light spectrum	FW (g)		DW (g)		%DW (g DW 100 g^{-1} FW)	
	Falcon F1	Viroflay	Falcon F1	Viroflay	Falcon F1	Viroflay
W	2.04±0.13 aB	1.95±0.03 aA	0.19±0.01 aB	0.18±0.02 aA	9.54±0.38 aB	9.30±0.17 aA
B	1.93±0.03 aA	1.95±0.04 aA	0.18±0.01 aA	0.18±0.01 aA	9.55±0.32 aB	9.24±0.13 aA
G	2.17±0.17 aB	2.09±0.18 aA	0.21±0.0 2 aB	0.19±0.0 2aA	9.60±0.34 aB	9.30±0.10 aA
R	2.59±0.20 bB	2.24±0.14 bA	0.25±0.01 bB	0.21±0.01 bA	9.58±0.20 aB	9.36±0.11 aA
Significance						
LED	*	*	*	*	NS	NS
Cv	*	*	*	*	*	*
LED x Cv	NS	NS	NS	NS	NS	NS

The values show means ± standard deviation (S.D.). W: White; B: blue; G: green; R: red. LED: LED light spectrum. Cv: Cultivar. Lowercase letters compared in the column and uppercase letters compared in the rows and indicate significant differences among LED light spectrum and cultivars, respectively. * p - value < 0.05. NS: not significant (p - value > 0.05).

Los valores muestran las medias ± la desviación estándar (S.D.). W: blanco; B: azul; G: verde; R: rojo. LED: Luz LED. Cv: Cultivar. Letras minúsculas comparan en columnas y letras mayúsculas comparan en líneas indicando diferencias significativas entre espectros de luces LED y cultivares, respectivamente. *valor p <0,05. NS: no significativo (valor de p > 0,05).

Table 4. Total phenolic content and antioxidant capacity using the DPPH and FRAP protocols of the leaves from two spinach cultivars (Falcon F1 and Viroflay) cultivated for baby leaves, under white, blue (468 nm), green (524 nm) and red (629 nm) LED light supplementation for 26 days.

Tabla 4. Contenido de fenoles totales y capacidad antioxidante por los métodos DPPH y FRAP de las hojas de dos cultivares de espinaca (Falcon F1 y Viroflay) cultivados para hojas baby, bajo suplementos de luces LED blanca, azul (468 nm), verde (524 nm) y roja (629 nm) durante 26 días.

LED light spectrum	Total phenolic content (µg GAE g^{-1} FW)		DPPH (µg TE g^{-1} FW)		FRAP (µg TE g^{-1} FW)	
	Falcon F1	Viroflay	Falcon F1	Viroflay	Falcon F1	Viroflay
W	1019.4±44.2 aA	1054.1±70.72 aB	904.7±34.5 aB	856.3±38.0aA	1070.3±57.1aB	987.2±33.2aA
B	1245.8±61.3 bA	1483.4±205.8 bB	998.8±25.0bB	949.5±39.0bA	1234.9±58.7bB	1115.9±65.8bA
G	1263.4±86.4 bA	1244.0±42.1bA	879.3±33.1aB	846.4±27.8aA	1041.9±39.4 aB	916.4±40.7aA
R	1036.1±113.0 aA	1143.7±41.48 aB	920.6±58.1bB	892.0±34.5bA	1022.1±46.9 aB	936.9±54.8aA
Significance						
LED	*	*	*	*	*	*
Cv	*	*	*	*	*	*
LED x Cv	NS	NS	NS	NS	NS	NS

The values show means ± standard deviation (S.D.). W: White; B: blue; G: green; R: red. LED: LED light spectrum. Cv: Cultivar. Lowercase letters compared in the column and uppercase letters compared in the rows and indicate significant differences among LED light spectrum and cultivars, respectively. * p – value < 0.05. NS: not significant (p – value > 0.05).

Los valores muestran las medias ± la desviación estándar (S.D.). W: blanco; B: azul; G: verde; R: rojo. LED: Luz LED. Cv: Cultivar. Letras minúsculas comparan en columnas y letras mayúsculas comparan en líneas indicando diferencias significativas entre espectros de luces LED y cultivares, respectivamente. *valor p <0,05. NS: no significativo (valor de p > 0,05).

Discussion

Physical parameters

According to Tadeo and Gómez-Cadenas (2008), the stress caused in plants by excessive radiation occurs when the light collector antennae of the photosystems absorb more light energy than they can use in photosynthesis. Light stress initially produces photoinhibition, which prevents oxidative damage of the photosynthetic apparatus by the generation of ROS. Should this process continue, it can trigger a lower dry matter accumulation and ultimately producing the wilting of the leaves. On the other hand, low radiation causes lengthening of the internodes, thinner stems, and wider and thinner leaves with little root development.

In case of almost no illumination or darkness, etiolation is provoked in the vegetal tissues (12). These results, along with those of Bula *et al*. (1991) and Lin *et al.* (2013) in lettuces, are consistent with our results, where spinach leaves were not damaged by this low radiation intensity supplementation. Additionally, Olle and Viršilé (2013) mentioned that LED light supplementation did not produce damage associated with high temperature.

From the results of the present study, none LED lights had a significant effect on color leaves. According to previous studies, high radiation can disrupt the photosynthetic apparatus, generating a mild yellowing coloration (44). Conversely, suboptimal levels of radiation cause losses of chlorophyll and a whitening coloration that could affect the color of the plants (12). Therefore, it seems that the increase in light intensity used in this work has not been sufficient to produce a photoinhibition of the photosynthetic apparatus and changes in the green color of the spinach leaves for both cultivars. These results are consistent with those of Lin *et al.* (2013) in lettuce, where they found similar contents of photosynthetic pigments such as chlorophyll a, b, a/b and carotenoids under different light treatments. Conversely, Son and Oh (2015) found changes in total chlorophyll concentration of lettuce cultivated under R- light with a PPFD of 173 ± 3 µmol m^{-2} s^{-1} for 4 weeks, which caused changes in leaf color due to a greater pigment synthesis. This result could confirm that LED supplementation with a PPFD of 26 µmol m^{-2} s^{-1} does not have a significant effect on the color parameters hue, C or L, because it does not induce a greater synthesis of pigments or stress capable of generating color changes in spinach leaves.

The effect of R-light on spinach leaves could be related to a high energy efficiency for a photosynthesis process from 600 to 700 nm (36). The results obtained are in agreement with those recorded by Johkan *et al.* (2010) and Borowski *et al.* (2015), who showed a significant increase in size of red and green lettuce leaves exposed to R- light compared to W and B-lights. Additionally, the same authors found no significant size differences between leaves treated with W and B-lights, following a similar trend to the one observed in our study in spinach leaves irradiated with B and W-lights. These results were contrary to those reported by Yanagi *et al.* (1996), where shorter lettuce leaves were reached under B-light (PPFD of 85 and 170 µmol m^{-2} s^{-1}) than W-light. In previous studies, plants irradiated with R- light demonstrated a greater growth of leaves in species like dill (14), potato (29) and lettuce (5, 17). R-light is considered an efficient energy source for plant photosynthesis (13, 35, 36). On the other hand, B-light causes the saturation of the photosynthetic apparatus, generating less accumulation of dry matter and less length. However, in our study a low PPFD supplementation (26 µmol m^{-2} s^{-1}) was used, and leaves were smaller than those treated with W- light. On the other hand, G- light supplementation at high intensity (300 µmol m^{-2} s^{-1}) promoted the growth and development of lettuce (23). Son and Oh (2015) also indicated that G-light generates an increase in the length of lettuce leaves compared to B and W-lights.

Previous studies have shown a significant increase in length and biomass of plants cultivated under monochromatic R- light (17, 22). This is in accordance with our work, in which R- light increased fresh and dry weights and length of the leaves. Using a PPFD of 130 ± 7 µmol m^{-2} s^{-1} for 23 days (photoperiod of 12 h), a significant increase in FW and DW of lettuce treated with R- light was observed compared to those exposed to B, G or W- lights (40). This may be because wavelengths between 600 and 700 nm (R- light) generate a higher level of photosynthesis per absorbed energy unit in plants, promoting greater vegetative growth (35, 36). On the other hand, plants exposed to wavelengths between 500 and 600 nm (G- light) reflect a large proportion of the G- light spectrum, thus becoming a spectrum of light that by itself could not sustain plant growth (38), which is also in agreement with the results obtained in this work. A combination of the G- light with another spectrum or alone as supplementary light, however, could have a positive effect on plants due to its photosynthetic efficiency per absorbed energy unit is similar to R- light (45). Additionally, PPFD of G- light higher than 100 µmol m^{-2} s^{-1} could increase FW and DW accumulation (23). The wavelengths of 400 to 500 nm had less photosynthetic efficiency per absorbed energy unit than 500 to 700 nm (36). Accordingly, higher PPFD must be used in plants treated with B-light to have the same photosynthesis values as R-light due to B-light being less efficient at increasing biomass. Son and Oh (2013) used combinations of R and B- lights with a PPFD of 173 ± 3 µmol m^{-2} s^{-1} for 4 weeks. Their results found that to achieve a greater biomass accumulation in lettuce, monochromatic B and R-lights must be used. Additionally, they found that a high R-light proportion favored higher FW and DW accumulation compared to B-light proportion. All these results are consistent with our study, and the highest biomass accumulation recorded in plants treated with R-light compared to B and W-lights.

Chemical parameters

The effect of the light treatments on spinach leaves were consistent with those described by Qian *et al.* (2016), who like us found an increase in TPC of cauliflower treated with B-light compared to those exposed to W-light. In similar conditions, Liu *et al.* (2016) found a significant increase in TPC of pea seeds under B-light compared to those treated with W-light. Lee *et al.* (2010) also found a significant effect of B-light on TPC in barley leaf compared to W-light. In this work, B and G-lights increased TPC in spinach leaves compared to those treated with W-light. In the case of B-light, probably was due to the capacity of this spectrum to stress plants due to its high energy content, which induces polyphenolic compound synthesis (10). The increase in TPC in plants is associated with the synthesis of the PAL enzyme, a key factor in the secondary metabolite biosynthesis pathway (40). In addition, other enzymes such as 3-deoxy-D-arabino-heptulosonate 7-phospate synthase (DS) and chorismate mutase (CM) can activate the synthesis of enzymes in this pathway (48). Plants produce a higher biosynthesis of the PAL enzyme in the presence of stress like B-light irradiation, which could be capable of saturating the photosynthetic apparatus due to its high energy content (2). Consequently, the PPFD of 26 μmol m^{-2} s^{-1} used in our study would enough to stimulate the phenolic synthesis without altering the visual appearance of the leaves. Regarding the G-light, there is no certainty of the role that it plays in biomass production such as antioxidant compound synthesis like phenols, because previous studies have noted that the green spectrum could be completely reflected by leaves. Nevertheless, studies carried out on lettuce have demonstrated that G-light could play an important role in phenol synthesis and vegetative growth when this spectrum is used as a supplementary light or with PPFD higher than 100 μmol m^{-2} s^{-1} (23). Furthermore, Fazal *et al.* (2016) found that G- light caused an increase in total phenolics and flavonoids production in callus cultures of *Prunella vulgaris* L. compared to the control. These conflicting results by different researchers might be due to differences in light sources, intensities and vary according to species (11). Moreover, previous studies have confirmed that R-light reduces TPC in vegetables like lettuce in comparison with those treated with W-light (22). In the present experiment, plants exposed to R-light did not present significant differences with those treated with W-light, because its energy content was not capable of saturating the photosynthetic apparatus by the PPFD used (38).

In previous studies, a significant increase in antioxidant capacity was found in plants exposed to B-light compared to R- light (24, 32, 41). In the present study, we also found a significant effect of B-light on the antioxidant capacity of spinach leaves; however, R-light had no significant impact compared to W-light, which could indicate that a PPFD of 26 μmol m^{-2} s^{-1} was not enough to cause significant changes in antioxidant capacity. Also, spinach leaves treated with G-light showed an antioxidant capacity similar to those exposed to W-light. According to other studies, there is no clarity regarding of the effect of G-light on the antioxidant capacity of plants. Broccoli stored under G-light (520 nm) with a PPFD of 12 – 13 μmol m^{-2} s^{-1} showed a significantly higher antioxidant capacity than W-light (21). It has also been reported that G-light could cause a positive effect in biomass accumulation when it was used as a supplementary light; however, there is no clear evidence that G-light can induce a high synthesis of antioxidant compounds in vegetables (45, 46).

Conclusion

R-light stimulates biomass production and B-light increases total phenolic content and antioxidant capacity in spinach leaves. On the other hand, G-light increase total phenolic compound concentration in comparison with W-light treatment. LED light supplementation could benefit both, the yield and antioxidant properties of spinach plants, without affecting their visual and color characteristics. Thus, the possibility of increasing the PPFD and exposure time remains open, making use of different spectrum to optimize yield and antioxidant compound accumulation in leafy vegetables like spinach.

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Soil compaction response to wheel traffic in the Rolling Pampas region of Argentina

Respuesta de la compactación del suelo al tráfico de ruedas en la Región de la Pampa ondulada de Argentina

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Abstract

The present work shows the effects of the different agricultural wheels traffic on the physical properties of a typical Argiudol soil worked under a no-tillage cropping system. Soil compaction produced by traffic was quantified through a series of parameters. These parameters were: a) cone index, b) rut depth and c) soil water content at the traffic moment. A grain chaser, a sprayer, a combine harvester and a tractor equipped with commonly used wheels were tested in the study area. The main results obtained showed that the tyres with the highest inflation pressure and tyre ground pressures produced the highest values of cone index and rut depth. A typical Argiudol soilis not able to constrain topsoil and subsoil compaction when wheeled by tyres with ground pressures greater than 77.6 kPa. This occurs when this soil is worked under a continuous no-tillage cropping system.

Keywords
tyre inflation pressure • cone index • soil bearing capacity

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Resumen

En el presente trabajo se muestran los efectos del tránsito de diferentes ruedas agrícolas sobre las propiedades físicas de un suelo Argiudol Típico trabajado bajo el sistema de no-labranza. La compactación del suelo producida por el tráfico se cuantificó a través de los parámetros: a) índice de cono, b) profundidad de huella y c) contenido de agua del suelo al momento del tránsito. Se ensayaron carro de granos, cosechadora, pulverizadora y tractor equipados con rodados de uso generalizado en la zona productiva bajo estudio. Los principales resultados obtenidos demostraron que los neumáticos con mayor presión de inflado y presión en el área de contacto rueda/suelo produjeron los mayores valores de índice de cono y profundidad de huella. El suelo Argiudol típico trabajado en forma continua bajo no-labranza no puede limitar la compactación superficial y subsuperficial del suelo cuando es transitado por ruedas con presiones en el área de contacto rueda/suelo mayores a 77.6 kPa.

Palabras clave
presión de inflado • índice de cono • capacidad portante del suelo

Introduction

According to the European Soil Framework Directive (2006), compaction, in addition to water and wind erosion, is one of the main causes of soil degradation. It has been estimated that more than half of the world's eroded area is caused by soil compaction and soil deformation. As a matter of fact, soil compaction and soil deformation are produced by incorrect soil management. Traffic compaction has adverse effects on the physical, chemical and biological properties of soil. This affects important soil processes and functions that govern the crop productivity (2).

Soil compaction causes a reduction in root growth and yield in many crops. Botta *et al.* (2004) applied 4 Mg tractor traffic in a field where a wheat-soybean double cropping rotation under no-tillage had been practised for seven years. Traffic was applied at intensities of 60 to 180 Mg km ha^{-1}. This treatment caused soil compaction up to 600 mm depth. As a result, there was a decrease in yield of the following soybean crop from 9.8% to 38%, respectively. Besides, Canarache *et al*. (1984) found that for each 1 kg/m^3 increase in bulk density in Romanian soils, a decrease in maize grain yields was 18% relative to the yield in a non-compacted plot.

According to Raper (2005), soil deterioration produced by agricultural traffic can sometimes be visible above-ground as soil deformation or it can be hidden below-ground. In either case, agricultural traffic can reduce crop production by causing a compacted soil condition that is not compatible with plant growth. Traffic-induced compaction in the subsoil (below 200 mm in our case) tends to be cumulative. This is because standard tillage operations are rarely performed at depths greater than about 25-30 cm (6, 17).

Compaction is caused by the high wheel loads and tyre ground pressures exerted on the soil by the tires of machinery used in no-tillage crop operations. Special emphasis should be placed on the impact of these operations when performed on wet clay soils or with high tire inflation pressure (between 140 and 218 kPa) (10).

It is difficult to generalise, globally, however there is a growing knowledge base that random traffic operations with heavy machinery on moist soil causes soil compaction specifically, and more generally, reductions in water use and fertiliser efficiencies and crop productivity, and off-site environmental problems particularly from increased runoff (26).

Threadgill (1982) noted that soils with a CI >1.5 MPa reduced root growth. In this regard, when soils are compacted with CI values higher than 2 MPa, the roots of most annual crops practically stop growing (7).

There are a number of techniques commonly utilized for the control and management of topsoil and subsoil compaction. These techniques are subsoiling and chiseling, controlled traffic farming (CTF), seasonal controlled traffic farming (SCTF) and axle load reduction (2). Deep soil compaction remediation can prove impractical, and often uneconomical, at depths greater than about 400-mm (9, 15, 18, 24, 25).

Note that permanent traffic lanes represent full adoption of the CTF. On the other hand, the SCTF refers to temporary tracks, where affected areas may be targeted for post-harvest remediation. Regarding the SCTF, Vermeulen and Mosquera (2009) found that the mean total and air-filled porosity at 10 kPa of topsoil water matric pressure increased on average from 0.468 to 0.492 and from 0.132 to 0.166, respectively. This was observed in comparison to the random traffic system for crops grown in a field study between 2002 and 2005. They also found that crop yields increased significantly by 31% in green peas in 2002, by 15% in spinach in 2004 and by 10% in planted onion sets in 2005. On the other hand, no differences were observed in carrot and sown onion when the SCTF system was used instead of the random traffic system.

The work presents two objectives. The first objective was to evaluate the effect of wheel traffic on the soil physical properties of a typical Argiudol soil in the Rolling Pampas region of Argentina. The second objective was to study the traffic layout in different agricultural tasks to reduce compaction in a soil under a continuous no-tillage cropping system. Our hypothesis was that, in a soil under a continuous no-tillage cropping system, compaction mitigation may be achieved by operating with low-loads, low contact pressure from tyres and reducing the trampled area.

Materials and methods

The site

The experiment was conducted in the east of the Rolling Pampa region, Buenos Aires State, Argentina. The soil is a typical Argiudol (22) worked under a no-tillage system. The soil physical and mechanical properties are given in table 1.

Table 1. Typical Argiudol soil profile characteristics.

Tabla 1. Características del perfil del suelo Argiudol típico.

	Ap	A12	B1	B21t	B22t	B23t	B3	Cca
Depth (mm)	5-10	16-25	25 -35	35 - 55	55 - 80	80 - 110	110-150	150 - 220
Organic Carbon (%)	2.05	1.44	0.95	0.61	0.55	0.32	0.20	0.11
Total nitrogen (%)	0.23	0.132	0.102	0.081	0.072	0.053	0.031	-----------
C/N ratio	8.9	10	9	8	8	6	6	-----------
Clay (<2 μ)	20.1	24.8	27.9	34.2	46.4	32.0	22.0	14.9
Silt (2-20 μ)	33.1	34.6	29.5	28.1	20.7	30.0	31.8	29.9
Silt (2-50μ)	75.6	70.8	67.2	61.3	50.0	63.0	72.7	79.9
Fine sand (100-250t μ)	0.3	0.2	0.3	0.4	0.4	0.4	0.5	0,4
Equvalent moisture (%)	26.6	28.5	26.8	28.7	35.2	31.9	27.0	23.5
pH	5.4	5.3	5.5	5.5	5.8	6.0	6.0	7.5
pH in H20 (1: 2.5)	5.8	5.8	6.0	6.2	6.5	6.4	6.4	7.9
Cation exchange (m.e. 100g)								
Ca^{2+}	11.4	12.7	12.0	13.8	18.3	17.2	16.5	-----------
Mg^{2+}	2.9	2.5	3.1	4.5	6.5	6.4	3.8	-----------
Na^{+}	0.2	0.1	0.2	01	0.2	0.2	0.3	0.5
K^{+}	1.4	1.0	0.9	1.3	2.3	2.4	2.3	2.4

Treatments

We performed five treatments. One of these treatments was used as a control with no traffic. The remaining four treatments were carried out with machines that had different tyre ground pressures. The modification of each treatment is determined by the agricultural machine and the tyre to be used respectively. Description of the machinery used is given in table 2 (page 112). The real work speed was calculated with the distance / time equation for each labour. The time the equipment took to travel 75 m of each plot was recorded.

Table 2. Characteristics of the machinery used in the trial.
Tabla 2. Características de la maquinaria usada en el ensayo.

Tractor FWA		Grain chaser (Two axle and single wheels)	
Engine power (CV/kW)	145/106.7	Front tyres	24.5 R 32
Front tyres	16.9 R 26	Rear tyres	24.5 R 32
Rear tyres	24.5 R 32	Inflation pressure, front tyre (kPa)	120
Inflation pressure, front tyre (kPa)	70	Inflation pressure, rear tyre (kPa)	120
Inflation pressure, rear tyre (kPa)	65	Overall weight loaded (kN)	196
Overall weight (kN)	79.80	Front weight (kN)	98
Front weight (kN)	31.75	Rear weight (kN)	98
Rear weight (kN)	48.05	Static load per front wheel (kN)	49
Static load per front wheel (kN)	15.875	Static load per rear wheel (kN)	49
Static load per rear wheel (kN)	24.025	Mean ground pressure per for front tyre (kPa)	77.6
Mean ground pressure per for front tyre (kPa)	37.63	Mean ground pressure per rear tyre (kPa)	78.7
Mean ground pressure per rear tyre (kPa)	40.02		
Distance between the tyres of the tractor and tyres of the grain chaser (mm)	3.380		
Combine Harvester		**Sprayer**	
Engine power (CV/kW)	255/187	Engine power (CV/kW)	142/104
Front tyres	600/70 R 30	Front tyres	12.4-36
Front tyres inflation pressure (kPa)	140	Front tyres inflation pressure (kPa)	285
Rear tyres	11.25 – 24	Rear tyres	12.4-36
Rear tyres inflation pressure (kPa)	145	Rear tyres inflation pressure (kPa)	285
Total weight loaded (kN)	152.5	Total weight loaded (kN)	108.7
Front axle weight (kN)	103.7	Front axle weight (kN)	43.48
Rear axle weight (kN)	48.8	Rear axle weight (kN)	65.22
Static load per front wheel (kN)	51.85	Static load per front wheel (kN)	21.74
Static load per rear wheel (kN)	24.4	Static load per rear wheel (kN)	32.61
Mean ground pressure per front tyre kPa)	135	Mean ground pressure per front tyre kPa)	233
Mean ground pressure per rear tyre (kPa)	198	Mean ground pressure per rear tyre (kPa)	254
Hedaer width (m)	7	Sprayer boom width (m)	21

The treatments were settled in plots of 100 m long by 20 m wide (2000 m^2), in four completely randomized replications plots (figure 1, page 113). A buffer zone of 10 m between plots was established according to the proposal of Maineri (2020).

The mean ground contact pressure (GCP) of the machines was measured with a Tekscan device. Tyre inflation pressures were adjusted according to the tyre manufacturers' recommendations for the load and the operation speed. Note that the soil water content (SWC) at the traffic moment was near but below the field capacity.

Parameters monitored

Cone index (CI), soil water content (SWC) and rut depth (RD) were measured on the same day as the traffic treatments were applied. The parameters (CI, SWC and RD) were measured along the central 50 m of each plot. The CI was measured with a recording penetrometer (4) and the procedure according to ASABE (2013). The average of 25 samples was taken as the datum for each plot at a depth range of 0–450 mm, measured at 25 mm depth intervals. The SWC was estimated according to Botta *et al*. (2002). The RD was measured using a profile meter consisting of a set of vertical metal rods of 700 mm long and 5 mm in diameter. These rods were spaced at 25 mm horizontal intervals, sliding through holes in a 1-m long iron bar. The bar was placed across the removed soil perpendicular to the direction of travel and the rods were positioned to conform to the shape of the depression. The removed area was calculated as the average depth of 20 reads on the 1-m bar.

Explanatory variables

The trampled area (TA) by agricultural machinery was determined using a PCS-215 Pentax total station. Finally, the maximum bulk density (BD) and the critical soil water content (SWC) were determined according to the standard Proctor method (5) described by Botta *et al.* (2012).

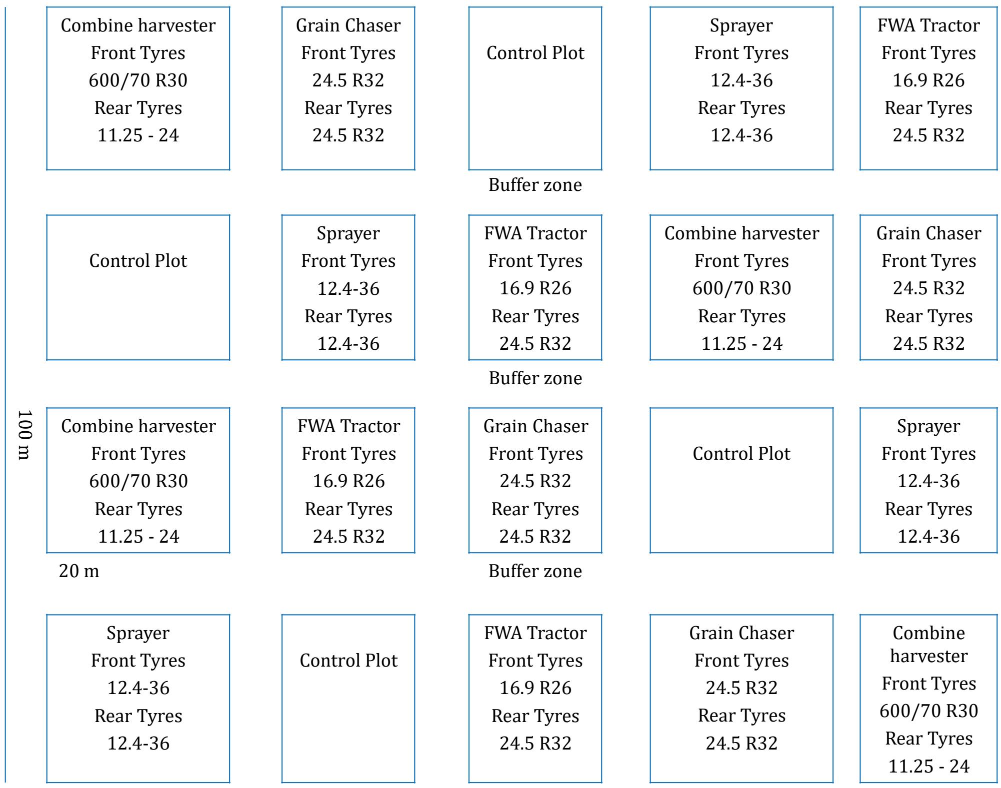

Figure 1. Experimental design. / **Figura 1.** Diseño experimental.

Statistical analyses

Statistical analyses were performed by the Statgraf program 7.1. An analysis of variance (ANOVA) was carried out and means were analyzed by Duncan's multiple range test. A priori we confirmed that the soil data followed a normal distribution, according to the Shapiro-Wilk test. When checking the normality of the deviations of each data with respect to the average of the respective treatment, the normality of the same was assumed (13).

Results and discussion

Soil water content and Cone index

The differences in the soil water content (SWC) were generally not significant between the different depth intervals on the day the traffic treatments were imposed on each sample (table 3). Therefore, the variations in CI in depth were not due to the SWC. This suggested that the cone index was a reliable indicator of the soil compaction degree as a function of the traffic treatment.

Table 3. Soil water content (w/w) at the traffic moment.

Tabla 3. Contenido de agua del suelo (m/m) al momento del tráfico.

Soil water content (w/w)	
Depth range levels (mm)	
0-150	21.1 ± 1.27 a
150-300	22.3 ± 1.24 a
300-450	22.9 ± 1.33 a

Average values ± standard deviation (n = 25).
Values with different letters are significantly different at each depth (P<0.01 Duncan's multiple range test).

Valores medios ± desviación estándar (n = 25).
Los valores con letras diferentes son significativamente diferentes para cada profundidad (P <0,01 Prueba de rango múltiple de Duncan).

According to table 3 (page 113), the SWC at the traffic moment was 21.1% in the topsoil (0–150 mm), 22.3% at 150–300 mm and 22.9% at 300– 450 mm. According to this, the SWC values at the time of testing were 0.9 points lower than the Proctor value (22.0%) in the 0 to 150 mm depth range. The SWC values were also 1.6 points lower than the Proctor value (23.9%) in the 150 to 300 mm depth range and 0.5 points minor (23.4%) in the 300 to 450 mm depth range respectively. From the mentioned Proctor values, it can be inferred that the SWC, at the traffic moment, was close to the value that can produce maximum soil compaction. This situation is very important when analyzing the compaction results due to traffic because it was the worst moment to carry out the traffic. However, these are normal values of soil water content when the farmers perform most of the agricultural work (7).

It is important to note that the typical tillage depths in Argentina are approximately 200 mm. Therefore, the Ap horizon is considered, in this experiment, 0–200 mm as the topsoil layer. The subsoil can be defined as the soil below the tillage layer (6).

The cone index data gave a clear indication of the initial soil condition in each treatment (table 4). The CI values reached their maximum at 200 mm depth (≈2653 kPa) in the topsoil of the control plot. On the other hand, in the subsoil the maximum CI value was found at 450 mm depth (≈3247 kPa).

Table 4. Average (n = 25) cone index values (kPa) for the traffic treatments.

Tabla 4. Valores medios (n = 25) de índice de cono (kPa) para los tratamientos de tráfico.

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).

Depth (mm)	Control plot (unloosened soil)	FWA Tractor	Grain chaser	Combine harvester	Sprayer
Topsoil (0 to 200 mm)					
0	1507 a	1723 b	1888 c	2200 d	2433 e
25	1682 a	1801 b	1980 c	2256 d	2525 e
50	1790 a	1904 b	2150 c	2334 d	2622 e
75	1880 a	2000 b	2187 c	2369 d	2740 e
100	2132 a	2250 b	2530 c	2698 d	2888 e
125	2167 a	2360 b	2563 c	2793 d	2951 e
150	2250 a	2450 b	2610 c	2804 d	2983 e
175	2369 a	2556 b	2690 c	2841 d	2990 e
200	2653 a	2700 a	2802 c	2959 d	3190 e
Subsoil (> 200 mm)					
225	2800 a	2900 a	3400 c	3234 b	3200 b
250	2901 a	2910 a	3457 c	3272 b	3196 b
275	2920 a	2940 a	3560 c	3230 b	3180 b
300	2956 a	2990 a	3601 c	3241 b	3200 b
325	2980 a	3010 a	3656 c	3287 b	3210 b
350	3001 a	3078 a	3678 c	3352 b	3265 b
375	3100 a	3140 a	3689 c	3390 b	3300 b
400	3145 a	3195 a	3699 c	3410 b	3309 b
425	3201 a	3233 a	3710 c	3429 b	3321 b
450	3247 a	3276 a	3745 c	3503 b	3400 b

These CI values in the control plot could tend to retard root growth in situations of low soil water content. However, there could also be an improvement in the soil bearing capacity that could moderate, together with the crop harvest residue on the soil surface, the negative effects of the agricultural traffic.

Besides, the treatments with the highest average ground pressure (combine harvester (> 135 kPa) and sprayer (> 233)) caused CI values exceeding 2000 kPa from the 0 mm depth level. Botta *et al.* (2018b) suggested that this value is a limitation, not only of the seed emergence, but also for the root development. It is important to mention that Threadgill (1982) indicates that CI values above 1500 kPa decrease root development, while CI values of 2100 to 2500 kPa can stop root growth.

For these treatments (the combine harvester and the sprayer), the CI values were higher than 2500 KPa in the subsoil, (300 mm to 450 mm), denoting over-compaction. Also for these treatments, the CI exceeded the critical soil strength values, above which root growth and expansion are significantly affected (*e.g.,* 11, 14, 18, 19).

According to Botta *et al.* (2019), subsoil compaction is due to several factors. These factors are the high wheel load, the tyre ground pressure, and the machinery traffic intensity used for crop protection and harvest operations, rather than for seeding. Special emphasis should be placed on the impact of these operations when performed on wet clayey soils or with high tyres inflation pressure (between 140 and 218 kPa).The induced soil compaction within this layer is cumulative, since no conventional tillage is performed at that depth.

Finally, the FWA tractor showed significant differences (in CI values) with respect to the control plot up to a depth of 175 mm (P<0.01). This indicated an increased subsoil carrying capacity compared to topsoil.

The rut depth measurements were significantly different for the sprayer machine and the combine harvester compared to the FWA tractor and the grain chaser (P<0.01). The sprayer and the combine harvester showed significantly higher RD values than the FWA tractor and the grain chaser. This was due to the high tyre ground pressure, being 23.7 and 20.1 mm for the sprayer and the combine harvester respectively (table 5). It should be noted that, despite the high tyre ground pressures values, the RD did not exceed 25 mm in any treatment. This probably occurred because that CI in the control plot already exceeded 2132 kPa at 100 mm depth. This indicated the high level of previous compaction it had due to the application of a continuous no-tillage cropping.

Table 5. Average values (n = 20) of rut depth (mm) in the four traffic treatments.

Tabla 5. Valores medios (n = 20) de profundidad de huella (mm) en los cuatro tratamientos de tráfico.

Sprayer	Combine Harvester	FWA Tractor	Grain Chaser
23.7 a	20.1 a	12.1 b	15.7 c

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).

As for the RD, Table 5 shows that the sprayer rut depth is 12.98% greater than the combine harvester rut depth. This should be taken into account even though there are no significant differences between the combine harvester rut depth and the sprayer rut depth. The results are in agreement with the results of Botta *et al*. (2019) and Raper (2005). These autors also indicated that the soil surface was the most vulnerable layer to both compression and displacement from the passage of the agricultural machinery.

In addition, there was a clear correlation (statistically significant) between RD and soil compaction (R^2 values were between 0.90 and 0.96 for CI) in the deeper subsoil (200 to 450 mm) for the combine harvester. This correlation was not clear (R^2 values were between 0.04 and 0.07 for CI) [P <0.01]) in the case of the grain chaser. In this treatment, the mean values of GCP per tyre and wheel load did not exceed 78.7 kPa and 49 kN, respectively.

The measurements of TA ($m^2\ ha^{-1}$) were significantly different for all traffic treatments (table 6, page 116). The smallest TA corresponds to the sprayer. This is easy to understand due to the wide case of the tyre that these kinds of machine use. However, despite this, it is important to remember that the tyre ground pressure RD was the highest for this machine. The average RD value was 23.7 mm. From this it can be noted that the tyre ground pressure exceeded 200 kPa in both axes.

Table 6. Average values (n = 20) of trampled area (m^2 ha^{-1}) in the four traffic treatments.
Tabla 6. Valores medios (n = 20) de área pisada (m^2 ha^{-1}) en los cuatro tratamientos de tráfico.

Sprayer	Combine Harvester	FWA Tractor *	Tractor and Grain Chaser
300.1 a	1713.6 c	1777.3 d	373.3 b

Values with different letters (horizontally) are significantly different at each depth (P<0.01) Duncan's multiple range test). * FWA Tractor and planter wheeled on the same track.
Los valores con letras diferentes (horizontalmente) son significativamente diferentes para cada profundidad (P <0,01 Prueba de rango múltiple de Duncan).

In the case of the FWA tractor, it should be noted that it produced a high trampled area per hectare. The value shown in table 6 seems high. Nevertheless, the trampled area produced by this tractor when it traffics on the same track as the grain planter caused the trampled area to be masked in that of the grain planter. This is very important for the annual traffic planning, as well as the track alignment of the machinery used as far as possible.

Finally, it was shown that when the machinery load increases on soils with high bearing capacity (soils under a long-term no-tillage system), the subsoil compaction problems increase. Hence, these data support the hypothesis. This hypothesis includes: In soils under a continuous no-tillage system, compaction mitigation may be achieved by operating with low-loads, low contact pressure from tyres and reducing the trampled area.

Conclusions

Given the experimental conditions of this study, the following conclusions can be drawn:

1) The area trampled by the agricultural machinery can be reduced by making a previous planning of the traffic as well as an adequate regulation of the wheel track width. In addition, track alignment of the agricultural machines can alleviate the compaction produced by the passage of the wheels with high load.

2) Soil under a no-tillage system does not limit topsoil and subsoil compaction when wheeled by tyres with ground pressures greater than 77.6 kPa.

3) Also, in relation to the machinery weight, it was established that agricultural machinery with a minimum weight of 79.8 kN (FWA tractor) can produce subsoil compaction with a single pass.

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Grain quality of common bean (*Phaseolus vulgaris* L.) cultivars under low and high nitrogen dose

Calidad de grano de los cultivares de frijol (*Phaseolus vulgaris* L.) común sobre baja y alta dosis de nitrógeno

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Abstract

This study aimed to evaluate the effects of low and high topdressing doses of nitrogen (N) on the qualitative attributes of common beans (*Phaseolus vulgaris* L.) cultivars and indicate cultivars with better grain technological characteristics. The experimental design was randomized blocks in split plots, with four replicates. Plots comprised 16 common bean cultivars of the commercial grain 'Carioca', while subplots comprised two N doses: 20 and 120 kg ha^{-1} applied as topdressing. The following evaluations were carried out: sieve yield (SY), relative grain production on sieves (RGPS), crude protein content (PROT), final water volume absorbed (FVabs), time for maximum hydration (TMH), hydration ratio (HR), cooking time (CT) and resistance to cooking (RC). The results were subjected to analysis of variance by F test and means were grouped by the Scott-Knott test. Multivariate principal component analysis was used to identify processes. Grain quality of common bean resulted to be more dependent on the genotype studied than on the agricultural management, such as nitrogen fertilization. Increasing N dose applied as topdressing increased grain size and protein content, but had little influence on grain hydration. FVabs and CT depended on the interaction between cultivar and N doses. Although low value, CT had an inverse correlation with PROT. The cultivars BRSMG Uai, IAC Alvorada, TAA Dama and TAA Bola Cheia showed the best grain quality characteristics.

Keywords
nitrogen fertilization • genotypes • grain technology • cooking time • protein content

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Resumen

Este estudio tuvo como objetivo evaluar los efectos de la baja y alta dosis de nitrógeno (N) en cobertura sobre los atributos cualitativos de los cultivares de frijol común (*Phaseolus vulgaris* L.) e indicar los cultivares con mejores características tecnológicas de grano. El diseño experimental fue bloques al azar en parcelas divididas, con cuatro repeticiones. Las parcelas comprendían 16 cultivares de frijol común del grano comercial "Carioca", mientras que las subparcelas comprendían dos dosis de N: 20 y 120 kg ha^{-1} aplicados como cobertura. Se llevaron a cabo las siguientes evaluaciones: rendimiento del tamiz (SY), producción relativa de granos en tamices (RGPS), contenido de proteína cruda (PROT), volumen de agua final absorbido (FVabs), tiempo para la hidratación máxima (TMH), relación de hidratación (HR), tiempo de cocción (CT) y resistencia a la cocción (RC). Los resultados se sometieron a análisis de varianza mediante la prueba F y las medias se agruparon mediante la prueba de Scott-Knott. El análisis de componentes principales multivariante se utilizó para identificar procesos. La calidad del grano del frijol común depende más del genotipo estudiado que del manejo agrícola como la fertilización nitrogenada. El aumento en la dosis de N aplicada como topdressing aumenta el tamaño de los granos de frijol comunes y su contenido de proteínas, pero con poca influencia en la hidratación del grano. FVabs y CT dependen de la interacción entre cultivar y N dosis. CT tuvo una correlación inversa con PROT, pero fue poco pronunciada. Los cultivares BRSMG Uai, IAC Alvorada, TAA Dama y TAA Bola Cheia tienen las mejores características de calidad de grano.

Palabras clave

fertilización nitrogenada • genotipos • tecnología de granos • tiempo de cocción • contenido de proteínas

Introduction

Brazil is the largest global producer of common beans (*Phaseolus vulgaris* L.), with a cultivated area of approximately 3.2 million hectares (4). The most consumed commercial grain at national level is the 'Carioca' type. The grains of this leguminous plant stand out for their high nutritional value, due to the high contents of protein with good digestibility, essential amino acids, potassium and low levels of fat (9, 14).

Common bean genetic improvement programs have sought to develop and select cultivars with high production potentials and also good acceptance in the market. Interesting characteristics for consumers and packing industries include shorter cooking time, higher and faster hydration capacity, larger size and high protein contents in the grains (14, 17).

These qualitative attributes are influenced by the interaction between genotype and environment. Pereira *et al.* (2017) observed that the environmental effect is greater than the genotypic effect on the expression of characters related to grain technology. Nitrogen fertilization management can cause differences in these characteristics, which depend on the production system adopted (5, 7). These authors found that increments in topdressing nitrogen (N) increased protein contents in common bean grains under both direct planting system and conventional soil tillage. However, cooking time showed different response patterns between the systems.

The relationships between grain qualitative attributes have not yet been well elucidated by the literature, and there are divergences of information. Pereira *et al.* (2017) and Mingotte *et al.* (2015) observed an inverse relationship between CT and protein content for some genotypes. Nonetheless, Farinelli and Lemos (2010) found increase in cooking time with the increment in protein content, for the conventional soil tillage system. Cooking time is affected by other factors which depend on genotypes, which makes it difficult to use the protein content for indirect selection of cultivars with shorter cooking time.

Given the above, it is necessary to conduct studies aiming to better understand the relationships between qualitative attributes and the influence of the environment on these grain characteristics. With the hypothesis that the genetic diversity among cultivars leads to differences in the grain quality and differentiated responses to topdressing N fertilization, the present study aimed to evaluate the effect of low and high topdressing N doses on the qualitative attributes of grains of common bean cultivars and indicate those with better grain technological characteristics.

Material and methods

Location, period and soil characterization

The experiment was conducted in the agricultural year 2016/17 in Jaboticabal, Sao Paulo State (SP), Brazil, (21°14'33'' S, 48°17'10'' W; average altitude of 586 meters) in soil classified as Oxisol (*Latossolo Vermelho eutroférrico*), with clayey texture, on a gently undulating relief with 6% slope. According to Köppen's classification, the climate of this region is Aw, humid tropical with rainy period in the summer and dry period in the winter.

The experimental area was in the first year of no-tillage system (NT) under millet ADR-300 (*Pennisentum americanum* L.) straw in the quantity of 5.1 t ha^{-1}. The chemical attributes of soil fertility and particle size were determined in the 0-0.20 m layer prior to common bean sowing. The results were: pH ($CaCl_2$) 6.0; organic matter (OM) = 29 g dm^{-3}; P (resin) = 50 mg dm^{-3}; K = 6.4 $mmol_c$ dm^{-3}; Ca = 33 $mmol_c$ dm^{-3}; Mg = 14 $mmol_c$ dm^{-3}; S = 8 mg dm^{-3}, B = 0.32 mg dm^{-3}; Cu = 1.1 mg dm^{-3}, Fe = 14 mg dm^{-3}, Mn = 20.1 mg dm^{-3}, Zn = 4.0 mg dm^{-3}, H+Al = 16 $mmol_c$ dm^{-3}; CEC = 70 $mmol_c$ dm^{-3} ; V = 77%, clay = 540 g kg^{-1}; silt = 230 g kg^{-1} and sand = 230 g kg^{-1}.

Conduction, experimental design and treatments

Common bean cultivars were manually sown under millet straw on June 8, 2017 at 0.45 m spacing between rows using 12 seeds per meter. Fertilization at sowing was applied using 210 kg ha^{-1} of the formulation 04-20-20 (5.1% Ca, 4% S, 0.05% B, 0.06% Mn and 0.27% Zn). Common bean plants were irrigated by a conventional sprinkler system with variable intervals according to crop requirements.

The experimental design was randomized blocks in split-plot scheme, with four replicates. Plots comprised 16 common beans cultivars of the commercial grain 'Carioca': ANFc 9, BRSMG Uai, BRS Estilo, BRSMG Madrepérola, Pérola, BRS FC402, IAC Alvorada, IAC Milênio, IAC Sintonia, IPR Andorinha, IPR Campos Gerais, IPR Curió, IPR Celeiro, IPR Maracanã, TAA Dama and TAA Bola Cheia. Subplots consisted of two levels of topdressing N fertilization: minimum level (N dose of 20 kg ha^{-1}) and maximum level (N dose of 120 kg ha^{-1}). Each plot had 22.5 m^2 and was consisted by two subplots which was formed by five 5-m-long rows of common bean with rows spacing of 0.45 m and evaluations were carried out using the three central rows, disregarding 0.5 m on each side. N doses were applied as topdressing at V_4 stage (presence of third trifoliate leaf) in continuous strip at 0.10 m away from the planting row, using coated urea as source, followed by irrigation with 20 mm water depth for incorporation.

Grain quality evaluations

After harvest, grain quality evaluations were carried out. To determine sieve yield (SY), grain samples were subjected to the set of sieves with oblong holes, in agitation for one minute, with oblong holes of 19.05 mm of major axis and the following minor axes size: 4.37 mm (S11); 4.76 mm (S12); 5.16 mm (S13); 5.56 mm (S14) and 5.96 mm (S15). The percentage of grains was calculated by the relationship between the mass of grains retained on each sieve and the total mass of the sample from each subplot. SY was given by the sum of the percentages of grains retained on sieves with holes larger than or equal to 12. These results were used to calculate the relative grain production on sieves (RGPS) according to the adaptation of the methodology of Carbonell *et al*. (2010), which establishes weights for the sieves. According to Carbonell *et al*. (2010), the notes for each sieve were defined based on the requirement of the packaging industry and the consumer market. For this definition of banknotes, the main packers in the State of São Paulo, Brazil, were visited to check which is the best type of grain depending on its size (sieve yield). In the present study, the weight 1 was considered for grains retained on the collector (S≤10, *i.e.*, smaller than or equal to 10). S10 had oblong holes of 19,05 mm at major axis and 3.96 mm at minor axis.

RGPS was obtained using the following equation:

$$RGPS = \frac{(S \le 10 \times 1) + (S11 \times 4) + (S12 \times 6) + (S13 \times 10) + (S14 \times 10) + (S15 \times 6)}{S \le 10 + S11 + S12 + S13 + S14 + S15}$$

Crude protein content (PROT) (g kg^{-1}) was determined by the equation: PROT = Total N x 6.25 (2), in which Total N represents the total N content in the grains from each subplot, obtained by sulfuric digestion. Samples for PROT determination came from the grains of five plants consecutively collected along the row, at physiological maturity. The other evaluations used grains retained on the S13. Grain hydration capacity was determined using a 500-mL graduated cylinder with precision of 5 mL and 250-mL beakers. Each beaker received a 50-g sample of previously selected grains and then 200 mL of distilled water. Every two hours, over a period of 16 hours, the volume of water not absorbed by the grains was evaluated, by pouring it from the beaker to the graduated cylinder. At the end of the expected time for hydration, excess water was drained and grains were weighed. Hydration ratio (HR) was determined by the ratio between final mass and initial mass of grains. Analysis of polynomial regression between time (hours) and hydration capacity (mL) was carried out to determine the time required for maximum hydration (TMH) of common bean grains. Along the test, water temperature was 25°C. The final water volume absorbed (FVabs) was given by the difference between the final and initial volumes measured in the TMH evaluation.

To determine the cooking time (CT) in minutes, common bean grains were subjected to Mattson cooker, which basically consists of 25 vertical plungers. The tip touches the grain during cooking and when it is cooked the tip penetrates it, hence displacing the plunger. The final time for cooking of the sample was obtained when 50% + 1, *i.e.*, 13 plungers had been displaced. For this determination, grains were hydrated in distilled water for a 12-h period. During the test, water temperature was 96°C. The scale of Proctor and Watts (1987) was adopted to verify the level of resistance to cooking.

Statistical analysis of the data

The data were subjected to analysis of variance by F test and means were grouped by the Scott-Knott test. When F was significant for the interaction between N doses and cultivar, it was further analyzed. Due to the dependency structure contained in the original set of variables, the data were submitted to multivariate analysis of principal components (PCs), which made it possible to project all the information contained in the original variables into new latent variables, which were the processes (7). Data were standardized, showing null mean and unit variance. The number of PCs was selected based on Kaiser's criterion, using those with eigenvalues above 1 (7).

Results and discussion

The percentage of grains retained on the sieves S11 to S15 was significantly influenced by the factors cultivar and N dose, except S12, for which the percentage was not affected by the increment of the nutrient (table 1, page 122). For S15, there was interaction between cultivar and N dose. The N dose of 20 kg ha^{-1} led to higher percentages on S11 and S13, whereas N application of 120 kg ha^{-1} resulted in higher percentage on S14 and S15.

The highest percentages on S11 were obtained for BRS FC402, IPR Curió, IPR Celeiro and IPR Maracanã, followed by BRSMG Madrepérola, IAC Milênio, IAC Sintonia and IPR Campos Gerais. The cultivars BRS FC402, IPR Curió, IPR Celeiro, BRSMG Madrepérola and IPR Maracanã also had higher percentages on S12. The genotypes BRSMG Madrepérola, BRS FC402 and IAC Sintonia had the largest amounts of grains retained on S13. On the other hand, the genetic materials with highest percentages on S14 were IPR Andorinha, TAA Dama and TAA Bola Cheia, followed by ANFc9, IAC Alvorada and IAC Milênio. For S15, at the lowest N dose, superiority was found for the cultivars IAC Alvorada, ANFc 9 and TAA Dama, whereas at N dose of 120 kg ha^{-1} the cultivars IAC Alvorada, followed by ANFc9, stood out. The cultivars ANFc9, BRSMG Uai, Pérola, IAC Milênio, IPR Campos Gerais and TAA Bola Cheia showed increased percentages of grains retained on S15 with the increment in N dose.

Table 1. Percentages of grains retained on the processing sieves S11 (4.37 mm), S12 (4.76 mm), S13 (5.16 mm), S14 (5.56 mm) and S15 (5.96 mm) as a function of cultivars and nitrogen (N) doses applied as topdressing.

Tabla 1. Porcentajes de granos retenidos en los tamices de procesamiento S11 (4,37 mm), S12 (4,76 mm), S13 (5,16 mm), S14 (5,56 mm) and S15 (5,96 mm) en función de los cultivares y las dosis de nitrógeno (N) aplicadas como cobertura.

Cultivars	S11 (%)	S12 (%)	S13 (%)	S14 (%)	S15 (%)	
					N20	N120
ANFc 9	4.2 c	12.6 d	27.7 e	38.9 b	10.0 bB	13.3 bA
BRSMG Uai	3.6 c	15.6 c	47.2 c	25.7 c	3.0 dB	4.8 eA
BRS Estilo	6.0 b	18.1 c	47.6 c	21.0 d	2.3 dA	1.4 fA
BRSMG Madrepérola	6.0 b	23.1 b	58.1 a	8.3 e	0.6 eA	0.9 fA
Pérola	4.8 b	16.3 c	38.1 d	30.4 b	2.3 dB	4.9 eA
BRS FC402	9.1 a	27.8 a	53.8 b	3.9 e	0.2 eA	0.2 fA
IAC Alvorada	4.4 c	14.5 c	29.0 e	33.3 b	15.2 aA	16.5 aA
IAC Milênio	4.9 b	15.6 c	32.9 d	36.1 b	5.5 cB	7.7 dA
IAC Sintonia	6.2 b	19.4 c	51.3 b	17.5 d	1.1 eA	1.3 fA
IPR Andorinha	3.2 c	13.3 d	34.5 d	42.5 a	3.9 dA	4.4 eA
IPR Campos Gerais	5.4 b	18.2 c	42.9 c	25.7 c	1.4 eB	3.0 eA
IPR Curió	9.9 a	29.3 a	44.4 c	9.0 e	0.6 eA	0.9 fA
IPR Celeiro	9.2 a	28.4 a	49.7 b	6.7 e	0.5 eA	1.4 fA
IPR Maracanã	9.0 a	24.2 b	42.9 c	16.9 d	2.9 dA	4.0 eA
TAA Dama	2.8 c	9.8 e	25.5 e	48.4 a	9.2 bA	10.4 cA
TAA Bola Cheia	2.5 c	9.2 e	26.2 e	48.2 a	9.1 bB	11.0 cA
CV% - C	29.01	19.93	14.32	26.36	45.75	
N doses (kg ha^{-1})						
N20	6.0 a	18.7 a	42.5 a	24.2 b	4.2 b	
N120	5.4 b	18.2 a	39.0 b	27.4 a	5.4 a	
CV% - N	26.12	18.35	11.76	21.88	22.90	
F test						
Cultivar (C)	16.91**	24.39**	25.60**	37.60**	35.83**	
N dose (N)	4.27*	0.64^{NS}	17.30**	10.57**	35.30**	
C X N	0.61^{NS}	1.00^{NS}	0.90^{NS}	1.22^{NS}	1.92*	

Means followed by different letters, lowercase in columns and uppercase in rows, differ by Scott-Knott test at 0.05 probability level. NS Not significant by F test. ** Significant by F test ($p < 0.01$). * Significant by F test ($p < 0.05$).

Las medias seguidas de diferentes letras, minúsculas en columnas y mayúsculas en filas, difieren según la prueba de Scott-Knott con un nivel de probabilidad de 0,05. NS No significativo por la prueba F. ** Significativo por prueba F (p < 0,01). * Significativo por prueba F (p < 0,05).

As a consequence of the highest percentages on S14, the cultivars with highest SY were ANFc 9, BRSMG Uai, BRSMG Madrepérola, IAC Alvorada, IAC Milênio, IPR Andorinha, TAA Dama and TAA Bola Cheia.

However, it is important to emphasize that all cultivars had SY above 70%, a reference value for packing industries to pay a bonus to the producer because the grains are large and with good acceptance in the market (3). The genotypes with highest levels of RGPS were ANFc 9, BRSMG Uai, BRS Estilo, BRSMG Madrepérola, Pérola, IAC Alvorada, IAC Milênio, IAC Sintonia, IPR Andorinha, IPR Maracanã, TAA Dama and TAA Bola Cheia. The means of RGPS for all genotypes were above 7, the value proposed by Carbonell *et al.* (2010) as grains of good acceptance in the market and packing industries. These results are interesting because they demonstrate the concern of genetic improvement programs about developing cultivars with larger grains. SY and RGPS were not significantly affected by N doses (table 2, page 123). The lack of significant difference for these variables is due to the fact that the N dose of 20 kg ha^{-1} led to higher percentages on S13 compared to S14. Both sieves are superior to S12 and have the same weight in the calculation of RGPS.

Table 2. Sieve yield (SY), relative grain production on sieves (RGPS), crude protein content (PROT) and final water volume absorbed (FVabs) as a function of the cultivars and nitrogen (N) doses applied as topdressing.

Tabla 2. Rendimiento del tamiz (SY), producción relativa de granos en tamices (RGPS), contenido de proteína cruda (PROT) y volumen de agua final absorbido (FVabs) en función de los cultivares y las dosis de nitrógeno (N) aplicadas como cobertura.

Cultivars	SY (%)	RGPS	PROT (%)	FVabs (mL)	
ANFc 9	90.9 a	8.3 a	18.2 a	68.7 bA	71.2 bA
BRSMG Uai	92.3 a	8.7 a	16.9 a	72.5 aA	75.0 aA
BRS Estilo	88.5 b	8.4 a	16.4 b	68.7 bA	73.7 aA
BRSMG Madrepérola	90.3 a	8.3 a	18.4 a	67.5 bA	67.5 bA
Pérola	88.4 b	8.3 a	16.8 b	70.0 bA	68.7 bA
BRS FC402	85.6 c	7.9 b	16.3 b	67.5 bA	68.7 bA
IAC Alvorada	92.7 a	8.2 a	17.8 a	67.5 bA	70.0 bA
IAC Milênio	91.1 a	8.5 a	15.9 b	70.0 bA	68.7 bA
IAC Sintonia	89.3 b	8.4 a	14.7 b	70.0 bA	65.0 bA
IPR Andorinha	94.4 a	8.9 a	16.6 b	66.2 bA	66.2 bA
IPR Campos Gerais	89.2 b	8.1 b	15.9 b	75.0 aA	67.5 bB
IPR Curió	83.5 c	7.7 b	17.8 a	73.7 aA	72.5 aA
IPR Celeiro	85.7 c	7.8 b	17.1 a	72.5 aA	73.7 aA
IPR Maracanã	87.5 b	8.5 a	17.3 a	73.7 aB	80.0 aA
TAA Dama	93.5 a	8.7 a	17.9 a	71.2 bA	76.2 aA
TAA Bola Cheia	93.8 a	8.7 a	17.4 a	77.5 aA	75.0 aA
CV% - C	3.65	5.65	9.14	5.00	
N doses (kg ha^{-1})					
N20	89.6 a	8.3 a	16.7 b	70.8 a	
N120	90.0 a	8.3 a	17.3 a	71.2 a	
CV% - N	3.37	5.26	8.05	5.26	
F test					
Cultivar (C)	7.45**	4.33**	3.13**	6.47**	
N dose (N)	0.55^{NS}	0.01^{NS}	5.72*	0.50^{NS}	
C X N	0.89^{NS}	1.16^{NS}	1.09^{NS}	1.91 *	

Means followed by different letters, lowercase in columns and uppercase in rows, differ by Scott-Knott test at 0.05 probability level. NS Not significant by F test. ** Significant by F test (p < 0.01). * Significant by F test (p < 0.05).

Las medias seguidas de diferentes letras, minúsculas en columnas y mayúsculas en filas, difieren según la prueba de Scott-Knott con un nivel de probabilidad de 0,05. NS No significativo por la prueba F. ** Significativo por prueba F (p < 0,01). * Significativo por prueba F (p < 0,05).

Crude protein contents increased with the N dose of 120 kg ha^{-1} as topdressing. After being absorbed, N associates with organic compounds, giving rise to proteins, which result in increase of grain weight (1, 13) and, consequently, increments in grain size.

The highest percentages were observed for ANFc 9, BRS MG Uai, BRSMG Madrepérola, IAC Alvorada, IPR Curió, IPR Celeiro, IPR Maracanã, TAA Dama and Bola Cheia. Protein contents varied from 14.7 to 18.4% (table 2). These values were below the mean contents of 20.2%, 21.6% and 20.3% found by Farinelli and Lemos (2010), Mingotte *et al*. (2015) and Miano *et al.* (2018), which is explained by the higher doses and splitting of topdressing N adopted in these studies.

FVabs was significantly influenced by the interaction between dose and cultivar. The grains which most absorbed water, under the lowest N dose, were those of BRSMG Uai, IPR Campos Gerais, IPR Curió, IPR Celeiro, IPR Maracanã and TAA Bola Cheia. Under the highest N dose, the largest volumes absorbed were found for BRSMG Uai, BRS Estilo, IPR Curió, IPR Celeiro, IPR Maracanã, TAA Dama and TAA Bola Cheia. With the increment in N, water absorption capacity decreased by 7.5 mL per 50 g of common bean in the cultivar IPR Campos Gerais and increased by 6.7 mL in IPR Maracanã.

Based on multivariate analysis of principal component (PCs) (figure 1, page 124), it can be observed that the first two PCs were responsible for 62.1% of the total data variability.

Numbers indicate cultivars and letters indicate N doses: 20 (A) and 120 (B) kg ha^{-1} of N. 1: ANFc 9; 2: BRSMG Uai; 3: BRS Estilo; 4: BRSMG Madrepérola; 5: Pérola; 6: BRS FC402; 7: IAC Alvorada; 8: IAC Milênio; 9: IAC Sintonia; 10: IPR Andorinha; 11: IPR Campos Gerais; 12: IPR Curió; 13: IPR Celeiro; 14: IPR Maracanã; 15: TAA Dama and 16: TAA Bola Cheia.

Los números indican cultivares y las letras indican dosis de N: 20 (A) y 120 (B) kg ha^{-1} de N. 1: ANFc 9; 2: BRSMG Uai; 3: BRS Estilo; 4: BRSMG Madrepérola; 5: Pérola; 6: BRS FC402; 7: IAC Alvorada; 8: IAC Milênio; 9: IAC Sintonia; 10: DPI Andorinha; 11: DPI Campos Gerais; 12: IPR Curió; 13: DPI Celeiro; 14: DPI Maracaná; 15: TAA Dama y 16: TAA Bola Cheia.

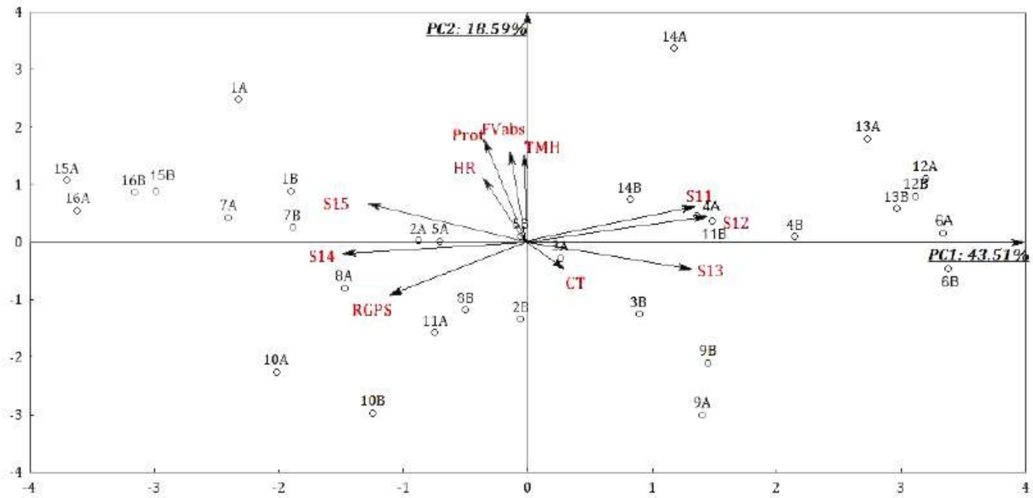

Figure 1. Biplot graph of the principal components for the distribution of common bean cultivars under nitrogen (N) doses as a function of grain technological variables.

Figura 1. Gráfico biplot de los componentes principales para la distribución de cultivares de frijol común sobre baja dosis de nitrógeno (N) en función de las variables tecnológicas del grano.

The variables which most discriminated PC1 were those related to the distribution of grains on the sieves. The high factorial scores of S11 (0.89), S12 (0.96), S13 (0.87), S14 (-0.97), S15 (-0.83) and RGPS (-0.72) with PC1 allowed the genotypes to be separated by grain size. An analysis of the biplot graph allows observing a positive correlation between the sieves with smallest holes (S11 to S13) and a negative correlation between these and the sieves with largest holes (S14 and S15). The variables with highest correlations with PC2 were protein content (0.75), FVabs (0.67) and TMH (0.63).

TMH was significantly affected only by the cultivars (table 3, page 125). The highest TMH was obtained for ANFc 9, which required 15 hours and 50 minutes for its maximum hydration. The common bean grains with fastest hydration were those of BRSMG Uai, BRS Estilo, IAC Alvorada, IAC Sintonia, IPR Andorinha and IPR Campos Gerais, varying from 11 hours and 54 minutes to 12 hours and 18 minutes. The results obtained for these materials, close to 12 hours, were satisfactory, since consumers usually subject common bean grains to soaking the night prior to cooking (5, 10).

HR values were higher for ANFc 9, BRSMG Uai, BRS Estilo, BRSMG Madrepérola, Pérola, IAC Alvorada, IPR Campos Gerais, IPR Celeiro, IPR Maracanã, TAA Dama and TAA Bola Cheia (table 3, page 125). Although the differences were significant between the cultivars, it is important to point out that all values were close to 2. These results corroborate those of Farinelli and Lemos (2010), who observed that common bean grains were able to absorb, in mass of water, approximately the equivalent to their initial masses. The increment of N via topdressing fertilization did not increase HR. Although the highest N dose increased protein content in the grains, the difference between fertilization managements was minimal (0.6%). Even though the protein is hydrophilic (5), with high water absorption capacity, this difference was not sufficient to increase TMH and HR under high N dose. In addition, the different proteins present in common bean grains have different levels of hydrophilicity, varying according to the genotype (11). Miano *et al*. (2018) cite that common bean hydration kinetics depends on other genetic factors, such as fat content, starch content, specific surface and morphological aspects of the grain.

Table 3: Time for maximum hydration (TMH), hydration ratio (HR), cooking time (CT) and resistance to cooking (RC) of common bean grains as a function of the cultivars and N doses applied as topdressing.

Table 3: Tiempo para la hidratación máxima (TMH), relación de hidratación (HR), tiempo de cocción (CT) y resistencia a la cocción (RC) de los granos de frijol común en función de los cultivares y dosis de N aplicadas como cobertura.

Cultivars	TMH (hh:mm)	HR	CT (mm:ss)		RC	
			N20	N120	N20	N120
ANFc 9	15:50 a	2.00 a	21:56 aB	24:32 aA	NR	NR
BRSMG Uai	11:54 c	2.03 a	23:37 aB	26:45 aA	NR	NR
BRS Estilo	11:55 c	2.01 a	17:47 bB	22:12 bA	MS	NR
BRSMG Madrepérola	13:16 b	2.02 a	21:09 aA	21:11 bA	NR	NR
Pérola	13:45 b	2.02 a	21:36 aA	21:45 bA	NR	NR
BRS FC402	13:40 b	1.98 b	21:37 aA	22:27 bA	NR	NR
IAC Alvorada	11:48 c	2.02 a	19:50 aA	19:07 cA	MS	MS
IAC Milênio	13:07 b	1.98 b	21:21 aA	20:26 cA	NR	NR
IAC Sintonia	12:01 c	1.97 b	21:53 aA	21:16 bA	NR	NR
IPR Andorinha	11:58 c	1.93 c	20:10 aA	20:22 cA	NR	NR
IPR Campos Gerais	12:18 c	2.00 a	17:16 bA	16:32 dA	MS	MS
IPR Curió	13:11 b	1.95 c	19:54 aB	23:17 bA	MS	NR
IPR Celeiro	13:02 b	2.01 a	21:06 aA	19:07 cA	NR	MS
IPR Maracanã	14:06 b	2.03 a	18:22 bA	16:57 dA	MS	MS
TAA Dama	13:23 b	2.02 a	17:58 bA	18:29 cA	MS	MS
TAA Bola Cheia	12:56 b	2.01 a	17:52 bA	18:29 cA	MS	MS
CV% - C	9.17	0.96	8.26		-	
N doses (kg ha^{-1})						
N20	13:00 a	1.99 a	20:48 a		MS	
N120	13:01 a	2.00 a	20:13 a		MS	
CV% - N	5.39	0.86	8.18		-	
F test						
Cultivar (C)	6.29**	18.72**	12.83**		-	
N dose (N)	0.08^{NS}	2.46^{NS}	4.00^{NS}		-	
C X N	0.81^{NS}	1.28^{NS}	2.43*		-	

Means followed by different letters, lowercase in columns and uppercase in rows, differ by Scott-Knott test at 0.05 probability level. ^{NS} Not significant by F test. ** Significant by F test (p < 0.01). * Significant by F test (p < 0.05). NR: Normal resistance; MS: Medium susceptibility.

Las medias seguidas de diferentes letras, minúsculas en columnas y mayúsculas en filas, difieren según la prueba de Scott-Knott con un nivel de probabilidad de 0,05. ^{NS}No significativo por la prueba F. ** Significativo por prueba F (p < 0,01). * Significativo por prueba F (p <0,05). NR: Resistencia normal; MS: Susceptibilidad media.

CT was significantly influenced by the cultivar and by the interaction between cultivar and dose (table 3). The lowest values of CT were obtained under the N dose of 20 kg ha^{-1}, for BRS Estilo, IPR Campos Gerais, IPR Maracanã, TAA Dama and TAA Bola Cheia. Under the highest N dose, the cultivars whose grains were cooked in the shortest time were IPR Campos Gerais and IPR Maracanã, followed by IAC Alvorada, IAC Milênio, IPR Andorinha, IPR Celeiro, TAA Dama and TAA Bola Cheia. In the genotypes ANFc 9, BRSMG Uai, BRS Estilo and IPR Curió, CT increased significantly with the increment in N dose.

According to the scale of Proctor and Watts (1987), the grains of the cultivars IAC Alvorada, IPR Campos Gerais, IPR Maracanã, TAA Dama and TAA Bola Cheia are grouped as materials of medium susceptibility to cooking (16 to 20 minutes), regardless of the N dose applied at common bean. After this group with greater facility for grains cooking, the materials ANFc 9, BRSMG Uai, BRSMG Madrepérola, Pérola, BRS FC402, IAC Milênio, IAC Sintonia and IPR Andorinha were classified as of normal resistance to cooking (21 to 28 minutes) and also had no influence of the N dose for changing the classification of susceptibility to grain cooking. The classification of BRS Estilo and IPR Curió changed from medium susceptibility to normal resistance as N doses increased. On the other hand, IPR Celeiro changed from normal resistance to medium susceptibility with the increment in topdressing N dose, although no significant difference was observed between the doses for CT (table 3). The scale of Proctor and Watts (1987) classifies the cooking resistance of grains in VS: Very susceptible, (less than 16 minutes), MS: Medium susceptibility (16 to 20 minutes), NR: Normal resistance (21 to 28 minutes), MR: Medium resistance (29 to 32 minutes), R: Resistant (33 a 36 minutes) and VR: Very resistant (more than 37 minutes). There were no common bean grains that cooked with less than 16 minutes or more than 28 minutes.

Based on PC2, there was a direct correlation between crude protein content in the grains, TMH, FVabs and HR (0.48), although for this last-mentioned variable the score was lower than 0.60. Moreover, it can be noted that CT has inverse correlation with these variables. However, such relationship is not very pronounced, since the score of CT with PC2 is low (-0.20). Similar results were reported by Pereira *et al.* (2017) and Mingotte *et al.* (2015), who observed inverse correlation between CT and protein content for some genotypes. However, in general, the correlations observed by these authors were not statistically significant (r = -0.19 and r = -0.22, respectively). Farinelli and Lemos (2010) found reduction in CT as a function of increasing protein content under no-tillage system. These results suggest that cooking time is not influenced only by protein content, but also affected by other factors which depend on the genotypes, which makes it difficult to use protein content for indirect selection of cultivars with shorter cooking time. The content of total soluble solids, water absorption capacity by the grains (14), iron content in the grains (16) and nitrogen fertilization (5, 18) also affect CT.

Conclusions

The grain quality of common bean is more dependent on the genotype studied than on agricultural management such as nitrogen fertilization. However, the increase in the N dose applied as topdressing increases the size of common bean grains and their protein content, but with little influence in grain hydration. The final water volume absorbed and cooking time by grains depend on the interaction between cultivar and N doses. Furthermore, the study of the interaction between the attributes, allows us to conclude that cooking time has a little pronounced inverse correlation with crude protein content in the grains. The cultivars BRSMG Uai, IAC Alvorada, TAA Dama and TAA Bola Cheia have the best grain technological characteristics.

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Pear and apple pomace compost in the production of tomato (*Lycopersicon esculentum* Mill.) seedlings

Compost de orujo de pera y manzana como alternativa en la producción de plantines de tomate (*Lycopersicon esculentum* Mill.)

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Abstract

Alternative substrates replacing non-renewable resources like peat, for growing media in horticulture, have gained importance. This work aimed to evaluate if pear and apple pomace compost could constitute an alternative to a commercial substrate for tomato seedlings production. Two experiments were carried out on trays with 100 cm^3 alveoli (experiment 1) and 30 cm^3 alveoli (experiment 2). In experiment 1, three substrates were used: commercial substrate (CS), mixture of CS and pomace compost (CS+C) and pure compost (C). In experiment 2 a fourth treatment with a mixture of compost and perlite (C+P) was incorporated. The obtained results indicated that seedling development using CS+C and CS as substrates, was similar in cells of 100 cm^3 and higher in cells of 30 cm^3. In addition, seedling growth on C+P in relation to CS, showed similar or higher values for some variables. These results indicate that replacing non-renewable resources such as peat in tomato seedling production, with a product obtained from a residue, would be feasible.

Keywords
aerial biomass • agroindustry wastes • *Lycopersicon esculentum* • nutrients • seedling production

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Resumen

La búsqueda de sustratos alternativos para reemplazar los recursos no renovables como la turba utilizada en medios de cultivo en horticultura, resulta importante. El objetivo de este trabajo fue evaluar si el compost de orujo de pera y manzana podría usarse como una alternativa a un sustrato comercial para la producción de plantines de tomate. Se realizaron dos experimentos en bandejas con alvéolos de 100 cm^3 (experimento 1) y 30 cm^3 (experimento 2). En el experimento 1, se usaron tres sustratos: sustrato comercial (CS), mezcla de CS y compost de orujo (CS + C) y compost puro (C). En el experimento 2 se incorporó un cuarto tratamiento con una mezcla de compost y perlita (C + P). Los resultados obtenidos indicaron que el desarrollo de los plantines usando CS + C y CS como sustrato fue similar en celdas de 100 cm^3 y mayor en celdas de 30 cm^3. Además, el crecimiento de los plantines en C + P en relación con CS mostró valores similares o más altos en algunas variables. Estos resultados indicaron que sería factible reemplazar el uso de recursos no renovables como la turba en la producción de plantines de tomate por un producto obtenido de un residuo.

Palabras clave

biomasa aérea • residuos agroindustriales • *Lycopersicon esculentum* • nutrientes • producción de plantines

Introduction

Plant substrate is any solid material other than soil, which allows root anchoring, water and nutrient absorption, and gas exchange from and to the root (27). Commercial substrates constitute mixtures of different products such as blond and black peat moss, pine bark of different granulometry, perlite, sand and humectants (16). Peat has been widely used as a growing medium in horticulture given its good physical properties and high nutrient exchange capacity (30). However, being a non-renewable, high-cost product, to replace it by cheaper and eco-friendly substitutes, (4, 5, 10) turns important.

In this sense, numerous studies on peat replacement by organic waste or compost have tested alternatives like substrates degraded by fungi (19, 23, 24), sludge from the paper industry (15), and compost made from urban, agroindustrial and green solid waste (9, 10, 11, 14). These substrates obtained by fungal degradation or waste composting resulted appropriate for total or partial replacement of peat, in the cultivation and production of seedlings of species like tomato (*Lycopersicon esculentum* Mill.), lettuce (*Lactuca sativa* L.), courgette (*Cucurbita pepo* L.), pepper (*Capsicum annum* L.), and geranium (*Pelargonium zonale* L.). Current requirements on organic waste usage have driven research lines based on the possible use of these wastes as components of substrates replacing peat. In this sense, the composting of pure agroindustrial wastes and as part of mixtures with other by-products, have proven to be similar or superior when used in the production of horticultural seedlings, compared to commercial products used for this purpose (7, 8).

Composting is a widely used technology for waste re-utilization. It allows the generation of biofertilizers and soil conditioners, and the production of gas, humus, and biofuels, among others. In this way, a rational use of these resources is promoted, while the negative impact of organic matter accumulation, is reduced (12).

This work aimed to evaluate pear and apple pomace compost as an alternative to a commercial substrate for tomato seedlings production (*Lycopersicon esculentum* Mill. Var. Flora Dade).

Materials and methods

Pear and apple pomace compost

Pear and apple pomace compost was produced by Jugos S.A. (Villa Regina, Río Negro-Argentina) using the windrow composting system. The pomace was arranged in pyramidal piles, 2 m wide x 1 m high x 150 m long, facilitating compost turning with rotovator for better homogenization and aeration during the process. The mixing frequency depended on temperature (never above 55 °C), moisture content (always over 40%), electrical conductivity, pH and organic matter. After 12 to 15 months, depending on the environmental conditions, the aforementioned physicochemical parameters were re-assessed.

Plant material and growth conditions

Two experiments were conducted in a laboratory under controlled conditions, 25-28 °C, illuminance of 7500 lux (using LED tubes of 18 W) and 12-hours photoperiod. Indeterminate growth Flora Dade tomato seeds (*Lycopersicon esculentum* Mill.) provided by Guasch Seeds, were used. Their germinative power, previously measured in Petri dishes, was 89.9%.

The experiments were carried out on different dates, using alveolate trays with dissimilar cell volumes. In both experiments, the commercial mixture Grow Mix Multipro (Terrafertil S.A., Argentina), composed of Sphagnum peat moss, bark compost, calcite lime, dolomite lime and wetting agents, was used as control substrate (CS) .

Experiment 1

Six alveolate trays of 25, 100 cm^3 cells, were used. Seedling development was evaluated on 3 substrates (CS, CS+C, and C), consisting of mixtures between the commercial substrate with pear and apple pomace compost (C) in the following proportions:

CS: Commercial substrate.

CS+C: Commercial substrate and Compost 1:1 v/v.

C: Compost.

For each treatment, two adjacent trays, were used. In the center of each cell, one seed was placed at a depth of approximately 2 mm.

Sowing took place on June 2nd, 2018. Seedling development was evaluated for 32 days. Daily irrigation by manual spray, was approximately 30 mL/cell.

Experiment 2

The second experiment was conducted in four alveolate trays of 128, 30 cm^3 cells, with the same variety and identical culture conditions as experiment 1. In addition, a fourth treatment, consisting of a mixture of compost and perlite (C+P) in a 3:1 v/v ratio, was incorporated.

Sowing was carried out on August 14th, 2018. Growth evaluation was carried out for 30 days. Daily irrigation was approximately 10 mL/cell.

Physicochemical analysis of substrates

The following physical and chemical properties of the tested substrates and mixtures were determined in triplicate:

- Percentage of organic matter (%OM) with an automatic carbon analyzer by dry combustion (IR).
- Total nitrogen (N) by semi-micro Kjeldahl.
- Available phosphorus (P) by the Bray-Kurtz method No. 1.
- Potassium by extraction with ammonium acetate at pH = 7 and determination by atomic emission spectrophotometry by induced plasma.
- Electrical conductivity (EC) and pH, with ASDW AD 8000 meter-conductivity pH, adapted from FAO (2009) using distilled water as an extractant in a 1:2.5 ratio v/v standing for 45 minutes.
- Total porosity (TP); aeration porosity (AP); water holding capacity (WHC); bulk density (BD) and particle density (PD), employing porometers of 15 cm height and 7.6 cm diameter, according to Pire *et al*. (2003).

Growth and development

After sowing, percentage of emergence was determined and date of appearance of the first, second and third true leaves for each treatment was daily recorded by direct counting.

Approximately one month after the sowing, 29 seedlings of the CS treatment, 27 of the CS+C and 23 of the C (pseudo-replicates) of experiment 1, and 44 seedlings for each treatment of experiment 2, were randomly selected. The number of randomly chosen individuals corresponded to a representative sample of each treatment, ± 10% and 90% confidence. In the CS+C and C treatments of experiment 1, the number of seedlings was lower given that finding 29 healthy individuals, was not possible.

For shoot fresh weight (SFW) and root fresh weight (RFW) determinations, seedlings were carefully extracted from the cells, placed in plastic mesh envelopes and submerged in water for five to ten minutes in order to carefully remove attached substrate. Water was drained on absorbent paper. Shoots were separated from roots by cross-section at neck level.

SFW and RFW were weighed with an Ohaus analytical digital balance (± 0.0001g). Total fresh weight of each plant (TFW) was obtained as SFW plus RFW. Shoot height (h) was measured from neck to apex, while stem diameter (d) was obtained at neck level with a digital SATA caliper (± 0.01 mm).

For shoot and root dry weight (SDW, RDW) determinations, samples were oven-dried at 60 °C until constant weight (72 h approx.). Dry weight was recorded with an analytical balance (± 0.0001 grams). Total dry weight of each plant (TDW) was determined as SDW plus RDW.

Statistics

In each experiment, means comparison was performed by one-way ANOVA and Tukey test ($p<0.01$) using InfoStat version 2016 (3). ANOVA assumptions (normality and homoscedasticity) were tested by the modified Shapiro-Wilks´ and Levene's tests, respectively. SFW, RFW, TFW, SDW, RDW, and TDW were square-root transformed, meeting these assumptions. Reported data correspond to untransformed values.

Results

Chemical and physical properties of the substrates

Table 1 shows organic matter, macronutrients and physicochemical properties of the tested substrates. The CS treatment had significantly higher OM. Total N was higher for those substrates containing compost, although not significant for C+P with respect to CS. Similar results were observed for P, twice higher, and K, 2.5 times higher in C with respect to CS. This treatment presented significantly lower pH and EC values than those containing compost (CS+C, C, C+P).

TP and WRC resulted considerably higher in the CS+C mix than in C. As for BD, the lowest value was obtained for CS (0.18 $Mg.m^{-3}$) and the highest for C (0.55 $Mg.m^{-3}$).

Table 1. Mean concentration and SD of the organic and inorganic components and physicochemical properties of the tested substrates (n=3).

Tabla 1. Concentración de los componentes orgánicos e inorgánicos y propiedades fisicoquímicas de los sustratos utilizados. Los valores representan la media y la DE (n=3).

	Treatment			
Properties	**CS**	**CS+C**	**C**	**C+P**
OM (%)	45.9 ±1.8^{a}	33.5 ± 3.1^{b}	22.6 ± 0.8^{c}	19.7 ± 0.5^{c}
N (%)	1.19 ± 0.08^{b}	1.70 ± 0.10^{a}	1.83 ± 0.04^{a}	1.35 ± 0.06^{b}
P (ppm)	76 ± 6^{c}	122 ± 10^{b}	142 ± 6^{a}	140 ± 8^{ab}
K (ppm)	667 ± 45^{b}	1576 ± 23^{a}	1880 ± 160^{a}	1652 ± 56^{a}
pH	5.70 ± 0.03^{c}	6.43 ± 0.06^{b}	7.04 ± 0.10^{a}	6.92 ± 0.06^{a}
EC ($dS.m^{-1}$)	0.53 ± 0.00^{c}	3.51 ± 0.09^{a}	3.38 ± 0.04^{a}	3.09 ± 0.03^{b}
TP (%)	66.3 ± 3.7^{ab}	67.7 ± 0.9^{a}	59.5 ± 0.3^{b}	63.6 ± 1.8^{ab}
AP (%)	7.4 ± 1.1^{ab}	3.9 ± 0.1^{bc}	2.7 ± 0.6^{c}	8.1 ± 1.7^{a}
WHC (%)	58.9 ± 2.7^{ab}	63.7 ± 1.0^{a}	56.8 ± 0.3^{b}	55.4 ± 1.2^{b}
BD ($Mg.m^{-3}$)	0.18 ± 0.00^{d}	0.44 ± 0.01^{b}	0.55 ± 0.03^{a}	0.38 ± 0.02^{c}
PD ($Mg.m^{-3}$)	0.52 ± 0.05^{c}	1.37 ± 0.02^{ac}	1.36 ± 0.07^{a}	1.04 ± 0.01^{b}

OM: organic matter, N: nitrogen, P: phosphorus, K: potassium, EC: electrical conductivity, TP: total porosity, AP: aeration porosity, WHC: water holding capacity, BD: bulk density, PD: particle density Different letters in each row show significant differences (Tukey´s test, p≤ 0.01).

OM: materia orgánica, N: nitrógeno, P: fósforo, K: potasio, EC: conductividad eléctrica, TP: porosidad total, AP: porosidad de aireación, WHC: capacidad de retención de agua, BD: densidad aparente, PD: densidad de partículas. Letras distintas en la misma fila indican diferencias significativas (prueba de Tukey, p≤0,01).

Growth and development in experiment 1

Seedling development in trays with 100 cm^3 alveoli showed marked differences in accumulated emergence and time of appearance of the first, second and third true leaves for treatments CS and CS+C with respect to C. Thus, emergence percentages for the mixtures containing commercial substrate reached a maximum of 94% at eleven days after sowing, while the seedlings developed on C, reached 74% after 16 days.

Appearance time of the first, second and third true leaves in 90% of the seedlings developed on CS and CS+C, was 15 and 26 days, respectively. In the C substrate, a maximum of 60-70% was obtained between day 24 and 25 for the first and second true leaves, while the third true leaf emerged on day 24, reaching only 10% at the end of the experiment (figure 1 A-D).

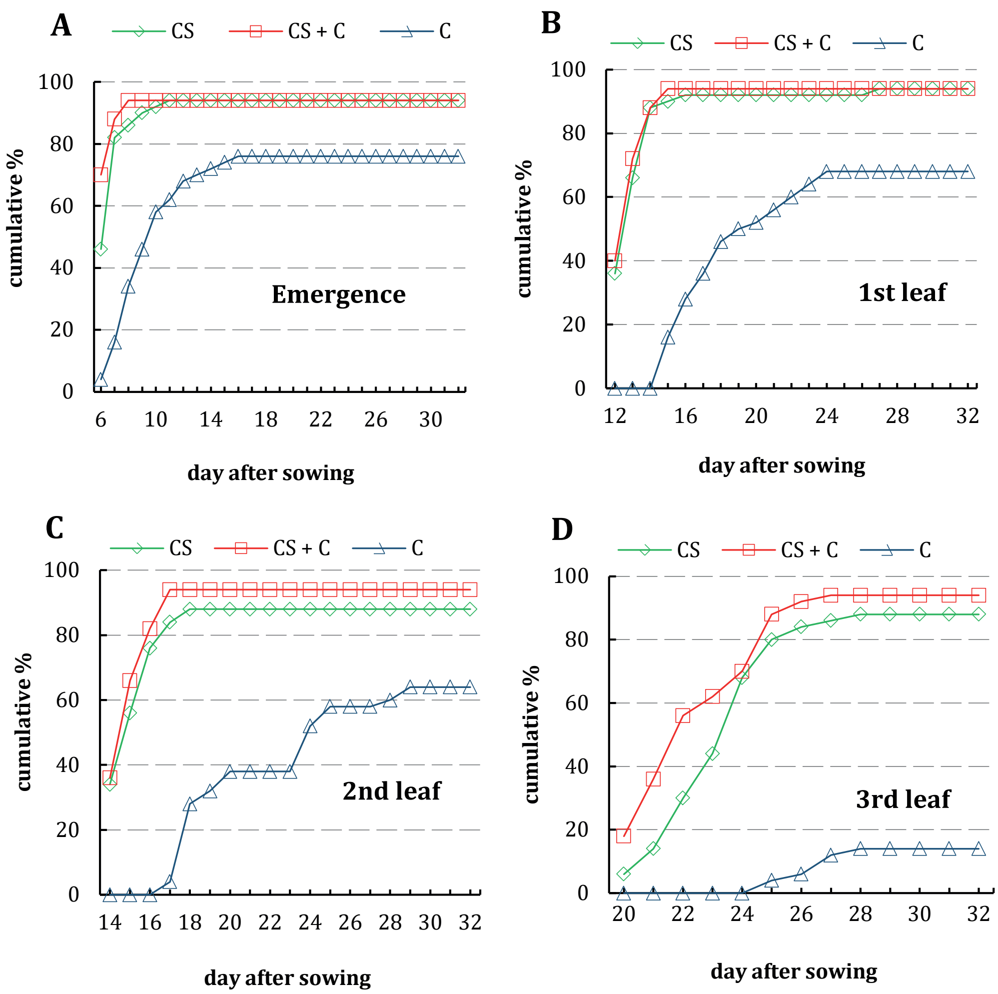

Figure 1. Cumulative emergent percentages (**A**) and appearance of the first (**B**), second (**C**) and third (**D**) true leaves of tomato seedlings grown on substrates CS, CS+C, and C as a function of time in days since sowing.

Figura 1. Porcentajes de emergencia acumulada (**A**) y aparición de la primera (**B**), segunda (**C**) y tercera (**D**) hoja verdadera de plantines de tomate cultivados en sustratos CS, CS+C y C en función del tiempo en días desde la siembra.

Developmental variables h, d, SFW, RFW, TFW, SDW, RDW and TDW, resulted similar between CS and CS+C treatments, and significantly higher than treatment C (table 2, page 133). Even though the precision balance weighted to 0.0001 g, standard deviations are indicated by one or two significant figures.

h: plant height, d: stem diameter, SFW: shoot fresh weight, RFW: root fresh weight, TFW: total fresh weight, SDW: shoot dry weight, RDW: root dry weight, TDW: total dry weight. Different letters in each row show significant differences (Tukey´s test, p≤ 0.01).

h: altura de la planta, d: diámetro del tallo, SFW: peso fresco de la parte aérea, RFW: peso fresco de la raíz, TFW: peso fresco total, SDW: peso seco de la parte aérea, RDW: peso seco de la raíz, TDW: peso seco total. Letras distintas en la misma fila indican diferencias significativas (prueba de Tukey, p≤0,01).

Table 2. Growth and development properties of the tomato seedlings measured in experiment 1 (100 cm^3 cells). Values represent mean and the SD (n=29, 27 and 23 for CS, CS +C and C, respectively).

Tabla 2. Propiedades de crecimiento y desarrollo de los plantines de tomate medidas en el experimento 1 (celdas de 100 cm^3). Los valores representan la media y la DE (n=29, 27 y 23 para CS, CS +C y C, respectivamente).

Properties	Treatments		
	CS	**CS+C**	**C**
	n = 29	n = 27	n = 23
h (mm)	74 ± 12^a	67.6 ± 9.2^a	44.6 ± 8.9^b
d (mm)	2.76 ± 0.58^a	3.00 ± 0.57^a	1.50 ± 0.47^b
SFW (g)	0.84 ± 0.38^a	1.07 ± 0.33^a	0.25 ± 0.14^b
RFW (g)	0.23 ± 0.11^a	0.210 ± 0.067^a	0.086 ± 0.038^b
TFW (g)	1.07 ± 0.48^a	1.28 ± 0.40^a	0.33 ± 0.17^b
SDW (g)	0.087 ± 0.042^a	0.100 ± 0.037^a	0.028 ± 0.015^b
RDW (g)	0.016 ± 0.010^a	0.011 ± 0.008^a	0.003 ± 0.003^b
TDW (g)	0.10 ± 0.05^a	0.11 ± 0.05^a	0.031 ± 0.018^b

Growth and development in experiment 2

Figure 2 (page 134) shows tomato seedling development in 30 cm^3 cells. Cumulative emergence percentages (figure 2A, page 134) and appearance of the first, second and third true leaves (figure 2B-2D, page 134) for the CS+C treatment, were the highest, followed by CS. Unlike the results obtained in the first test with 100 cm^3 cells, for C+P treatment the third leaf was anticipated with respect to CS. Development in C produced the lowest values for all the variables analyzed.

Unlike experiment 1, the values obtained for h, d, SFW, RFW, TFW, SDW, and TDW for the CS+C treatment resulted higher, except for RDW. In the case of the seedlings developed on CS and C+P, statistically identical values were obtained in most of the variables, except for SFW, TFW, and TDW. Compost (C) restricted plant development, obtaining significantly lower values for all variables except for RDW, which was similar to that obtained in the CS treatment, as shown in table 3 (page 134).

Comparison of development between experiments

When comparing developmental variables according to cell size (experiments 1 and 2), seedling h resulted higher in the larger cells for the CS treatment, but showed no significant differences in the CS+C and C treatments (figure 3A, page 135).

SFW and d resulted higher for the bigger cells of the CS treatment, while for C, larger diameters and weights resulted in the smaller cells (figure 3B-3C, page 135).

RFW developed on CS+C was notably higher in the smaller cells, while no differences were found for the CS and C treatments (figure 3D, page 135).

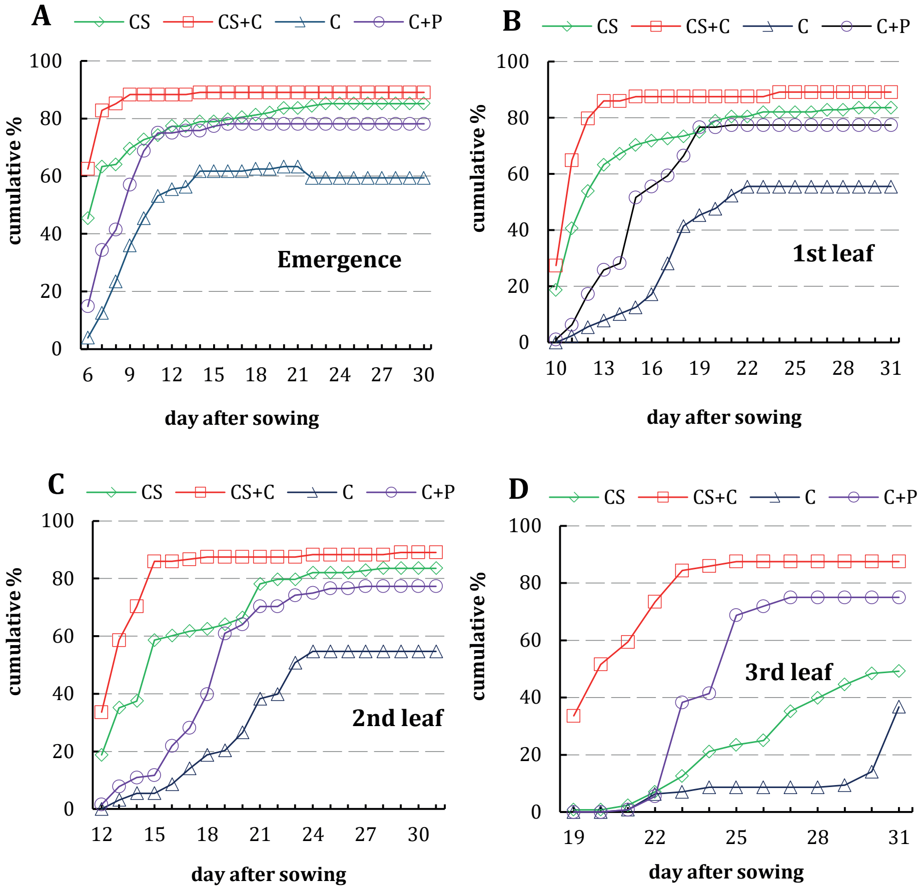

Figure 2. Cumulative emergent percentages (**A**) and appearance of the first (**B**), second (**C**) and third (**D**) true leaves of tomato seedlings grown on substrates C, CS+C, C and C+P as a function of time in days since sowing.

Figura 2. Porcentajes de emergencia acumulada (**A**) y aparición de la primera (**B**), segunda (**C**) y tercera (**D**) hoja verdadera de plantines de tomate cultivados en sustratos C, CS, CS+C y C en función del tiempo en días desde la siembra.

Table 3. Growth and development properties of tomato seedlings measured in experiment 2 (30 cm^3 cells). Values correspond to means and SD (n= 44).

Tabla 3. Propiedades de crecimiento y desarrollo de los plantines de tomate medidas en el experimento 2 (celdas de 30 cm^3). Los valores representan las medias y las DE (n= 44).

Properties	Treatments			
	CS	CS+C	C	C+P
	n=44	n=44	n=44	n=44
h (mm)	45.5 ± 5.5^{b}	60.6 ± 6.7^{a}	39.7 ± 5.9^{c}	43.8 ± 6.6^{bc}
d (mm)	2.08 ± 0.25^{b}	2.78 ± 0.30^{a}	1.86 ± 0.22^{c}	2.24 ± 0.25^{b}
SFW (g)	0.49 ± 0.15^{c}	1.20 ± 0.25^{a}	0.47 ± 0.13^{c}	0.69 ± 0.13^{b}
RFW (g)	0.31 ± 0.11^{b}	0.60 ± 0.22^{a}	0.18 ± 0.10^{c}	0.39 ± 0.12^{b}
TFW (g)	0.80 ± 0.23^{c}	1.81 ± 0.42^{a}	0.65 ± 0.22^{c}	1.08 ± 0.23^{b}
SDW (g)	0.065 ± 0.023^{bc}	0.131 ± 0.033^{a}	0.07 ± 0.11^{c}	0.079 ± 0.052^{b}
RDW (g)	0.025 ± 0.009^{ab}	0.053 ± 0.057^{a}	0.024 ± 0.044^{b}	0.054 ± 0.090^{a}
TDW (g)	0.090 ± 0.030^{c}	0.184 ± 0.070^{a}	0.10 ± 0.12^{c}	0.13 ± 0.10^{b}

h: plant height, d: stem diameter, SFW: shoot fresh weight, RFW: root fresh weight, TFW: total fresh weight, SDW: shoot dry weight, RDW: root dry weight, TDW: total dry weight. Different letters in each row show significant differences (Tukey´s test, p≤ 0.01).

h: altura de la planta, d: diámetro del tallo, SFW: peso fresco de la parte aérea, RFW: peso fresco de la raíz, TFW: peso fresco total, SDW: peso seco de la parte aérea, RDW: peso seco de la raíz, TDW: peso seco total. Letras distintas en la misma fila indican diferencias significativas (prueba de Tukey, p≤0,01).

Different letters in each treatment show significant differences (Tukey´s test, p≤ 0.01).

Las barras de error representan una DE. Letras distintas para el mismo tratamiento indican diferencias significativas (prueba de Tukey, p≤0,01).

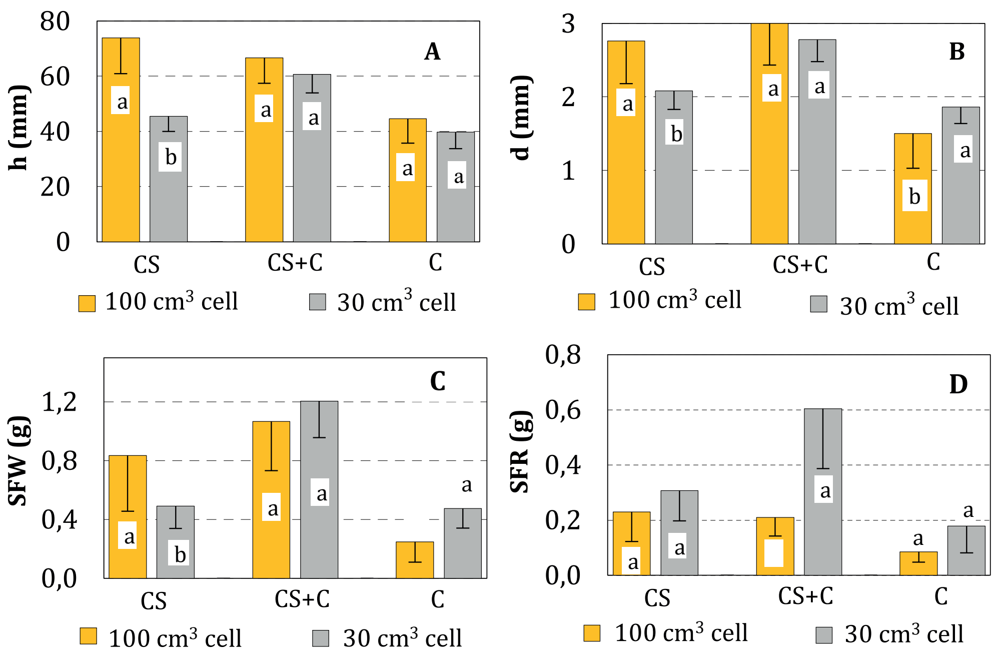

Figure 3. Seedling height (h, **A**), stem diameter (d, **B**), shoot fresh weight (SFW, **C**) and root fresh weight (RFW, **D**) for commercial substrate (CS), mixture of CS and pomace compost (CS+C) and pure compost (C) treatments in trays with large cells (100 cm^3) and small cells (30 cm^3). Error bars represent one SD.

Figura 3. Altura de los plantines (**A**), diámetro de los tallos (**B**), peso fresco de la parte aérea (**C**) y peso fresco de la raíz (**D**) para los tratamientos CS, CS + C y C en bandejas con celdas grandes (100 cm^3) y celdas pequeñas (30 cm^3).

Discussion

Experiment 1

In experiment 1 (trays with 100 cm^3 alveoli) seedling emergence and appearance time of the first, second and third true leaves revealed that the commercial substrate (CS) and the mixture with compost (CS+C) allowed the highest accumulated percentages in terms of days after sowing. In treatment C, these variables resulted in lower values (figure 1, page 132).

In this regard, the reduced seedling emergence percentages in treatment C (74% at 16 days) compared to treatments CS and CS+C (94% emergence at 11 days) could be explained by the low TP and AP values (table 1, page 131), quite different to the optimum for tomato seedlings (TP≤ 85% and AP from 20 to 30%) (1). Both factors are important for seedling germination/emergence since porosity is related to water holding and aeration capacities, allowing oxygen in the root media (26). Additionally, treatment (C) resulted to have high electrical conductivity (2.38 $dS.m^{-1}$), indicating moderate to high saline content for this species (2, 28), and consequent lower quality of easily usable water for seed imbibition and seedlings initial growth.

Appearance time of the first, second and third true leaves was markedly anticipated in CS and CS+C with respect to C, indicating that TP and AP substrate physical factors are directly related to seedling development (18), especially when associated with high OM levels, as observed in table 1 (page 131).

The highest cumulative leaf appearance percentages in CS and CS+C treatments corresponded, as expected, to the highest values of d, h, SFW, SDW, RFW and DRW. Seedling development in C, resulted significantly lower in relation to CS and CS+C (figure 1, page 132 and table 2, page 133). Similar results were obtained by Romero Aranda *et al.* (2001) when analyzing salinity effects on dry matter production, height and number of leaves of two different tomato varieties.

Experiment 2

In experiment 2 (30 cm^3 cell trays), the incorporation of a fourth treatment (C+P) formulated from the combination of compost and perlite (in relation to volumes 3:1) allowed appreciating increased total porosity and aeration porosity percentages, and a decrease in bulk density with respect to C. This resulted in significantly higher seedling developmental variables, allowing similar or superior results to those of CS.

In accordance, at the end of the experiment, 75% of plants under C+P treatment had three true leaves, lower than the 88% obtained with the CS+C treatment, and higher than the percentages obtained in CS and C treatments (49% and 37%, respectively). This could have been due to the notably higher phosphorus and potassium contents in C+P with respect to CS. Despite the complete and balanced CS formulation, after 25-30 days of growth in a volume of 30 cm^3, macronutrients content (NPK) turns deficient. According to Santacruz Oviedo *et al*. (2012), larger volume containers showed greater plant retaining capacity, benefiting late transplant. This would explain experiment 1, considering nutrient content in the CS treatment, in 100 cm^3 cells, enough for 30 days of seedling growth, and third leaf development.

The higher RDW, SDW, d and h in the C+P treatment with respect to CS and C, but not with respect to CS+C, is noticeable. This allows inferring that improvements in physical characteristics TP, AP and BD, in addition to high levels of available phosphorus and potassium, as those reported by Martínez *et al*. (2017), are necessary for plant development (table 3, page 134).

Development with different volumes of cells

The SFW, d, and h of CS treatment were significantly higher as cell size increased. These results agree with those obtained by Vagnoni *et al*. (2014), who studied, among other variables, height, leaf, stems and root dry weights, and leaf area in three different cell volumes (20 cm^3, 40 cm^3 and 120 cm^3). Similarly, Wilches Rojas *et al*. (2008) found that maximum heights occurred in trays with larger alveolar volume. According to these authors, this is given by a bigger and deeper rhizosphere with better nutrient and water absorption capacities.

In the CS+C treatment, h, d and SFW showed no important differences between cell sizes. Differences in RFW resulted in 30 cm^3 volume cells. This could be given by restricted root exploration due to smaller cell size and a subsequent greater, partitioning of biomass towards the roots with respect to the shoots, as indicated by Mugnai *et al*. (2011).

For treatment C, in 30 cm^3 cells, seedlings developed higher d and SFW than those in 100 cm^3 cells. The physical and chemical characteristics (BD, TP, AP, and EC) of this treatment, could have differentially affected seedling development in larger cells than in smaller ones.

Conclusions

Results obtained in 30 and 100 cm^3 alveolate trays allow considering pear and apple pomace compost as a complementary source of commercial substrate replacement. From the environmental point of view, usage of non-renewable resources such as peat, part of the composition of the commercial substrate, may be replaced by an eco-friendlier product, obtained from pear and apple pomace waste.

In 30 cm^3 cell trays of the compost and perlite treatment (3:1 v/v), obtained seedling quality would enable a high post-transplant survival percentage.

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Environmental factors affecting seed quality and germination of *Mimosa ephedroides* (Fabaceae), an endemic shrub from Monte Desert, Argentina

Factores ambientales que afectan la calidad y germinación de semillas de *Mimosa ephedroides* (Fabaceae), un arbusto endémico del Desierto de Monte, Argentina

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Abstract

Mimosa ephedroides is an endemic shrub from western Argentina. Knowledge on its germination response to different environmental factors contributes to an efficient use of this species in ecological restoration projects. This study aimed to examine different aspects of the species germination. Seed quality and dormancy, optimum germination temperature, effects of water and saline stresses (by using Mannitol and NaCl as osmotic agents, respectively) and of seed storage time on seed viability and germination, were evaluated. Experiments were carried out in controlled growth chambers. Germination percentage and mean germination time were calculated. Results showed that seeds of this species have high viability, low pre-dispersal loss and are non-dormant. Regarding abiotic factors, optimum germination temperature ranged from 20 to 30 °C, while high germination percentages were observed even at moderate and severe water stress (-1.12 MPa). Germination percentages decreased when salinity increased over 300 mM NaCl. For short periods (up to 36 months), seed storage at room temperature (18 °C) was adequate and did not affect seed viability or germination. These results constitute important contributions to the autecological aspects of this endemic species, supporting its incorporation in restoration projects and allowing efficient use of its seeds in direct seeding or seedling production.

Keywords

seed dormancy • viability • temperature • water stress • salinity • storage • restoration

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Resumen

Mimosa ephedroides es un arbusto endémico del oeste de Argentina. El conocimiento de la respuesta germinativa de esta especie ante distintos factores ambientales contribuye a una utilización eficiente de la misma en proyectos de restauración ecológica. Este estudio tuvo por objetivo examinar diferentes aspectos de la germinación de esta especie. Se evaluaron la calidad de las semillas y la dormición, la temperatura óptima de germinación y los efectos del estrés hídrico y salino (usando Manitol y NaCl como agentes osmóticos respectivamente) y del tiempo de almacenamiento de las semillas en su viabilidad y germinación. Los experimentos se llevaron a cabo en cámaras de crecimiento controlado. Se calculó el porcentaje de germinación y el tiempo medio de germinación. Los resultados mostraron que las semillas de esta especie presentan alta viabilidad, bajas pérdidas pre-dispersión y son no durmientes. En lo que respecta a los factores abióticos, la temperatura óptima para la germinación se encontró entre 20 y 30 °C, presentando altos porcentajes de germinación incluso con estrés hídrico moderado y severo (-1,12 MPa). Los porcentajes de germinación disminuyeron con el aumento de los niveles de salinidad a partir de 300 mM NaCl. Durante períodos cortos (hasta 36 meses), el almacenamiento de las semillas a temperatura ambiente (18°C) fue adecuado y no afectó la viabilidad o la germinación de las semillas. Estos resultados constituyen un aporte al conocimiento de los aspectos autoecológicos de esta especie endémica, apoyan su incorporación para proyectos de restauración y permiten el uso eficiente de sus semillas en la siembra directa o la producción de plántulas.

Palabras clave

dormición de semillas • viabilidad • temperatura • estrés hídrico • salinidad • almacenamiento • restauración

Introduction

The Fabaceae family presents a wide variety of adaptations and lifestyles. Many species of this family have been broadly used in restoration activities given their multiple ecosystemic benefits like food supply, carbon sequestration, soil fertility improvement, and under-canopy favourable microclimatic conditions for other species establishment (18, 35, 40). The genus *Mimosa,* belonging to the sub-family Mimosoideae and the Fabaceae family, comprises over 530 species worldwide. Thus far, only 5% of these species have been studied, and many aspects of their biology and ecology are still unknown (29). Only 56 *Mimosa* species have been recorded in Argentina, most of which grow in the Mesopotamian region (30).

Mimosa ephedroides (Gillies ex Hook. & Arn.) Benth. is one of the few endemic species of *Mimosa* that belongs to the phytogeographical province of Monte. Known by the common names of “pichana negra” and “prendedor”, it constitutes a perennial aphyllous shrub with photosynthetic stems and ephedral aspect, generally found in plains and dune areas, and occasionally, in saline environments (25). Its geographical distribution covers the Argentinean provinces of Catamarca, La Rioja, San Juan, San Luis and Mendoza, constituting this last province, its southern dispersion limit (2). This xerophytic and psammophytic plant may contribute to dunes fixation, and as a fodder and melliferous shrub (8, 12). Therefore, its incorporation in restoration and conservation projects in arid areas is considered a matter of interest. Unfortunately, information about its autecology is scarce.

In arid and semiarid areas, plant community recovery of degraded environments is usually slow and unpredictable (5, 15, 28). Native shrubs play key roles in such ecological restoration processes, since they contribute to the ecosystemic structural and functional stability (19). Their use in restoration activities is recommended, as such species are highly adapted to environmental difficulties, especially drought, low soil fertility, high temperatures, and salinity (14). Furthermore, the nursery effect generated by these shrubs facilitates other species propagule recruitment (37).

Seed germination is a critical stage in plant life cycle, since along with environmental conditions (such as climate and safe site), it constitutes a determining factor in the successful establishment of seedlings (1, 16, 28). In seed-based restoration efforts, one of the main factors limiting seedling efficient production and establishment is related to limited knowledge on seed biology of native species (20, 25, 38). In arid lands, transplanting seedlings into the field is one widely used technique due to direct-sowing low success (7).

Seed dormancy is an extended adaptive strategy of native shrubs from arid lands, increasing the possibilities of seedling survival in unpredictable environments (1, 14, 18). A dormant seed does not germinate even under favourable conditions for germination (1). Different authors have verified the existence of physical dormancy in seeds of *Mimosa* species, due to impermeable seed coats (3, 4, 33, 38). Scarification is the most effective technique for overcoming this type of dormancy (5, 29, 33). In this sense, the fact that species of the same genus have similar dormancy traits, is often assumed. However, the presence of physical dormancy should be tested for each species, since it may vary within families, genera, and populations (41). Identifying dormancy and its proper rupture method, is fundamental to efficiently spread a species. For this purpose, and for proper dormancy classification, seed imbibition tests must be performed before pre-germination treatments (1, 17).

Seed quality determinations should be carried out before dormancy testing. Seed quality assessment is aimed at obtaining information about the collected material concerning viability, percentage of damaged seeds, and seed mass, among others (22). Regarding non-viable seeds, abortion and predation are biotic factors that may represent large pre-dispersive losses for some species (46). Consequently, these factors might limit availability of quality material for restorative tasks. In *Mimosa* species, pre–dispersal seed predation is mostly due to insects of the *Bruchidae* family (3, 42, 44).

Seed mass constitutes a critical trait in plant life. Strong correlations among this trait and seed germination, plant survival, and establishment have been identifyed (22). It may vary significantly among different species within a genus, and among different populations within a species (28). Therefore, to study this trait in local seed provenances, becomes important for restoration tasks.

In arid areas, abiotic factors like temperature, water stress and salinity, trigger germination responses (5, 6, 27, 47). Knowledge on tolerance limits to these factors is essential to understand the adaptive capacity of the species under such conditions (11, 28), contributing to efficient restoration improvement.

The range of germination temperature is related to the temperature intervals that characterize the most favourable establishment time of year. This may vary throughout the distribution area of the species (27). Water availability is the main environmental filter affecting germination. Endemic and native species of arid and semiarid lands, typically present strategies to tolerate or avoid such environmental filtering at their different stages of development (10, 11, 28). Water potentials required for germination may vary greatly among species from such areas. While a group of native plants produces seeds with low moisture thresholds adopting risk-tanking strategies, others only germinate at high water potentials (11). With regards to salinity effect on germination, osmotic effects and ionic toxicity are generally evaluated with NaCl, most frequently found salt in saline environments (6, 26, 47).

Other factors influencing germination are seed storage periods and conditions (1, 17, 43). In ecological restoration projects, having available quantity and quality of plant material, is necessary. As a result, in many cases, drawing on to ex-situ conservation of the seeds for variable periods, is required. Species from the Fabaceae family do not significantly reduce their viability or germination when stored in dry environments at room temperature (~18°C) for short periods (< 5 years) (17, 43).

In order to ensure the most efficient use of native seeds in restoration activities, understanding the various factors that affect germination responses and seed quality, turns necessary. Therefore, this study aimed to characterize *M. ephedroides* seeds, evaluate the presence of seed dormancy and the effect of temperature, water and saline stresses on germination percentages, as well as the effect of storage time at room temperature on germination and seed viability.

Material and methods

Seed collection

Fresh mature fruits of *M. ephedroides* were randomly harvested in March 2016 from more than 20 adult specimens. The collection area corresponds to the farmer household of "La Majada", located in Lavalle department, NE of Mendoza province (32° 21' 59.4" S 67° 54' 26.9" W), phytogeographical province of Monte. Its climate is arid, with an average annual rainfall of 159 mm, occurring mostly in summer (12). The average annual temperature is 18.3 °C, the absolute minimum temperature in the coldest month (July) is -10 °C, and the absolute maximum temperature in the warmest month (January) is 48 °C. Soils are typically torrid.

Characterization of the collected material

Seeds were manually separated from the pods and stored in wooden paper bags in dry environment at room temperature (18± 2°C). Five grams of Carbaryl were included in each bag to prevent insect damage.

Seed mass, percentage of aborted and predated seeds, and seed viability were measured to assess seed quality. Seed mass was determined by weighing six samples of 100 seeds. Pre-dispersal seed loss was evaluated after randomly harvesting samples of four replicates with 50 seeds. Seeds were classified as follows (44): well-formed and healthy (without any evidence of predation and fully developed) (figure 1a); aborted (non-fertilized ovules or underdeveloped seeds) (figure 1a), and predated (damaged tissues caused by insects) (figure 1b). To ensure that well-formed seeds were not predated, they were cut in half, getting rid of not emerged insects. Averages were expressed in percentages. Insects found in the seeds were well preserved for later identification. Seed viability was assessed by the Tetrazolium topographical test (TZ), with four replicates of 20 seeds each. All seeds were hydrated for 12 h and then split in halves. Only those halves containing the embryo were soaked in a 1.5% solution of 2.3.5 triphenyltetrazolium and then placed in a growth chamber at 40 °C and darkness, for 1h. Finally, assisted by a stereoscopic magnifying glass, those seeds with red-colored embryos were classified as viable.

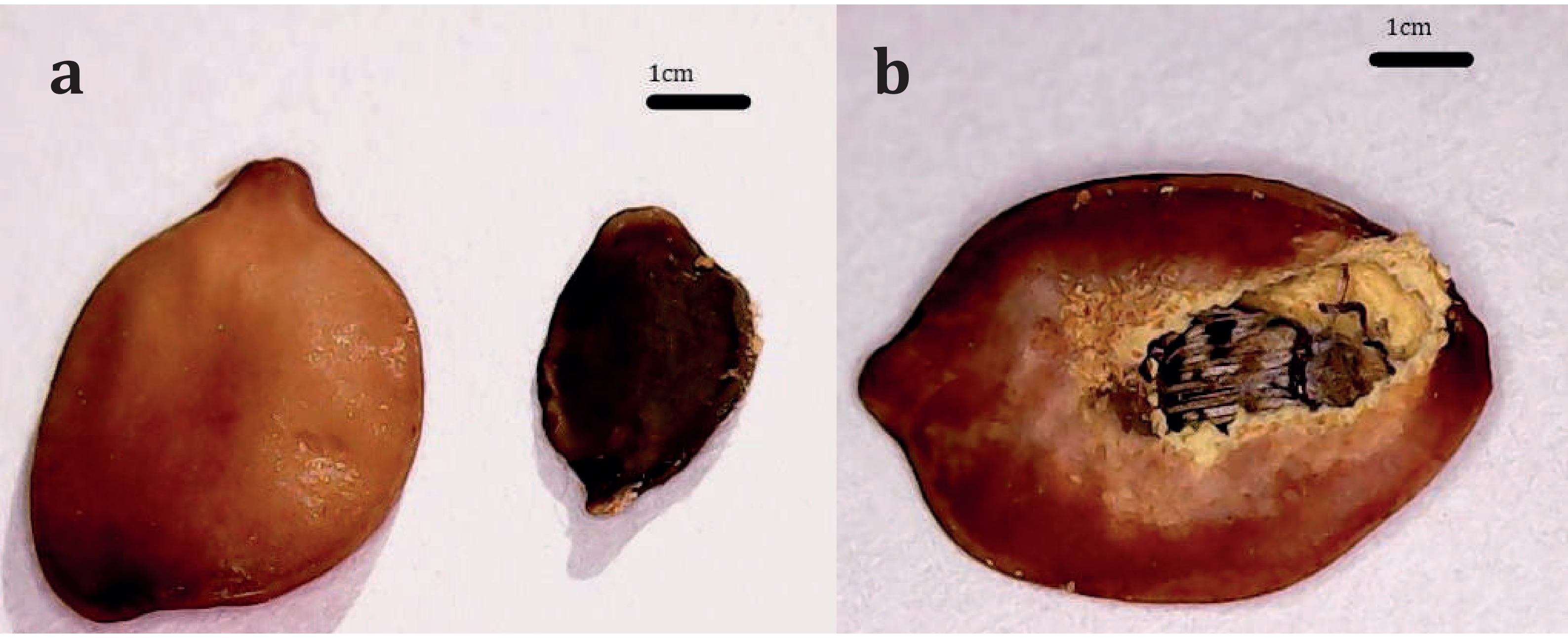

Figure 1. a) Well-formed and healthy seed (left), and an aborted seed (right) of *M. ephedroides* b) *M. ephedroides* seed without endocarp and an adult bruchid inside.

Figura 1. a) Semilla bien formada y sana (izquierda), y una semilla abortada (derecha) de *M. ephedroides* b) Semilla de *M. ephedroides* sin endocarpio y con un brúquido adulto en el interior.

Imbibition test

At the beginning of the experiment, dry seeds were weighed. Then, non-scarified and mechanically scarified seeds (using a fine 220-grit sandpaper) were placed in Petri dishes with tissue paper discs soaked in distilled water and incubated at 20 °C. Four replicates of 20 seeds each, were used. Later, all seeds were removed from the Petri dishes, dried with tissue paper, and re-weighed on a precision analytical balance, at 2-hours intervals for 10 h and then, after 20 h from the beginning of the assay. Seed moisture content was determined gravimetrically (15).

Effect of abiotic factors on seed germination

General conditions

Germination assays were performed in 90 mm diameter sterile Petri dishes. They were conditioned with three layers of filter paper saturated with the corresponding solutions for each test. Seeds were disinfected with sodium hypochlorite (2 g/l of active Cl) by complete immersion for ten minutes and then rinsed three times with distilled water (20). Only those well-formed and healthy seeds (figure 1a, page 142), were used. No pre-germination treatments were carried out. Germination was assessed on a daily basis. Four replicates of 20 seeds each, were used for all treatments. Seeds were considered germinated when their radicle reached 2 mm long (15). All germination trials lasted 15 days. Preliminary trials had determined that seeds germinate equally under light or dark conditions (data not shown), thus experiments were conducted under continuous darkness and constant temperature.

Final germination percentage (G) and mean germination time (MGT) were assessed for each treatment. MGT was calculated as Merino-Martín *et al.* (2017):

$$\mathbf{MGT = (\Sigma\, n * T) / \Sigma n}$$

Where:
n = number of germinated seeds per day
T = time measured in number of days

Temperature

Temperature tests were carried out simultaneously in four growth chambers with four levels: 10, 20, 30 and 40 °C. Paper discs in Petri dishes were saturated with distilled water.

Water stress

Water stress was simulated by Mannitol solutions with the following water potentials: 0.0, -0.14, -0.56, -1.12,-1.50, -1.72 and -2.2 MPa. The desired concentration of Mannitol solutions was calculated according to Fernández and Jhonston (1986). Petri dishes were incubated at 20°C, optimal temperature already determined in the temperature test.

Saline stress

Salinity effects on germination were evaluated with NaCl as osmotic agent. Solutions were prepared with the following concentrations: 0 (control); 100; 200; 300; 400 and 500 mM. Petri dishes were incubated at the determined optimal temperature (20°C). After 15 days, viability of non-germinated seeds was checked by TZ test.

Effect of storage time

Seeds stored in a dry environment and at room temperature (18 ± 2°C) were extracted at 0; 6; 12; 24 and 36 months after harvest. Effects of storage time on viability, G and MGT were tested according to the above-mentioned methods.

Data analysis

The experimental design was completely randomized. G and MGT data were analyzed by one-way ANOVA. In order to meet analysis assumptions, germination percentages at the end of the assay, were arcsine transformed. If significant differences were detected by ANOVA, Tukey's test with p≤ 5% was chosen for multiple comparison test. Analyses were performed using Infostat Professional 2018 software (9). Untransformed data is shown in all figures and tables.

Results and discussion

Characterization of the collected material

Characterization of collected material constitutes a useful tool for restorative tasks (15), allowing to estimate available seed quantity according to pre-dispersal loss, viability, and seed weight. Table 1, shows such parameters for mature seeds of *M. ephedroides,* at harvest time.

Table 1. Mature seeds mass (g), viability (%,) and pre-dispersal seed loss by abortion and predation (%) of *M. ephedroides* collected material (S.E. between parentheses).

Tabla 1. Masa de semillas maduras (g), viabilidad (%) y pérdida pre-dispersión por aborto y depredación (%) del material recolectado de *M. ephedroides* (D.E. entre paréntesis).

Parameter	Value
Seed mass	0.025 (0.02)
Viability of well-formed seeds	97.5 (2.8)
Aborted seeds	12.0 (2.5)
Predated seeds	18.0 (1.5)

Concerning seed mass, environmental factors like rainfall, photoperiod, and temperature, may influence this feature (24). *M. ephedroides* seed mass (table 1) was higher than that recorded for other species of the same genus and from arid areas, such as *M. luisana*, *M. aculeaticarpa var aculeaticarpa*, *M. calcicola* and *M. lacerata* (3, 29). Leishman and Westoby (1994) pointed out that under low humidity, larger seeds present better seedling establishment. Moreover, Rubio de Casas *et al.* (2017) suggested that species with non-dormant seeds would probably have heavier seeds than those with physical dormancy. Consequently, this trait might have important implications for restorative tasks.

The Tetrazolium viability test is a useful tool for seed quality determinations. Due to lack of seed testing procedures for some native species, to adjust concentrations of the agent used, staining time, or seed pre-treatment, turns necessary (34). The chosen methodology resulted to be adequate for viability assessment of *M. ephedroides* seeds. This species presents high viability values (table 1), coinciding with other species of the same genus (29, 33, 38).

Insects collected from fruits and seeds of *M. ephedroides* were identified as bruchid beetles (Chrysomelidae: Bruchidae) (Roig, pers. comm 2019) (figure 1b, page 142). For different *Mimosa* species, bruchid damaged seeds may vary between 25-70% (3). Unlike its congeners, *M. ephedroides* presented relatively low parasitized seed percentages (table 1), (38, 42, 44). However, bruchid predation on *M. ephedroides* seeds, can significantly affect germination (data not shown). For species of the *Mimosoideae* subfamily, pre-dispersal seed predation by other insects of the Coleoptera order, such as apionids weevils, has also been cited (46). Nevertheless, these insects were not found in the collected material. Concerning pre-dispersal seed loss by abortion (table 1), this fact resulted to be similar to that found in other species of the *Mimosoideae* subfamily from Monte desert (46).

Imbibition test

After 20 h, seed mass of non-scarified and scarified seeds showed substantial weight increases of 61.90±1.42 % and 62.80±0.62 % respectively, but without significant differences between them ($F=1.36$; $p=0.2873$). After reaching this moisture content, the radicle emerged. According to Parsons (2012), *M. ephedroides* is a fast-germination species since under suitable conditions it germinates in less than 24 h after imbibition, meaning that, *M. ephedroides* seeds have permeable coats, and thus, scarification treatments are not required. These results were consistent with those found by Galindez *et al.,* (2016) for other Argentinean native legumes with no physical dormancy, but differed from those observed in most species belonging to *Mimosa*, with physical dormancy given by one or more layers of palisade cells (1, 3, 4, 26).

Effect of abiotic factors on seed germination

Temperature

Temperature had statistically significant effects on germination (F= 17; p<0.0001) and MGT (F= 44.30; p<0.0001). Germination occurred from 10 to 40°C, in accordance with the fact that *Mimosa* species germinate at a wide range of temperatures (3, 4, 26, 30). At 10°C, the lowest germination percentage and the major MGT were recorded, showing significant differences with the remaining temperatures (figure 2). Slower germination is probably related to slower metabolic activity at that temperature (13).

a) Temperature; b) Water stress simulated with Mannitol; c) Saline stress simulated with NaCl and d) Seed storage time at dry conditions and room temperature. Different letters show significant differences for Tukey at p < 0.05.

a) Temperatura; b) Estrés hídrico simulado con Manitol; c) Estrés salino simulado con NaCl y d) Tiempo de almacenamiento de las semillas en condiciones secas y a temperatura ambiente. Letras diferentes muestran diferencias significativas para Tuket a p<0,05.

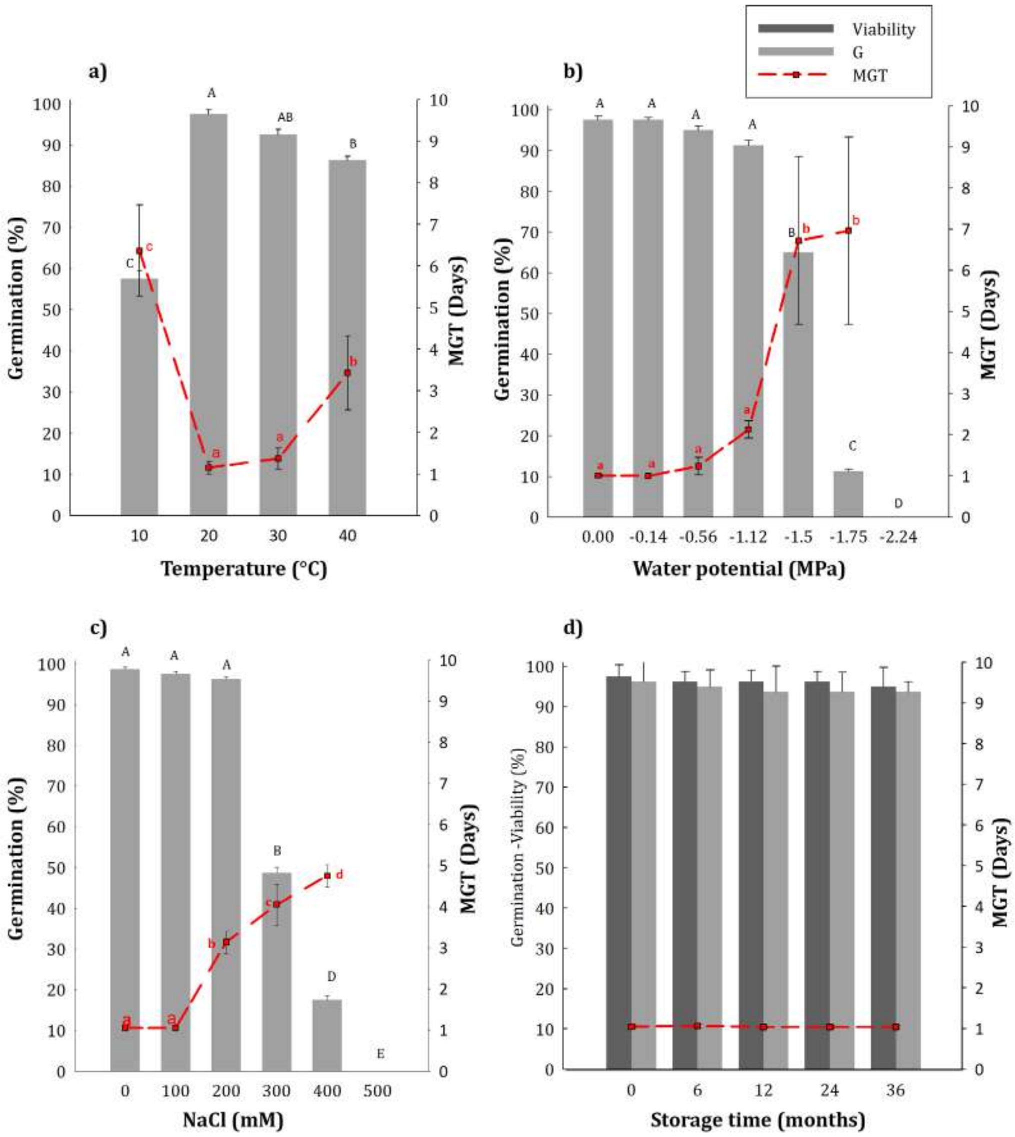

Figure 2. Effect of abiotic factors on germination percentage (G) (mean ± SE) and mean germination time (MGT) (mean ± SE) of *M. ephedroides.*

Figura 2. Efecto de factores abióticos sobre el porcentaje de germinación (G) (media ± EE) y el tiempo medio de germinación (MGT) (media ± EE) de *M. ephedroides*.

Optimum temperatures are those in which the highest germination percentages are obtained, in the shortest time. For *M. ephedroides,* optimal temperatures range from 20 to 30°C. This range occurs during summer, with the highest water availability in the Monte desert, allowing maximum seedling survival and establishment. These results were consistent with those obtained for other native shrubs in the Monte Province (14, 15, 27, 37) and other *Mimosa* species from arid lands (3, 4, 29).

Water stress

Water stress had a significant effect on germination ($F=78.69$; $p<0.0001$) and MGT ($F=20.78$; $p<0.0001$) of *M. ephedroides*. Germination percentage significantly decreased for osmotic potentials lower than -1.5 MPa, while the highest MGT was observed at those potentials. At-2.24 MPa, germination was completely inhibited.

Above −1.12 MPa, moderate to severe water stress, germination was greater than 90%, evidencing remarkable tolerance to this stress factor (figure 2b, page 145). As for MGT, at -1.12 MPa, germination almost doubled the control value (0.00 MPa), and even occurred in a short period of time (MGT< 2 days). Rapid germination under drought allows for efficient water use during the short period of availability. This germination-related trait has an important influence during seedling establishment in restoration activities (21). According to Duncan *et al.,* (2019), this species presents a risk-taker strategy to achieve germination. Therefore, the ability of *M. ephedroides* to germinate even under low water availability makes it a promising species for seed-based restoration efforts in arid lands, where water is the main limiting factor.

Similarly, drought tolerance during germination has been reported for other species of the Mimosoideae subfamily, from the same distribution area of *M. ephedroides*. For instance, germination of some *Prosopis* species is only significantly affected by osmotic potentials under -1.4 MPa (6, 41). On the other hand, unlike the results obtained in the present work, studies carried out with other *Mimosa* species of subtropical distribution indicate that their germination is strongly affected under decreasing water potentials, suggesting probable environmental adaptation. Germination of *M. pudica* and *M. invisa* decreases significantly from -0.8 MPa and it is completely inhibited at -1.12 MPa (4), while for *M. bimucronata* germination is significantly affected from -0.49 MPa (40). Li *et al.,* (2013), postulated that seed drought resistance could be linked to the accumulation of organic osmolytes, allowing cell membrane functionality to be maintained.

Salt stress

Seed germination response of *M. ephedroides* to increased concentrations of NaCl, indicated that germination ($F=182.25$; $p<0.0001$) and MGT ($F=141.69$ $p<0.0001$) were significantly affected by saline stress. High germination percentages were observed up to 200 mM (figure 2c, 145). The salinity threshold, above which germination percentage and MGT were significantly affected, was of 300 mM. Nasr *et al.,* (2012), pointed out that saline stress during germination affects cell division and elongation, and therefore, mobilization of essential reserves for this stage. Furthermore, under saline stress, seed reserves that could be used for growing embryonic axis are used to repair damaged cells (45). Saline stress increases MGT and reduces germination percentages, as shown by this study.

Likewise, under saline stress, other moderate salt tolerant legumes present similar germination behaviors. For instance, germination of *Mimosa invisa* and *Prosopis argentina* was significantly affected from 300 mM NaCl and inhibited at 400 mM NaCl (4, 47). Conversely, high salt-tolerant species from *Mimosoidea* subfamily such as *Prosopis alpataco* and *Prosopis alba* were able to germinate at >85% under 400 mM, while germination was still not inhibited even at 600 mM NaCl (26, 47).

Seeds of glicophyte and halophyte species generally show higher germination rates at low salt concentrations. However, seeds of halophyte species may remain viable at high salt concentrations, constituting an adaptive characteristic (16). In the present study, seeds that did not germinate at 300 mM NaCl or more, did not remain viable at the end of the experiment, suggesting a glycophytic type of behaviour regarding this trait.

Effect of storage time

In the present study, both recently harvested seeds and those stored at room temperature for 6, 12, 24 and 36 months, presented high viability and germination percentages at optimum temperature (figure 2d, page 145). These variables did not register statistically significant differences from those at different storage periods (F=0.50; p=0.20). Seed storage conditions of *M. ephedroides* caused no seed deterioration, neither secondary dormancy processes. Besides, MGT was not affected by storage period (F=0.27; p=0.89). These results match with those found by other authors for other species of *Mimosoideae*, where seeds stored at room temperature remain viable for several years (20). For some species of *Mimosa,* storage at room temperature for long periods can increase germination or reduce MGT (32, 36, 47). These differences are probably due to physical dormant seeds of some species that may become permeable to water after long storage periods (2, 36). Nevertheless, in the present study, no differences were observed among these variables.

Conclusions

Several factors affect germination of *M. ephedroides.* Concerning the biotic factors studied in this research, bruchid predation and abortion did not prevent the obtention of quality and quantity material of this endemic shrub. Mature seeds of this species exhibit high viability, have a permeable coat and germinate at high percentages under a wide range of conditions. Therefore, physical dormancy or any other type of primary dormancy, were ruled out. Moreover, dry storage at room temperature was an effective method for ex-situ conservation of these seeds, at least, for up to 36 months. These characteristics facilitate restoration programs with this species seeds.

Regarding germination responses of *M. ephedroides* to different abiotic factors, this species resulted able to germinate in a wide range of temperatures and under moderate and severe water stress. This constitutes an interesting adaptive strategy to the local environment. In the Monte desert where rainfall is scarce and unpredictable, these features, plus its germination behaviour could evidence that this species takes advantage of small pulses of precipitation to germinate and probably, establish. However, *M. ephedroides* tolerates low salt concentrations during germination, thus, multiplying it under more than 200 mM NaCl, is not recommended. Besides, its usage in saline environments, would be restrictive.

Since *M. ephedroides* mature seeds are non-dormant, present high viability, and rapid germination in a wide range of temperatures and water availability, this shrub is considered a promising species for ecological restoration tasks, in arid lands. These findings constitute an essential contribution to the knowledge of basic autecological aspects of this endemic legume shrub from the Monte Desert. Moreover, they could assist an efficient use of native seeds for direct seeding or seedling production. Future studies on seed provenances, factors affecting direct seeding, and seedling pre-conditioning and transplanting techniques in degraded environments, are required to improve the use of this species in restoration tasks.

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Key factors affecting the technical efficiency of bee farms in the province of La Pampa (Argentina): A two-stage DEA approach

Factores clave que afectan la eficiencia técnica de las granjas apícolas en la provincia de La Pampa (Argentina): un enfoque DEA en dos etapas

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Abstract

This paper analyzes how decision-making, management capacity and technology adoption by beekeepers, affect the technical efficiency (TE) of Argentinean beekeeping through the case study of the province of La Pampa (Argentina). The assessment of TE is currently receiving ever-growing attention as an indicator of sustainability and usage of sufficient natural resources in beekeeping activities. This study aimed to identify the key factors affecting the technical efficiency of bee farms in the province of La Pampa. The study included a sample of 40 bee farms and estimated their TE score through an input-oriented Data Envelopment Analysis (DEA) model. In a second stage, Tobit regression was determined to evaluate the technical inefficiency determinants. This paper found that most beekeeping production units have low TE levels. Only 25 % of bee farms produce either at or close to the frontier. The Tobit model revealed that variables such as marital status, educational level, primary family income, source information usage, planning and health area, affect positively on pure technical efficiency. These results are considered to be of great interest for structured beekeeping systems on small-scale and family farms, as well as for political decision-makers, regarding a public program in apiculture.

Keywords
sustainability • bee farm management • decision-making • DEA • Tobit model

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Resumen

En este documento se analiza cómo la toma de decisiones, la capacidad de gestión y el nivel de adopción tecnológica por parte de los apicultores, afectan a la eficiencia técnica (ET) de la apicultura argentina a través del estudio de caso de la provincia de La Pampa. La evaluación de la ET está recibiendo una atención cada vez mayor como un indicador de la sostenibilidad, así como en el uso adecuado de los recursos naturales en las actividades apícolas. El objetivo de este estudio fue identificar los factores clave que afectan a la eficiencia técnica de las explotaciones apícolas de la provincia de La Pampa. El estudio incluyó una muestra de 40 unidades apícolas situadas en la provincia de La Pampa para estimar su nivel de ET mediante la metodología de Análisis de la Envolvente de Datos (DEA) orientado a los *inputs*. En una segunda etapa se empleó la regresión Tobit para evaluar los factores determinantes de la ineficiencia. En este trabajo se comprobó que la mayoría de las unidades de producción apícola tienen niveles bajos de ET. Solo el 25% de las unidades apícolas producen en la frontera de producción o cerca de ella. El modelo Tobit reveló que variables como, estado civil, nivel educativo, principal fuente de ingresos familiares, uso de información, planificación y el área de salud, son las que afectan positivamente a la ET pura. Estos resultados se consideran de interés para los sistemas apícolas de pequeña escala y familiares, y pueden resultar útiles para los responsables de las políticas sobre un programa público de apicultura.

Palabras clave

sostenibilidad • manejo de la granja apícola • toma de decisiones • DEA • modelo Tobit.

Introduction

The Food and Agriculture Organization of the United Nations (FAO) has the commitment to eradicate hunger and reduce poverty in the world by ensuring food security and improving livelihoods. Besides, FAO has five strategic objectives, one of them being to "Make agriculture, forestry and fisheries more productive and sustainable", likewise including a more productive, sustainable, and efficient apiculture (20).

Argentinian beekeepers own over 3.5 million beehives distributed by 30,000 production units, which produce more than 80,000 t per year, with an income of 180 million US$ the last year by international trade. Thus, Argentina is the leading country in America, exporting honey wordwide (32). An insufficiently developed domestic consumer market, favourable monetary exchange rates and the existence of official traceability schemes to guarantee the quality of the final product, are factors contributing to this scenario (21). Moreover, ecological conditions prevailing in Argentina ensure the availability of flowers from wild species and cultivated plants. Production of honey is concentrated in the Pampean region, contributing 70% of the total production in Argentina (47).

In the last years, various approaches to decision-making and managerial capacity have been widely used to quantify and compare the performance of agricultural and livestock systems (34, 36, 38, 39, 51, 53). Furthermore, the concept of efficiency has been awidely used tool for evaluating technical and economic success (6, 7, 15, 29, 41). So far, several studies have been carried out on beekeeping all over the world. Some researchers have focused on the technical side of beekeeping, while the rest have been interested in the economic dimension of this activity (2, 3, 42). However, the studies focused at a beekeeper level have been rare all over the world since the process of collecting information requires more time (12). Some researchers have focused only on production indicators by ignoring the details of data management. Relatively few researchers have studied some aspects using detailed data management at a beekeeper level (28, 33, 46). It is noteworthy that Lema and Delgado (2000) investigated the sources of technical efficiency (TE) including decision-making and managerial capacity variables in producers from the province of Buenos Aires. Besides, Bragulat *et al.* (2018) studied the influence of decision-making and managerial capacity of La Pampa honey producers in the economic feasibility. The well-fitting regression model was constructed with variables related to decision-making knowledge and evaluated the principal lack of beekeepers in the management of their productive units.

A key indicator of resource optimisation within farming systems is the assessment of TE, which measures the amount of physical output attainable from a given set of inputs. The approaches estimating TE are divided into two methodologies: the parametric methods, including the construction of stochastic (8) or deterministic (6, 51, 52) frontier models; and non-parametric methods. Recently, the measurement of productive efficiency with the non-parametric approach has used the Data Envelopment Analysis (DEA) method. DEA involves linear programming to calculate the frontier of an economic unit and identify the proportion of inputs with the maximum efficiency, or the portion of maximum output with the limited inputs (7, 28). DEA developed by Charnes *et al.* (1978), has also been widely used to estimate the TE score of livestock (13).

Using this procedure, the most efficient beekeeping production unit in a sample can be used as the main reference to measure the relative efficiency of each unit and to determine the causes of inefficiency. This approach, commonly used in economics, has proven to be useful when evaluating livestock production systems. Therefore, its application to a system such as beekeeping could lead to better knowledge for the improvement of sustainability on bee farms. Based on this background, our study aimed to identify the key factors affecting the technical efficiency of beekeeping production units of La Pampa (Argentina). The method was based on a two-stage analysis approach, with productivity measures derived in the first stage using input-orientated DEA. In the second stage of the analysis, the influence of apiarist managerial capacity and farmer decision-making on the derived efficiency was examined through the Tobit regression model. It is worth to note that this study is the first to apply the DEA method of apiculture sector in Argentina. Understanding this issue will be beneficial for planning actions to alleviate inefficiency and to improve the agricultural production efficiency in Pampean beekeeping.

Material and methods

Study area and data collection

The study was carried out in the province of La Pampa in Argentina, which is composed of 1,500 bee farms, distributed in four of the province´s 22 administrative departments (45). La Pampa is located in the geographic centre of Argentina and covers an area of about 143,440 km^2 (approximately 5.2% of the country). The soil, which shows a slight slope towards the East, and mild undulations from North to South, is constituted by sandy sediments, ranging from 1m, in the West, to over 6m depth in the East, with no rocky patches (27). The climate of the area is characterized by mild winters and summers, with seasonal rains during the spring. The average annual precipitation and temperature are 724 mm and 15°C, respectively.

According to Milán *et al.* (2011), a randomized sampling design, stratified per department with proportional allotment, was used (0.95 confidence level, 0.1 precision and 0.5 estimated true proportions). The selected sample comprised 40 bee farms (which constituted a 5.3 % of the studied population) and included beekeepers ready to provide information.

Information related to management was obtained by direct interviews with the apiarist or the manager, and by direct observation on farms during the period 2011-2013. This was based on the methodology described by Perea-Muñoz *et al.* (2011), which was appropriately adapted to beekeeping.

Information that was gathered from the questionnaire included 18 management variables, which were defined under the hypothesis that they may explain differences in farm technical inefficiency. In a similar way, Perea *et al.* (2014), studied the effect of management variables in dairy farm viability. Management variables used are shown in table 1 (page 153).

Nine variables were selected to represent the influence of the apiarist personal aspects (table 1, page 153): family size, civil state, age, experience, educational level, personnel training, continuity of activity, primary source of family income and primary source of income for the apiarist. The influence of the decision-making process was studied through nine variables representing two aspects: access and use of information by the manager, and the formality of the process.

Table 1. Definition of variables as influencing technical efficiency of bee farms.
Tabla 1. Definición de variables que influyen en la eficiencia técnica de las explotaciones apícolas.

Variable	Definition
Apiarist´s personal aspects	
Family size (FS)	Number of family members
Civil state (CS)	Dummy = 1 If the producer is married or divorced, 0 if he is single
Age (A)	Manager age
Experience (E)	Number of years of managerial experience
Educational level (EL)	Dummy = 1 If it is secondary or higher, 0 if it is primary level.
Personnel training (PT)	Dummy = 1 If it is has taken beekeeping courses, 0 if there is not
Continuity of activity (CA)	Dummy = 1 If it is continuing with the long-term activity, 0 if there is not
Primary source of family income (PSF)	Dummy = 1 if only beekeeping, 0 if there are another source of family income
Primary source of apiarist income (PSA)	Dummy = 1 if only beekeeping, 0 if there are another source of apiarist income
Decision–making process	
Record (R)	Dummy = 1 If data are periodically recorded, in an organized manner, 0 if there is not
Record use (RU)	Dummy = 1 If records are used formally, 0 if there is not
Information use (IU)	Dummy = 1 If external information is used, 0 if there is not
Economic advisors (EA)	Dummy =1 If periodic economic advisers are available, 0 if there is not
Technical advisors (TA)	Dummy =1 If periodic technical advisers are available, 0 if there is not
Associationism (As)	Dummy =1 If the production unit is integrated in some producers' association, 0 if there is not
Objective (O)	Dummy = 1 If there is a clear a consistent planning with corporate aims, 0 if there is not
Planning (P)	Dummy = 1 If there are ongoing plans coherent with the objectives, 0 if there is not
Evaluation (E)	Dummy = 1 If the objectives in the assessment procedure used by the manager to evaluate the outcome of his plans, 0 if there is not
Technologies adoption level	
Equipment area (% T1)	Percentage of technologies that maximize the use of the infrastructure
Health area (% T2)	Percentage of technologies that allow to mitigate the risks associated with animal health, and enhance and guarantee the quality of the products
Feeding area (% T3)	Percentage of technologies that identify and optimize the feeding system

The information obtained by the manager was studied through the following variables: records, economic advisors, technical advisors and associationism. Regarding the use of information, apiarists were asked to classify it in each of the provided sources. The usage of records and information involves variables indicate the regular use of the archives, and the frequent use of external information sources, respectively. The formality of the decision-making process was evaluated according to three aspects: objective, planning and evaluation. The apiarists were requested to describe the goals of their business, their ongoing plans and whether if they knew their programmes worked.

Furthermore, technology adoption level was analysed (table 1, page 153). To do this, three variables that indicate the level of adoption in the main technological areas, were evaluated. These technological areas were equipment (% T1), health (% T2) and feeding (% T3). Each area has a set of technologies and innovations allocated called items. The technological area indicates the proportion of the items adopted by each production unit over the total of items assigned in the area. Identification of technologies and innovations relevant to the apicultural system and their allocation in areas, was achieved through a participatory process of discussion and consensus, following the methodology described by García-Martínez *et al.* (2016). A similar procedure was applied by Freitas *et al.* (2004) to evaluate the technological level of beekeeping in Brazil. The equipment area comprised six items: basic beekeeping facility, basic apiculture machine, basic beekeeping equipment, honey extraction capacity, honey collection capacity and wax melting capacity. The health area included seven items: disease prevention program, ability to diagnose pathologies, antiparasitic rotation, and application of routine treatments against Varroasis, American foulbrood, European foulbrood and Nosemosis. Finally, the feeding area consisted of five items: winter feeding, protein supplementation, productive incentive, transhumance of hives and queens rearing.

Data Envelopment Analysis and Tobit regression analysis

Data Envelopment Analysis (DEA), a non-parametric method, whose was first proposed by Farrell (1957) and then improved by Charnes *et al.* (1978), has become a popular tool for measuring the technical efficiency of larger application field. In economics, a decision-making unit (DMU) is considered to refer to an individual or entity. Thus, DEA measures the efficiency of each DMU relative to that frontier, attributing all observed deviations from that frontier to inefficiencies. DMU is a commonly used term in DEA analysis, and it corresponds to a bee farm in La Pampa Province.

DEA model needs an orientation, either input or output-oriented. Pampean beekeepers tend to have greater control over their inputs than they have over their output. For that reason, an input-oriented model was applied in order to reduce the inputs. DEA model was constructed by 40 DMU of the sample considering one output and four inputs. Table 2 shows the output and inputs, their units, means, standard deviations and coefficient of variation. The output used was honey production (kg/year) as the main product coming from bee farms. According to our literature review, the chosen inputs were investment (ARS), the number of hives, feed cost (ARS/year) and labour cost (ARS/year).

Table 2. Descriptive statistics of the inputs and outputs used to estimate technical efficiency.

Tabla 2. Estadísticas descriptivas de insumos y productos utilizados para estimar la eficiencia técnica.

Variable	Mean	Standard deviation	Minimum	Maximum
Output				
Honey production (kg/year)	7,398.75	13,745,53	30	56,000
Inputs				
Investment (ARS)	276,645.87	401,566.79	17,200	1,819,550
Number of hives	339.75	477.30	20	2,000
Feed costs (ARS/year)	2,901.62	4,775.67	0	18,000
Labour costs (ARS/year)	3,894.5	9,928.06	0	54,000

1 USD = 4,4 Argentinian pesos (ARS).

1 USD =Pesos argentinos (ARS).

The number of DMUs has an essential impact on the degree of freedom; Cooper *et al.* (2011) provided a rough rule to solve this problem in the DEA model. The recommendation is to select a value of *n* that satisfies $n \geq \{m \times s; 3(m + s)\}$ where n is the number of DMUs, m is the number of inputs, and s is the number of outputs. Therefore, the number of DMUs in our sample satisfies the rule in this study.

The data for all bee farms were represented by the K ×N input matrix (X) and M × N output matrix (Y). Using piecewise linear construction of technology, an input-oriented measure of efficiency can be calculated for the i-the beekeeping farms the solution to linear programming (LP), as follows:

$$\text{Minimising}_{\theta\,\lambda}\,\theta$$

Subject to

$$-y_i + Y_\lambda \geq 0$$

$$\theta_{xi} - X\ \lambda \geq 0$$

$$\lambda \geq 0$$

$$N1\lambda = 1$$

where θ is the TE score having a value between 0 and 1, this means that the maximum proportional reduction of all inputs is (1 - θ) while holding output constant; the vector λ is an N ×1 vector of weights which defines the linear combination of the peers of the i-th beekeeping farms; N1 is a vector of ones and the restriction N1 λ = 1 allows for variable returns to scale.

Overall technical efficiency or technical efficiency at constant return to scale (TEcrs) for each DMU, can be broken down into pure technical efficiency (technical efficiency at variable return to scale, TEvrs) and scale efficiency (SE). TEcrs does not include the portion of any inefficiency that is the result of not operating at the optimal scale. The SE of each DMU is given by the ratio TEcrs/TEvrs (17), where EE=1 indicates constant returns to scale and EE<1 shows scale inefficiency. SE is due to either increasing or decreasing returns to scale, which can be determined by inspecting the sum of intensity variables (9). Finally, SE quantifies the impact of scale on the productivity of a DMU. When the SE is equal to one, the constant returns to scale prevails for that DMU; and if the SE is less than one, then either increasing (IRS) or decreasing (DRS) return to scale exists.

The efficiency analysis was executed under CRS as well as VRS, as the scientific literature shows no clear preference concerning the assumptions of scale economics in beekeeping. However, some beekeeping´s researchers have focused its studies on VRS specification (28).

Once the model was determined, and the level of efficiency of the beekeeping farms was calculated, a set of exogenous or explanatory variables were related to the efficiency scores in order to determine the causes of inefficiency. Explanatory variables used are shown in table 3.

Table 3. Explanatory variables used in Tobit models.

Tabla 3. Variables explicativas usadas en los modelos Tobit.

Dependents variables	Apiarist´s personal aspects	Decision–making process	Technology adoption level
TE_{VRS} TE_{CRS} SE	Family size (FS) Age (A) Experience (E) *Dummies* Civil state (CS) Educational level (EL) Personnel training (PT) Continuity of activity (CA) Primary source of family income (PSF) Primary source of apiarist income (PSA)	*Dummies* Record (R) Record use (RU) Information use (IU) Economic advisors (EA) Technical advisors (TA) Associationism (As) Objective (O) Planning (P) Evaluation (E)	% T1 Equipment area % T2 Health area % T3 Feeding area

According to Ceyhan *et al.* (2017), the Tobit model was used to analyse the effects of management, decision-making variables and technologies adoption level affected the efficiency of beekeeping in the second stage of efficiency analysis. The standard Tobit model can be expressed as follows:

$$Y_i^* = \beta\, X_i + \mu_i;\ \mu_i \sim \left(0, \sigma_{\mu_i}^2\right);\ \ i = 1, 2, \ldots, n$$

$$Y_i = Y_i^*\ if\ Y_i^* > 0$$

$$Y_i = 1\ if\ Y_i^* \leq 0$$

where Y_i is the technical efficiency score for beekeeping farms measured using a latent variable, Y_i^* for positive valued and censored otherwise, β are the parameters of the model, X_i is a vector of explanatory variables, μ_i is a commonly and independently distributed error, which submits to $\mathrm{N}\left(0, \sigma_{\mu_i}^2\right)$ (49).

Statistical analysis

All the statistical analyses were carried out with software SPSS version 20 for Windows. The program used to calculate the model was DEAP Version 2.1: A Data Envelopment Analysis (Computer) Program, whose detailed operation is described in Coelli (1996). Finally, Eviews 8.0 was used to determine the canonical censored regression model, known as the Tobit.

Results and discussion

Profile of the Pampean beekeeper

Table 2 (page 154), shows the descriptive statistics for output and input variables of bee farms included in our sampling during the period 2011-2013. Research results obtained present a wide variation in all variables (inputs and output) across the different farms. The honey production, as the main output, varies from a minimum of 30 kg per year to a maximum of 56,000 kg per year, indicating high variability. The productivity of beekeeping is a measure of honey yield per beehive, and the honey yield is a significant factor affecting the profitability of unit production (5). A beekeeping production unit has, on average, 339.75 hives that produce an average of 13.9 kg of honey. Comparable results were observed by Aksoy *et al.* (2018) in Turkey, which obtained an average of 14 kg per hive in 2015. Nonetheless, higher values of productivity were found by Magaña-Magaña *et al.* (2016) in Mexico. According to Bragulat *et al.* (2018) in La Pampa there is coexistence of non-technified and small traditional with specialized models that incorporate technology achieving high productivity.

In the region of La Pampa, beekeepers have an average experience and age of 16.5 and 41.4 years, respectively. The mean family size was 2.97 individuals, with a range of 1-5. Studies carried out in Mexico, Romania, and Turkey showed similar results (18, 43, 46). It should be noted that most beekeepers have completed at least secondary education levels and have the intention to continue pursuing their beekeeping activity (table 4, page 157). The age of the producer and his experience in the beekeeping activity are considered factors of interest for the management capacity and the adoption of new technologies. According to findings by Contreras-Escañero *et al.* (2013), our results suggest a low level of technological adoption (in equipment, health and feeding area) in the region of La Pampa.

Technical efficiency score

The results of the technical efficiency obtained using inputs-orientated DEA are shown in table 5 (page 158). The overall technical efficiency (TE_{CRS}) may be decomposed into the two parts of pure technical efficiency (TE_{VRS}) and scale efficiency (SE), providing some ideas about the source of inefficiency.

Table 4. Descriptive statistics of explanatory variables to determinate of inefficiency causes.
Tabla 4. Estadística descriptiva de variables explicativas que determinan las causas de ineficiencia.

	Mean	Standard deviation	Minimum	Maximum
Continuous variables				
Family size (FS)	2.97	1.14	1	5
Age (A)	41.15	11.32	26	65
Experience (E)	16.5	7.14	9	34
% Equipment area (% T1)	54.16	20.23	16.67	100
% Health area (% T2)	54.64	19.91	28.57	100
% Feeding area (% T3)	51.5	24.34	0	100
Categorical variables	**Description**	**Number**	**Percentage**	
Civil state (CS)	0	17	57.5	
	1	23	42.5	
Educational level (EL)	0	7	17,5	
	1	33	82,5	
Personnel training (PT)	0	22	55	
	1	18	45	
Continuity of activity (CA)	0	3	7.5	
	1	37	92.5	
Primary source of family income (PSF)	0	23	57.5	
	1	17	42.5	
Primary source of apiarist income (PSA)	0	6	15	
	1	34	85	
Record (R)	0	30	75	
	1	10	25	
Record use (RU)	0	21	52.5	
	1	19	47.5	
Information use (IU)	0	19	47.5	
	1	21	52.5	
Economic advisors (EA)	0	34	85	
	1	6	15	
Technical advisors (TA)	0	35	87.5	
	1	5	12.5	
Associationism (As)	0	19	47.5	
	1	21	52.5	
Objective (O)	0	21	52.5	
	1	19	47.5	
Planning (P)	0	28	70	
	1	12	30	
Evaluation (E)	0	23	57.5	
	1	17	42.5	

Table 5. Statistical summary of technical efficiency.

Tabla 5. Resumen estadístico de la eficiencia técnica.

	TE_{CRS} Overall efficiency	TE_{VRS} Pure efficiency	SE Scale efficiency
Mean	0.406	0.572	0.664
Standard deviation	0.002	0.264	0.346
Minimum	0.03	0.2	0.05
Maximum	1	1	1
Number of efficient DMU	4	7	5
% of firms DRS[1]			7.5
% of firms IRS[2]			80

DRS [1]: Decreasing returns of scale.
IRS [2]: Increasing returns of scale.
DRS [1]: Rendimientos decrecientes de escala.
IRS [2]: Rendimientos crecientes de escala.

The minimum and the maximum TE_{CRS} and TE_{VRS} scores were estimated as 0.3-1 and 0.20-1, respectively. These differences observed between the maximum and minimum values indicate a considerable degree of variation in the efficiency of beekeeping systems in La Pampa. On average, the overall and pure technical efficiency score of the sample of beekeeping farmers in the studied area was estimated at 0.406 and 0.572, respectively. Our results are relatively similar to the findings of Lema and Delgado (2000) in Buenos Aires, but lower compared to Ceyhan *et al.* (2017) value of 0.62 from the Turkish beekeepers´ association. The results indicated that bee farms in La Pampa could reduce their level of input used by approximately 42.8% assuming a variable return to scale, while maintaining the same output levels and production technologies, provided that farms adopt best-observed practices. Thus, this implies that there is quite a room for improvement.

Some farms operate either on or close to the frontier of efficiency, but a significant number of beekeeping production units show technical efficiencies below 50%. Only thirteen farms and twenty-one farms have TE_{CRS} and TE_{VRS} over 0.50, respectively. Our results suggest that a significant proportion of the studied bee farms have a low honey production, which is reflected in low yields, probably due to the fact that farms are operating below their optimal scale. Figure illustrates the frequency distribution of TE_{CRS} and TE_{VRS} in bee farms in La Pampa. Most farms showed efficiency results within the ranges 0.20 to 0.40 (37.5%) and 0.40 to 0.60 (40%) under CRS and VRS assumptions, respectively.

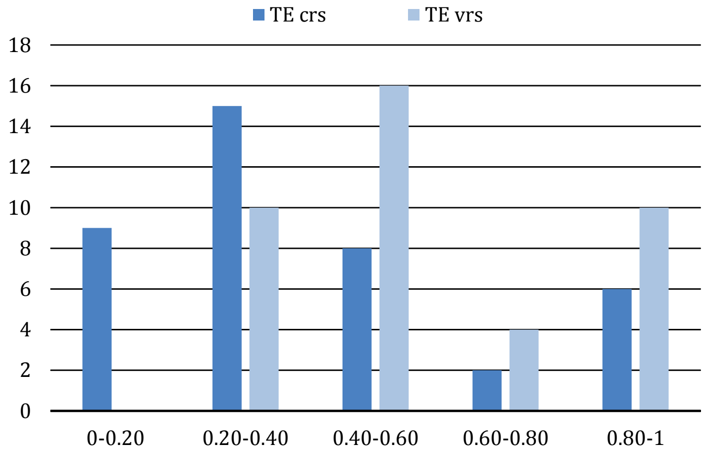

Figure 1. Frequency distribution of overall (TEcrs) and pure efficiency (TEvrs) level.

Figura 1. Distribución de frecuencias del nivel de eficiencia global (TEcrs) y pura (TEvrs).

Scale efficiency (SE) scores could be interpreted, allowing some interesting remarks. On average, the SE of the sample in the study area was estimated at 0.664, which implies that the average size of bee farms in La Pampa is far from the optimal size. SE (TE_{CRS}/TE_{VRS} ratio) measures potential productivity gains for a farm operating at optimal scale (17). Thus, an additional 33.6% productivity gain would be feasible to provide they adjusted their farm operation to an optimal scale, close to best practices. The SE analysis provides valuable information about returns to scale; either farm is operating below or above to their optimal scale (17, 44). Overall, 7.5% were operating under decreasing return scale (DRS), and most scale-inefficient farms (80%) displayed were operating under increasing returns scale (IRS). This implies an opportunity to gain additional honey production through carefully planned growth. This finding is consistent with the results reported in American beekeeping by Jones-Ritten *et al.* (2018).

The efficiency analysis of Pampean apiculture farms suggests that the latter are small farms that need to increase their size, in order to achieve best practice´s level (table 6). Our findings are consistent with the overall picture of the Buenos Aires bee farms showed by Lema and Delgado (2000). They reported that several hives given as size indicator, affected the productivity of farms, and probably this will imply a negative effect on economic performance.

[a,b,c] Different letters denote significant differences with $p \leq 0.05$.

a,b,c Las letras diferentes denotan diferencias significativas con $p \leq 0,05$.

Table 6. Farm size (in number of hives) according to return to scale.

Tabla 6. Tamaño de las explotaciones de acuerdo con su rendimiento de escala.

Returns of scale	Farms (%)	Mean	Standard Deviation
Decreasing returns of scale	7.5	1766.67 [c]	1766,67
Increasing returns of scale	80	197.18 [a]	172,327
Constant returns of scale	12.5	1100 [b]	839,643

The second stage: determinants of technical efficiency

The effects of influencing factors on inefficiency showed in table 3 (page 155), are evaluated by using the Tobit model. The results of the regression analysis are shown in table 7 (page 160).

Assuming variables scale returns model how the best fit (Log-likelihood = 18.568; AIC = 0.260), the crucial determinants that positively affected to technical efficiency in bee farms in La Pampa were civil state (married), educational level, the primary source of family and apiarist income, information usage, planning and health area. On the other hand, personnel training, continuity of activity and objective affected negatively on TE_{CRS} ($p < 0.05$).

Regarding manager personal aspects, it can be highlighted that, contrary to what was expected, both experience and age did not affect technical efficiency. These variables are aspects that are usually considered always in the management and decision-making in the company, but are also found to be very controversial (39). The explanation for this is probably the low average technical efficiency score and the sample size. On the other hand, results obtained also show how the marital status (married) positively affects the efficiency level, what highlights the vital role that women play in beekeeping and the rural areas (42, 43). The fact that families have apiculture as the primary source of income was also positively associated, which may be linked to a higher level of innovation and adoption of technology that could favour the development of the beekeeping activity (10).

In terms of the aspects related to access and use of information and formality of the decision-making process, the use of external information, as well as setting objectives and appropriate planning, have a positive impact on the efficiency score. These results agree with the works of Mujuni *et al.* (2012) in Uganda and Adgaba *et al.* (2014) in Saudi Arabia.

Regarding technologies adoption level, only health area had a positive effect on technical efficiency. It is understood that those apicultural units that adopt a more significant number of health items also make management more efficient. Our findings are in complete agreement with Ferrier *et al.* (2018) which suggested that the livelihood of beekeepers depends on the health and productivity of his apiaries. In this way, it would be crucial to identify those beekeeping units that carry out the best practices and detect critical points by applying benchmarking techniques (25, 28).

Table 7. Results of the Tobit analysis: technical inefficiency determinants.
Tabla 7. Resultados del análisis de Tobit: determinantes de la ineficiencia técnica.

Variable	TE_{CRS}		TE_{VRS}		SE	
	Coefficient	P	Coefficient	P	Coefficient	P
Constant	-0.992720	0.0943	0.086719	0.8768	-0.305242	0.7001
Family size (FS)	0.015141	0.7069	-0.028394	0.4544	0.004818	0.9286
Civil state (CS)	-0.036462	0.7911	0.259462	0.0455	-0.207605	0.2589
Age (A)	0.022020	0.0361	-0.001898	0.8480	0.025055	0.0742
Experience (E)	-0.020177	0.0164	-0.005866	0.4592	-0.019351	0.0849
Educational level (EL)	0.166749	0.2657	0.282259	0.0456	-0.117131	0.5583
Personnel training (PT)	-0.330231	0.0007	-0.192362	0.0369	-0.196430	0.1327
Continuity of activity (CA)	-0.146536	0.3791	-0.442170	0.0049	-0.014255	0.9489
Primary source of family income (PSF)	0.020374	0.7843	0.210233	0.0027	-0.097165	0.3286
Primary source of apiarist income (PSA)	0.134705	0.3134	0.176255	0.1617	-0.118791	0.5057
Record (R)	-0.163369	0.2055	-0.071772	0.5551	-0.142141	0.4096
Record use (RU)	-0.296037	0.0570	-0.292748	0.3700	-0.160594	0.2615
Information use (IU)	0.414225	0.0000	0.387486	0.0000	0.364925	0.0066
Economic advisors (EA)	-0.591872	0.0017	-0.286902	0.1066	-0.374265	0.1375
Technical advisors (TA)	0.185498	0.1851	0.070671	0.5922	0.053399	0.7752
Associationism (As)	-0.141698	0.1512	-0.103677	0.2652	-0.249247	0.0587
Objective (O)	-0.199455	0.0454	-0.288988	0.0021	-0.115305	0.3866
Planning (P)	0.554160	0.0007	0.370894	0.0156	0.490463	0.0241
Evaluation (E)	0.209191	0.0313	0.120220	0.1892	0.173326	0.1817
Equipment area (% T1)	-0.002354	0.2672	-0.001463	0.4645	0.000344	0.9033
Health area (% T2)	0.013947	0.0000	0.011399	0.0000	0.009562	0.0011
Feeding area (% T3)	0.002906	0.1594	0.001913	0.3258	0.002546	0.3563
Log likelihood	16.557		18.568		6.711	
Avg. Log likelihood	0.486		0.546		0.197	
Akaike info criterion	0.379		0.260		0.958	

TE_{CRS} = Overall technical efficiency; TE_{VRS} = pure technical efficiency; SE = scale efficiency.
TE CRS = Eficiencia técnica media ; TE VRS = Eficiencia técnica pura ; SE = Eficiencia de escala.

According to table 7, the most important variables that positively affected SE were information use, planning and health area. The results of our study suggest that lower units of production might improve the SE by adopting technologies, particularly in the area of management and health. Usually, the adoption of technologies is associated with a better organizational structure, which occurs in larger production units. The importance of size, in several hives, was confirmed as the decisive variable for the improvement of scale efficiency (table 6, page 159). Similar results were obtained in apicultural units from Buenos Aires (30).

Although this method used a robust methodology to calculate technical efficiency scores and a checked regression approach, the small sample size likely limited our ability to identify the more statistically significant variable. Future studies should include an emphasize on getting larger samples of productions units from different locations, to understand better the roles of management factors (11, 28, 50).

Policy implication and conclusions

On average, bee farmers in La Pampa (Argentina) are operating below the production frontier, which indicates there is still scope for improvement. Beekeepers have greater control over their inputs that outputs, thus the input orientated model was used. The scale of the bee farms in La Pampa does not reach the optimal levels required to achieve productive efficiency, and it would be convenient to increase their scale.

The government should promote to help inefficient bee farms in order to achieve their best practice by assuming benchmarking techniques. Beekeepers should also be motivated by the government to improve their efficiency through incentives, such as training programs to ease decision-making and management, therefore enhancing productivity.

Most of the explanatory variables in the Tobit model have expected sign but statistically insignificant. Despite these limitations and the sample size, our study has contributed to the existing literature on studies of technical efficiency in bee farms. However, more definite conclusions will be possible when additional studies focus on further analysis of heterogeneous production frontiers (according to size or location) and the quantification of total slacks in inputs set by applying benchmarking techniques.

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Preliminary use of a fulvic acid, as a strategy to improve water use in saline soils

Uso preliminar del ácido fúlvico, como estrategia para mejorar el uso del agua en suelos salinos

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Abstract

An evaluation of a fulvic acid (FA) was made in a Loam soil, by selecting the best dose to achieve salt displacement under a drip emitter. In trial 1, PVC columns were filled with a loam soil and irrigated with a KCl solution of electrical conductivity (EC) of 12.5 dS m^{-1}. Once the soil solution EC reached the value of the KCl solution, FA doses of 0, 2.1, 5.3 and 10.5 kg ha^{-1} were applied. The bulk electric conductivity and soil chemical properties were evaluated after 6 irrigation cycles. In trial 2, the same soil salinized with the KCl solution was placed in 0.8 m^3 containers. Two irrigations treatments were performed: a control and the best FA dose from trial 1. The displacement of the salt bulb created from irrigation with a dropper in the soil profile was characterized. In trial 1, the dose of 5.3 kg ha^{-1} reached the lowest EC after the third irrigation. In trial 2, the selected dose reduced EC until 3.75 dS m^{-1} at 0.3 m depth at the third irrigation, saving 246 L of water compared to control. Additionally, the salinity bulbs were more horizontally extended in the FA treatment.

Keywords
soil conditioner • humic acid • soil electric conductivity • leaching fraction

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Resumen

Se evaluó el uso del ácido fúlvico (FA) en un suelo franco, seleccionando la mejor dosis para lograr el desplazamiento de sal. En el ensayo 1, columnas de PVC se rellenaron con suelo franco y se regaron con una solución de KCl conductividad eléctrica (EC) 12,5 dS m^{-1}. Cuando la EC de la solución suelo alcanzó el valor de la solución de KCl, se aplicaron dosis de FA de 0; 2,1; 5,3 y 10,5 kg ha^{-1}. Se evaluó la EC aparente y las propiedades químicas después de 6 ciclos de riego. En la prueba 2, el mismo suelo salinizado con la solución de KCl se colocó en recipientes de 0,8 m^3. Se realizaron dos tratamientos de riego: control y la mejor dosis de FA obtenida del ensayo 1. Se caracterizó el desplazamiento del bulbo salino creado a partir del riego por goteo en el perfil del suelo. En el ensayo 1, la dosis de 5,3 kg ha^{-1} alcanzó la EC más baja después del tercer riego. En el ensayo 2, la dosis seleccionada redujo la EC hasta 3,75 dS m^{-1} a 0,3 m de profundidad en el tercer riego, ahorrando 246 L de agua. Además, los bulbos de salinidad se extendieron más horizontalmente en el tratamiento de FA.

Palabras claves

acondicionador de suelos • ácido húmico • conductividad eléctrica del suelo • fracción de lixiviación

Introduction

The management of saline soils for agricultural use is mostly based on the application of excessive volumes of irrigation water (above the water demand of the crop) so that salts are dissolved and leached out of the root zone. Such management is contradictory, precisely because water in arid and semi-arid regions, where soil salinization processes occur, is low in quantity as well as quality. Worldwide, over 50 million hectares of agricultural land have salinity problems, which, when added to the scarcity of water resources, forces us to seek new management perspectives for these soils. This is how different types of amendments and conditioners have been documented for the recovery of saline soils, such as gypsum, organic acids and different types of polyacrylamides, compost, etc., with positive results being reported (3, 10, 12, 20, 24). Soil conditioners are naturally or chemically synthesized substances, which can improve soil quality and facilitate plant growth (1, 2, 9). Accordingly, the incorporation of soil conditioners rich in organic matter (OM), of different types and origins, is becoming a common practice for soils affected by salts. The literature has reported different responses of saline soils to organic matter applications, which aimed to favor the movement of salts. These responses may be physical, such as the increase in coarse porosity and hydraulic conductivity (5, 15, 18), chemical, due to interactions between the active part of certain functional groups, particularly carboxylic acids with salts (4) and biological, such as improvements in nutrient uptake (N, Ca, P, K, Mg, Fe, Zn and Cu) (3, 14, 15). Thus, various mechanisms of OM reduce the negative effects of salinity, making it clear that it is a complex system, where different OM fractions can act in different ways, improving physical, chemical or biological conditions and promoting plant growth.

The agricultural supplies industry has recently put products on the market called "salt shifters", soil conditioners that combine soluble organic acids (humic and fulvic), polysaccharides and / or soluble polymers, which can adsorb cations and take them out of the root zone together with the irrigation water, leaching more salts with less water; however, they are poorly documented (24). On the other hand, the application of humic substances and their positive effect on the remediation of saline soils has been associated more with the indirect effect of fulvic acids, such as improving soil physical and chemical properties (15, 19), rather than their leaching effect. A chelating effect, in particular of fulvic acids, could mobilize the salts of the soil exchange complex and, with good irrigation management, produce effective leaching. In this regard, Osman and Ewees (2008) suggested that the charged functional groups of the organic acids (COO-) could retain and chelate cations, becoming them inactive or moving them deeper.

Although there is a series of investigations related to the effects of the application of amendments on the chemical and physical properties of saline and sodic soils and their effect on crop production (3, 9, 19), few studies have monitored the electrical conductivity in different soil layers after the application of amendments (26). Many of these studies have been conducted for the remediation of saline-sodic soils under field and laboratory conditions, but the spatial and temporal evaluation of the movement of solutes in the field is difficult, so the soil is generally evaluated in columns and under controllable conditions (13).

In this context, the aim of this study was to develop a preliminary evaluation to guide future research on soil salinity reclamation based on more efficient irrigation strategies with the use of fulvic acid (FA). The latter was accomplished through two trials; in the first one, it was tested three different doses of FA in a saline soil to select the best dose for salt leaching purposes. In the second trial it was used the dose selected previously to observe its effect on salt displacement in soil depth, with the use of drip irrigation.

Materials and methods

Two trials were carried out in a protected environment in the Irrigation and Soil Physics laboratories of the Faculty of Agronomy of the University of Chile, located in Santiago, Chile, coordinates 33° 34´11"S, 70°37´50" W.

Treatments and experimental design

Trial 1: A commercial conditioner derived from Leornardite was used, presented as soluble powder, pH 4.0 to 5.0 and a mass base composition of: FA (700 g kg^{-1}), magnesium (5000 - 6000 mg kg^{-1}), sulfur (5000 - 6000 mg kg^{-1}), iron (4000 mg kg^{-1}), zinc (2.5 mg kg^{-1}), manganese (2500 mg kg^{-1}) and copper (1000 mg kg^{-1}), the manufacturer claims that the minerals are added to the final product as chelates. The experimental unit corresponded to a PVC column 0.2 m in diameter and 0.25 m in length with a fine net of 85 mesh at its base to allow free drainage, filled with a loam soil. The soil was sieved at 4mm and it was composed of 15% clay, 40% silt and 45% sand, particle density 2.53 Mg m^{-3} and 0.011 g kg^{-1} soil organic matter (SOM). Prior to salinization, the soil had an electrical conductivity measured in saturated paste (ECes) (22) of 1.63 dS m^{-1}, a cation exchange capacity (CEC) of 19.8 cmol kg^{-1} (extraction in ammonium acetate) (22), and a pH of 8.16 (suspension and potentiometric determination) (22). The filling of the columns included a 5 cm layer of sand at the bottom, placing 15 cm of soil over this layer. The soil was settled through the application of successive loads of water, until reaching a bulk density of 1.4 Mg m^{-3}. The column was salinized by the addition of potassium chloride (KCl) in solution with an EC of 12.5 dS m^{-1}. The experimental unit was irrigated several times with this solution until obtaining the same EC of the solution in the drainage water. No preferential flow was detected during the essay, so homogeneous condition of the soil was assumed. To verify the effectiveness of soil salinization without altering the samples, its porous electrical conductivity (ECp) was obtained based on its permittivity data measured by a FDR sensor (GS3, Decagon Devices, WA, USA) previously parameterized with the parameters from Hilhorst (2000). The results showed that the ECp was on average 12.1 ± 0.94 dS m^{-1} (n = 12).

The experimental design was completely randomized, with three treatments plus one control, with three replicates each, totaling twelve soil columns. The treatments corresponded to FA based applications of 2.1 (T1), 5.3 (T2) and 10.5 (T3) kg ha^{-1} plus the control (T0) without application, which correspond to 6.6 (T1), 16.7 (T2) and 33 (T3) mg per column. The T1 dose was that recommended by the manufacturer (2.1 kg ha^{-1}) and the other two corresponded to progressive increases close to 100% of the previous treatment. It should be noted that the product is marketed as a stimulant for plant development, so there is no a specific recommended dose to promote the mobilization of salts.

Trial 2: The experimental unit was a container, with inner dimensions of 1.14 m length, 1.14 m width and 0.62 m height. This was filled with 50 cm of the soil described in Trial 1, over a layer of 5 cm of sand to allow adequate drainage. The soil was settled by the application of successive loads of water (until it equilibrated to a bulk density of approximately 1.4 Mg m^{-3}) and salinized with potassium chloride (KCl) in solution with an

EC of 12.5 dS m^{-1}. It was irrigated with a volume of 1.6 m^3 of solution, totaling five times the pore volume of the soil, until obtaining an EC of the soil solution similar to the one applied, which was obtained through suction lysimeters (SSAT, Irrometer, Riverside, USA). The experimental design was completely randomized, with two treatments (FA application and control), with three replicates each, totaling six containers. The dose of treatment (Ta) corresponded to the most effective in the leaching of salts according to the results obtained in Trial 1, while the other treatment (Tb) corresponds to the control without any kind of amendment.

Material and methods

Trial 1: Previously calibrated FDR sensors (soil water content and bulk electric conductivity, GS3) were placed in the center of each column, which made it possible to obtain bulk EC (EC_b) and water content in the first 5 cm; all information was recorded in a datalogger (EM50, Decagon Devices, WA, USA).

The dose of FA from treatments T1, T2 and T3 was applied partially, 50% in the first irrigation and 50% in the second, and then four irrigations were carried out. Each irrigation was applied for 17 minutes with a dripper of 4 L h^{-1} located at the center of the column, enough time to completely moisten the column. The volume of water applied for each irrigation was 1.15 L (approximately half the total porosity of the soil in the column). Once watered, it was allowed to drain freely for 48 h before the next irrigation. Additionally, the EC of the water used prior to irrigation was monitored, remaining stable at 1.04 dS m^{-1}. To test the effect of the treatments, the EC_b and soil water content were determined 48 h after each irrigation. Once the whole test was finished, a soil sample was taken from each column (0-10 cm depth) and soil extractable cations (extraction by ammonium acetate), CEC (extraction by ammonium acetate), pH (suspension and potentiometric determination) and SOM (calcination) were evaluated following the methodologies proposed by Sadzawka *et al*. (2006).

Trial 2: To extract the soil solution from the container, at the moment of applying the soil filling, five suction lysimeters (SSAT, Irrometer, Riverside, USA) were installed, 12 cm apart in depth and 15 cm apart laterally as it is shown in figure 1, establishing a grid that made it possible to monitor the movement of the salts in the profile according to the symmetry of the wet bulb in homogeneous porous media (8).

The letters A to E indicate the position of the five installed lysimeters.

Letras A a la E indican la posición de los 5 lisímetros instalados.

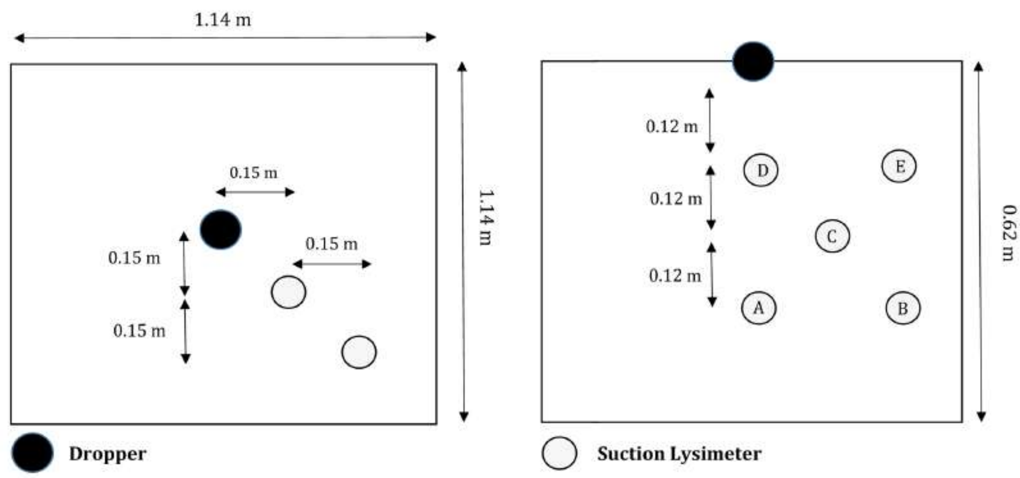

Figure 1. Suction lysimeters grid. On the left, flat view (width: 1.14 m; length: 1.14m) ; right, longitudinal view (height: 0.62 m).

Figura 1. Disposición de los lisímetros de succión. A la izquierda, vista plana; a la derecha, sección longitudinal.

Once the assembly was made and the soil salinized at an approximate EC of the soil solution of 12.5 dS m^{-1}, 6 irrigations 48 h apart were applied using a dripper of 4 Lh^{-1} located at the center of the container. The first irrigation was used for the application of FA (5.3 kg ha^{-1}, most effective dose selected from Trial 1), while the remaining 5 were salt leaching irrigations. The volume of water applied in each irrigation was approximately 25% of the total soil porosity (82 L). After each irrigation, the soil was allowed to drain freely for 48 h to reach the field capacity; simultaneously, to avoid the contribution of water by precipitation and its loss from the soil by evaporation, the surface of the container was covered with a plastic cover. After 48 h, the soil solution was extracted with the aforementioned lysimeters with a vacuum application between -70 and -80 cb for 1 h. The EC of the extracted solution was determined with a CON510 conductivity meter (Oakton, Illinois, USA). As a control, the water EC was monitored prior to irrigation, fluctuating between 0.87 and 1.33 dS m^{-1}. Based on the EC spatial data obtained from the 3 replicates, their average was interpolated using kriging, assuming a mirror image distribution and a soil profile of 0.4 m (depth) and 1 m (width). To visualize the displacement of salt (salinity contour lines) in the container after each irrigation, the software Surfer 13 was used. In addition, to numerically visualize the impact of each irrigation period in the displacement of salt, an analysis of the surface was carried out, taking as a reference the soil profile and its salinity contour lines obtained through the interpolation. This analysis shows the fraction of the area of the soil profile, expressed as a percentage of the total area, which is within a range of EC, obtained as a result of an irrigation.

Statistical analysis

The results of the measurements of the Trials 1 and 2 were analyzed statistically through an analysis of variance (ANOVA) with a confidence level of 95%. To establish differences, the Tukey test was used with a 5% significance, comparing the means among treatments for an irrigation period (Trial 1) for salinity, the means among treatments for all the irrigation periods (Trial 1) for soil water content, and the means between treatments for a lysimeter´s position and a determined irrigation period (Trial 2).

Results

Trial 1: Average soil water content was 0.36 ±0.013 m^3 m^{-3} did not show differences among treatments and irrigation period (P>0.05). Under this condition, the differences in EC_b between treatments are mainly due to the variation in the ion concentration of the soil solution. Table 1 (page 169), shows the evolution of EC_b values by treatment measured 48 hours after irrigation. Statistical significant differences were found from the third irrigation, where the lowest values of EC_b were obtained in T2 and T3, with no statistical differences between them, however T3 did not show differences with T0 and T1 while T2 did, showing in average a better performance. From irrigation 4 to 6, the statistical differences between treatments were maintained, but T1 differed from T3, showing it to be the least effective treatment. On the other hand, T3 exhibited the same behavior as T0, which in turn showed no difference with T1. T2 (5.3 kg ha^{-1}, FA dose) showed until the end to be statistically the most effective.

No significant pH differences were found in the soil columns at the end of the trial, standing around pH 9.0 (table 2, page 169). No differences were found in the SOM content or CEC as well (table 2, page 169). These results show that doses lower than 10.5 kg ha^{-1} of FA applied under the conditions and soil used in this test did not affect the buffer capacity of the soil.

The distribution of extractable cations in the soil at the end of the trial is given in table 3 (page 169), which shows that there are no statistical differences between treatments.

Note: T1, T2 and T3 correspond to doses of 2.1, 5.3 and 10.5 kg ha^{-1}

[a] Different letters indicate statistically significant differences between treatments in the same irrigation according to the Tukey test with a 5% significance.

Nota: T1, T2 y T3 corresponden a dosis de 2.1, 5.3 y 10.5 kg ha^{-1}

[a] Las diferentes letras indican diferencias estadísticamente significativas entre los tratamientos en el mismo riego de acuerdo con la prueba de Tukey con una significancia del 5%.

Table 1. Bulk Electrical Conductivity (EC_b´s values after 48 hours of irrigation). Average value and its standard deviation per treatment are presented.

Tabla 1. Conductividad eléctrica aparente (valores de EC_b después de 48 horas de un riego). El valor promedio y su desviación standard son presentados.

EC_b (dS m^{-1})			
Treatments	**Irrigation 1**	**Irrigation 2**	**Irrigation 3**
T0	0.945 ± 0.086 a[a]	0.589 ± 0.060 a	0.517 ± 0.040 b
T1	0.945 ± 0.005 a	0.599 ± 0.003 a	0.538 ±0.005 b
T2	0.945 ± 0.120 a	0.515 ± 0.088 a	0.406 ±0.051 a
T3	0.945 ± 0.126 a	0.533 ± 0.043 a	0.460 ±0.019 ab
Treatments	**Irrigation 4**	**Irrigation 5**	**Irrigation 6**
T0	0.493 ± 0.038 bc	0.474 ± 0.042 bc	0.464 ± 0.042 bc
T1	0.513 ± 0.009 c	0.497 ± 0.007 c	0.484 ± 0.007 c
T2	0.373 ± 0.042 a	0.351 ± 0.035 a	0.336 ± 0.034 a
T3	0.428 ± 0.008 ab	0.411 ± 0.015 ab	0.399 ± 0.017 ab

Note: T1, T2 and T3 correspond to fulvic acid doses of 2.1, 5.3 and 10.5 kg ha^{-1}.

[a] Different letters indicate statistically significant differences according to the Tukey test (5% significance).

Nota: T1, T2 y T3 corresponden a dosis de ácido fúlvico de 2.1, 5.3 y 10.5 kg ha^{-1}.

[a] Letras diferentes indican significancia estadística de acuerdo con el test de Tukey (5% de significancia).

Table 2. Average values of pH, organic matter, CEC and its standard deviation by treatments once the trial was finished.

Tabla 2. Valores promedio de pH, materia orgánica, CEC y su desviación estándar para cada tratamiento una vez terminado el ensayo.

Treatment	**pH**	**Organic Matter**	**CEC**
	---	g kg^{-1}	cmol kg^{-1}
T0	8.96 ± 0.08 a[a]	0.0103 ± 0.0003 a	22.19 ± 1.15 a
T1	9.12 ± 0.01 a	0.0107 ± 0.0011 a	19.78 ± 1.57 a
T2	8.96 ± 0.25 a	0.0101 ± 0.0041 a	19.37 ± 0.20 a
T3	8.92 ± 0.11a	0.0106 ± 0.0010 a	21.56 ± 1.34 a

Note: T1, T2 and T3 correspond to fulvic acid doses of 2.1, 5.3 and 10.5 kg ha^{-1}

[a] Different letters indicate statistically significant differences between treatments according to the Tukey test (5% significance).

[a] Letras diferentes indican significancia estadística entre tratamientos de acuerdo con el test de Tukey (5% de significancia).

Table 3. Average values of pH, organic matter and CEC by treatments once the trial was finished.

Tabla 3. Valores promedio de pH, material orgánica y CEC para cada tratamiento una vez terminado el ensayo.

Treatment	**Ca^{2+}**	**K^+**	**Mg^{2+}**	**Na^+**
	-------------- cmol kg^{-1} --------------			
T0	16.70 ± 5.09 a[a]	0.52 ± 0.04 a	0.25 ± 0.12 a	0.39 ± 0.02 a
T1	17.75 ± 7.71 a	0.55 ± 0.01 a	0.22 ± 0.10 a	0.40 ± 0.01 a
T2	14.90 ± 4.24 a	0.41 ± 0.02 a	0.23 ± 0.05 a	0.34 ± 0.01 a
T3	16.15 ± 0.64 a	0.53 ± 0.05 a	0.16 ± 0.06 a	0.38 ± 0.06 a

Trial 2: The most effective treatment (T2= 5.3 kg ha^{-1}) from Trial 1 was selected to perform Ta in Trial 2. Table 4 shows the average soil solution EC and its standard deviation extracted by the lysimeters in each irrigation period. Statistical differences between treatments were observed ($p<0.05$) from the first irrigation (lysimeter B and E), position according figure 1 (page 167). These differences ($p<0.05$) were more consistent from the third irrigation involving a higher proportion of the soil profile (lysimeters A, B, C, E). The differences were maintained until the fifth irrigation, where Tb (Control) begins to equate the effects of Ta (5.3 kg ha^{-1}of FA) in the position of the C lysimeter, however differences were reached even in the sixth irrigation in the B and E lysimeters´ positions. Therefore, it was observed from the first irrigation and until the end of the test, substantively lower EC values in the containers treated with FA (Ta), reaching differences of up to 8.6 dS m^{-1} (lysimeter E, Irrigation 2, table 4).

Table 4. Average EC values and its standard deviation of the solutions obtained with the lysimeters for each irrigation period (Ri), for Tb (Control) and Ta (5.3 kg ha^{-1}of fulvic acid).

Tabla 4. Valores promedio de la EC y su desviación estándar de las soluciones obtenidos con los lisímetros para cada periodo de riego (Ri), para Tb (Control) y Ta (5.3 kg ha^{-1} de ácido fúlvico).

	R1		R2		R3	
	Tb	Ta	Tb	Ta	Tb	Ta
	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}
A	11.83 ± 1.50 a	11.85 ± 0.54 a	10.97 ± 1.81 a	9.44 ± 1.15 a	8.37 ± 2.35 b	5.37 ± 0.68 a
B	12.53 ± 0.38 b	11.64 ± 0.36 a	12.96 ± 0.02 b	7.29 ± 0.96 a	10.51 ± 2.62 b	2.68 ± 0.44 a
C	11.37 ± 2.12 a	12.36 ± 0.16 a	11.84 ± 1.45 b	9.72 ± 0.66 a	8.07 ± 3.62 b	4.30 ± 0.81 a
D	4.72 ± 1.58 a	4.94 ± 1.41 a	2.82 ± 0.41 a	2.20 ± 0.53 a	1.55 ± 0.30 a	1.66 ± 0.10 a
E	13.47 ± 0.66 b	9.38 ± 1.94 a	12.65 ± 1.06 b	3.97 ± 0.64 a	8.48 ± 4.51 b	1.50 ± 0.36 a
	R4		R5		R6	
	Tb	Ta	Tb	Ta	Tb	Ta
	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}	EC dSm^{-1}
A	5.10 ± 1.66 b	2.77 ± 0.15 a	4.00 ± 1.15 b	2.17±0.34 a	3.89 ± 1.63 a	2.07 ± 0.77 a
B	8.81 ± 4.82 b	1.98 ± 0.01 a	6.31 ± 3.16 b	1.65 ± 0.03 a	4.18 ± 0.81 b	1.92 ± 0.40 a
C	6.17 ± 2.84 b	3.17 ± 1.04 a	4.62 ± 2.07 a	2.51 ± 0.85 a	3.41 ± 1.57 a	2.72 ± 0.41 a
D	1.49 ± 0.15 a	1.56 ± 0.11 a	1.32 ± 0.22 a	1.44 ± 0.08 a	1.44 ± 0.31 a	1.63 ± 0.20 a
E	7.15 ± 5.30 b	1.60 ± 0.07 a	5.17 ± 2.37 b	1.34 ± 0.27 a	3.55 ± 1.57 b	1.54 ± 0.30 a

Note: R1 to R6= irrigation period, A to E = lysimeters´ position (figure 1, page 167).

[a] Different letters indicate statistically significant differences according to the Tukey test (5% significance), between treatments for each irrigation period and each lysimeter position.

Nota: R1 a R6= periodo de riego, A to E = posición de lisímetros (figura 1, page 167).

[a] Letras diferentes indican significancia estadística de acuerdo con el test de Tukey (5% de significancia), entre tratamientos para cada periodo de riego y cada posición de lisímetro.

Figure 2 (page 171), shows the evolution of the salinity of the soil solution as a function of the irrigations, the figure is consistent with the results from table 4, and also depicted the greater opening of the salinity bulb generated with the FA treatment. By comparing the dispersion of EC values in-depth between treatments, the impact of the use of FA is accentuated if a more efficient use of water available for irrigation is considered. For example, it is observed that the dispersion of the EC obtained at the sixth irrigation in the Tb (control) is similar to that achieved in the third irrigation with the application of the FA (figure 2, page 171). A similar conclusion could be inferred from table 1 (page 169), (Trial 1) where after the third irrigation with 5.3 kg ha^{-1} dose, soil EC_b is 0.406 dS m^{-1}, which is below the level of 0.464 dS m^{-1} obtained in the Control with six irrigations.

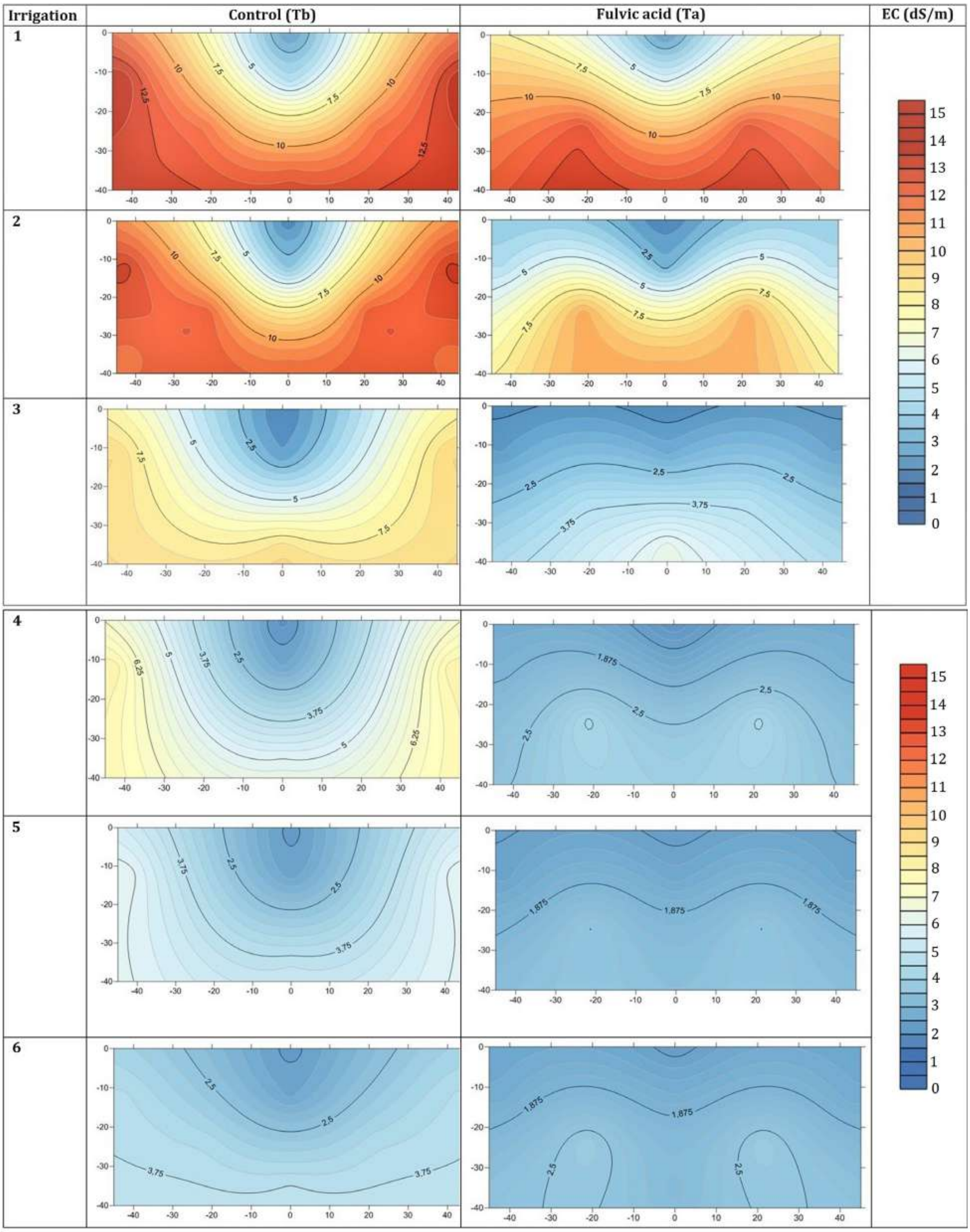

Figure 2. Isosalinity contour lines. As a result of the interpolation from the average EC values of the soil solutions obtained with the lysimeters for each irrigation period. Tb (Control) and Ta (5.3 kg ha^{-1} of fulvic acid).

Figura 2. Curvas de isosalinidad. Como resultado de la interpolación de los valores de CE promedio de las soluciones de suelo obtenidas con los lisímetros para cada período de riego. Tb (Control) y Ta (5,3 kg ha^{-1} de ácido fúlvico).

Therefore, based on the observation from figure 2 (page 171) and under the conditions of this test, it may be inferred that a decrease in EC from 12.5 to 3.75 dS m^{-1} at 30 cm depth for a surface of 1.3 m^2 is achieved either by applying 449.8 L of irrigation water (EC = 1.04 dS m^{-1}) or by irrigating with 203.5 L of the same water plus an application of 5.3 kg ha^{-1} of FA, generating a water consumption equivalent to 45.2% of the control. This result supposes that an application of 5.3 kg ha^{-1} of FA would be equivalent to 246.3 L of water in the washing of salts for that surface.

Table 5 presents the fraction of the area of the soil profile expressed as a percentage of the total area for a range of EC obtained as a result of irrigation. The results are consistent with table 4 (page 170); figure 2 (page 171) , and it is observed that from the first irrigation the fraction of the area with lower EC ranges increased more rapidly using FA. For example, it is inferred that in irrigation 3, for the treatment with FA (Ta), 97.9% of the profile´s cross section has EC values lower than 5 dSm^{-1} while Tb (control) only 25, 8%.

Table 5. Fraction of the area of the soil profile expressed as a percentage of the total area for an EC (average) range obtained as a result of an irrigation period. Tb (Control) and Ta (5.3 kg ha^{-1} of fulvic acid).

Tabla 5. Fracción del área del perfil del suelo expresada como porcentaje del área total para un rango de EC (promedio) obtenido como resultado de un período de riego. Tb (Control) y Ta (5,3 kg ha^{-1} de ácido fúlvico).

CE	Tb						Ta					
(dSm^{-1})	R1	R2	R3	R4	R5	R6	R1	R2	R3	R4	R5	R6
0-2.5	1.0	2.1	7.1	10.3	16.2	21.7	0.7	5.2	44.8	58.2	100	86.5
2.5-5	6.9	7.5	18.7	37.4	77.9	78.3	6.2	28.5	53.1	41.8	-	13.5
5-7.5	12.9	10.9	40.6	52.4	5.9	-	13.9	28.0	2.2	-	-	-
7.5-10	16.1	18.2	33.6	-	-	-	27.3	38.2	-	-	-	-
10-12.5	63.0	61.2	-	-	-	-	52.0	-	-	-	-	-
Total	100	100	100	100	100	100	100	100	100	100	100	100

Note: Tb (Control) and Ta (5.3 kg ha^{-1} of fulvic acid); R corresponds to the irrigation period.

Nota: Tb (Control) y Ta (5,3 kg ha^{-1} de ácido fúlvico); R corresponde al periodo de riego.

Discussion

In trial 1, the T1 dose (2.1 kg ha^{-1}) was not enough to show positive effects on the soil, whereas the T3 dose (10.5 kg ha^{-1}), being much higher, was able to cause the FA to complex with each other, being dragged in masse, with little participation of other cations. Norambuena *et al*. (2014), working with a sandy loam soil with no salinity problems, demonstrated that increasing doses of humic+ gypsum substances generate an initial increase in water infiltration, with a subsequent decrease depending on the interaction of the organic amendment with the gypsum; the explanation may be a dispersion effect generated by high doses, considering the high reactivity of these substances, which generates a chemical seal that hinders the movement of water.

The fastest decrease of EC by the action of fulvic acid in the T2 treatment could be explained by its capacity to generate organo-mineral soluble complexes, where the selectivity of the cation to be transported is given by its ionic radius and the electrochemical affinity of the ligands (4, 6, 23). The most obvious cause of this phenomenon corresponds to the neutralization of charges (23). The metal-organic matter complexes, once formed, follow three routes: (1) they are sorbed at exchange sites, (2) they coprecipitate, (3) the metal competes with other metals in the complex, some of which are able to precipitate as hydroxides and carbonates; these last two cases are hardly reversible (23). Ettler *et al*. (2009) found such organo-mineral complexes in soil leachates after the application of organic acids, which suggests that organic acids of low molecular weight (citric, acetic, malic acids, etc.) and those of high molecular weight (fulvic and humic acids) could increase the mobility of metal cations in the soil.

This phenomenon is favored at lower pH values due to the variable charge present in organic molecules (3, 6). In our study, irrigation water had no influence on the pH increase (table 2, page 169), and this may be responding to the result of the SOM stabilization process during its microbiological decomposition, as noted by Rowley *et al*. (2018) for the first stages of decomposition, where SOM occlusion process is generated at macroscale level (aggregates 250-2000 μm). As a complement, Mahmoodabadi *et al.* (2013) state that the movement of cations will be given by the initial condition of the soil (concentration and type of cations present) and the type of SOM. In the present assay, no differences were observed between the extractable cations (table 3, page 169), being Ca^{+2} the dominant in the exchange complex, despite soil was treated with KCl. Rowley *et al*. (2018) highlight the dominance of Ca^{+2} in the exchange complex, with an apparent occlusion capacity of organic matter. In this sense, soil presented 0.011 g kg^{-1} of SOM, with no differences between treatments at the end of the trial; thus, SOM content could interfere in the EC results.

The treatments with respect to the control did not generate an effect of decreasing the concentration of a particular cation (table 3, page 169), which can be explained by the high retention force of these elements attributable to a high pH of the soil where OH- anions predominate. The absence of statistically significant differences between treatments indicates that there was no tendency for FA to displace any particular cation, so they all moved according to their participation in the exchange complex.

Figure 2 (page 171) shows the effect of the application of FA (concentration of 5.3 kg ha^{-1}) on the composition of salinity bulbs (Trial 2), which are much more horizontally extended, with less curved isolines of less curvature with respect to the dripper (located in 0,0 coordinate) when compared to the control without application. Moreover, from the third irrigation, relatively flat isolines are generated, an effect enhanced by the surfactant characteristics of this organic acid, allowing for a better soil wetting (11).

On the other hand, the Ta (5.3 kg ha^{-1} of FA) modified the distribution of the solutes in such a way that when comparing the salinity bulbs (figure 2, page 171), it is observed that the contour lines for the same EC value circumscribe a larger area in the containers treated with organic acids. This is evident in table 5 (page 172), where the percentage of the profile area is shown by EC sections in each irrigation as a percentage of the total area of the profile. The action of the organic acid is not determined exclusively by chemical aspects, but also by an improvement in the physical properties of the soil, mainly a greater stability and porosity (16), which determine a greater water flow capacity (17), optimizing the leaching of salts. However, as pointed out by Wuddivira and Camps-Roach (2007), the final effect depends on the content and type of clay, having dispersion processes in 2:1 minerals in high doses of organic amendments. This is how the Ta already has a profile with an EC of 5 dS m^{-1} or less at the fourth irrigation, while the Tb only has an area under these conditions of 47.7%. Following this same analysis Ta reaches the total soil profile under 2.5 dS m^{-1} at the fifth irrigation, while Tb at the sixth irrigation only reaches 21.7% of the area of the container below this threshold. Our results poses new challenges for future studies in which it is desired to use humic amendments for the recovery of soils degraded by salinization processes.

Conclusions

Application of FA (5.3 kg ha $^{-1}$) reduced the water use by 50% compared to leaching carried out exclusively with water. The effective dose selected for salt leaching was found to be more than twice the recommended dose (2.1 kg ha $^{-1}$) and it turned out this latter did not show differences with the control (only water). These results impose some additional cost challenges to use this product more widely in agriculture. These challenges must be contrasted with the cost of irrigation water use that is becoming scarce, especially in arid and semi-arid regions. Future research should be conducted in order to know the persistence of the soil properties imposed by the product, to obtain a broader perspective of its use for future users. In general, these preliminary results reflect the use of fulvic acid as an alternative to consider in the recovery of saline soils under drip irrigation when irrigation water is scarce.

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Reuse of treated domestic sewage for biquinho pepper cultivation

Reúso de aguas domésticas tratadas en el cultivo de la pimienta de pico

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Abstract

This study aimed to evaluate the reuse of treated domestic sewage in biquinho pepper (*Capsicum chinense L.*) cultivation under different irrigation regimes. The experiment was carried out in a greenhouse, in a randomised block design with a 3 x 4 factorial scheme, with four replications. Forty-eight pepper plants were subjected to three treated domestic sewage concentrations (0, 50, and 100%) and four irrigation depths (75, 100, 125, and 150% of the crop evapotranspiration - ETc). Data for ETc estimation were obtained from an automatic meteorological station, which was installed in the greenhouse. The results showed that irrigation depths corresponding to 125 and 150% of the ETc at a concentration of 100% treated domestic sewage resulted in higher plants. Moreover, longer fruits were obtained when plants were subjected to 100% ETc at 100% treated domestic sewage. The studied irrigation depths influenced pepper fruit total weight and yield. No treated domestic sewage concentrations significantly influenced the variables analysed. Treated domestic sewage reuse can constitute an alternative for quality water saving and for chemical fertilisation of biquinho pepper crop.

Keywords
Capsicum chinense L. • water reuse • evapotranspiration • greenhouse

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Resumen

Este estudio tuvo por objeto evaluar el reúso de aguas domésticas tratadas en el cultivo del pimiento de pico (*Capsicum chinense L.*) sometido a diferentes niveles de reposición de agua. El experimento se llevó a cabo en un invernadero en un diseño de bloques aleatorios en un esquema factorial 3 x 4, con cuatro repeticiones. Cuarenta y ocho pimientas fueron sometidas a concentraciones de 0, 50 y 100% de reúso de aguas domésticas tratadas y cuatro niveles de reposición de agua correspondientes al 75, 100, 125 y 150% de la evapotranspiración del cultivo (ETc). Los datos para la estimación de la evapotranspiración de los cultivos se obtuvieron de una estación meteorológica automática instalada dentro del invernadero. Los niveles de reposición de agua del 125 y 150% de la evapotranspiración de los cultivos con una concentración del 100% de reúso de aguas domésticas tratadas proporcionaron mayores alturas de las plantas. Se obtuvieron mayores longitudes del fruto del pimiento cuando se sometió a una ETc del 100% con una concentración del 100% de reúso de agua domésticas tratadas. Los niveles de reposición de agua influyeron en la producción total y la productividad de los frutos del pimiento. Las concentraciones de reúso de agua domésticas tratadas no influyeron significativamente en las variables analizadas. El reúso de agua domésticas tratadas puede ser una alternativa para reducir el uso de agua de mejor calidad y la fertilización química en el cultivo del pimiento de pico.

Palabras clave

Capsicum chinense L. • reúso de agua • evapotranspiración • invernadero

Introduction

Pepper cultivation has assumed great importance for the Brazilian and world populations. Biquinho is a pepper variety (*Capsicum chinense* L.) widely grown by small farmers due to its high yield potential, gastronomic value, market acceptance, and financial return (3). It stands out for adding commercial value depending on the region of sale and has been increasingly valued in the consumer market, especially for consumption in the form of pickles and for a mild flavour and absence of pungency (9). Crop evapotranspiration knowledge during different phenological stages helps determine crop water requirements and water use efficiency (5).

The cultivation of peppers in north-eastern Brazil is affected by local water scarcity due to irregular rainfall and high evapotranspiration rates (13). In this sense, irrigation constitutes a valuable alternative for its production in this region. However, due to low water availability, crop irrigation is not always possible (18). Thus, reusing treated water in irrigated agriculture is a way of providing good quality water in optimum amounts for this purpose, seeking to save water for human and animal consumptions (14).

Reuse of water for irrigation purposes can benefit crops in terms of nutrient supply, reducing the need for synthetic fertilisers (2). Queiroz *et al.* (2015) observed that, despite several benefits, unrestricted irrigation with urban effluent may imply health and environmental risks. Besides supplying organic matter and nutrients to the soil, it also increases salt contents, which, depending on the concentration, can compromise crop quality and reduce soil osmotic potential, thus limiting productive capacity.

Several studies on wastewater reuse in agriculture have been conducted on chilli pepper (18), okra (15), papaya (10), white oats (4), and lettuce (8). Therefore, this study aimed to evaluate the effect of reusing treated domestic sewage in biquinho pepper (*Capsicum chinense L.*) growth under different irrigation regimes.

Material and methods

The experimental area is located at 30 m above sea level. According to Köppen's classification, the local climate is humid tropical with a dry season in the summer. The region has an average annual temperature of 25.2°C and rainfall of 1300 mm, which is concentrated from April to September. The soil used is classified as Ultisols (17).

Forty-eight biquinho pepper (*Capsicum chinense L.*) plants were grown in 21-L plastic containers arranged on a bench inside a greenhouse. The experiment was carried out in a randomized block design and arranged in a 3 x 4 factorial scheme, with three treatments and four replications. The plants were subjected to three treated domestic sewage concentrations (0, 50, and 100%) and four irrigation regimes (depths), which corresponded to 75, 100, 125, and 150% of the crop evapotranspiration (ETc).

The plants were fertilised at transplanting time according to soil analysis, applying: urea (1 g pot^{-1}), P_2O_5 (4.2 g pot^{-1}), and K_2O (1.5 g pot^{-1}). After 30 days, topdressing was performed by applying urea (0.5 g pot $^{-1}$) and K_2O (0.75 g pot^{-1}). After transplantation, the plants were supplied with 100% water. Weather data used for reference evapotranspiration were obtained from an automatic meteorological station, which was installed in the greenhouse. Crop coefficients of greenhouse-grown pepper used to determine crop evapotranspiration were 0.74, 1.38, 1.40, 1.17, and 1.02 (16).

The parameters evaluated were plant height, fruit length, and stem diameter, which were measured with a millimetre ruler and a pachymeter. Fruit weight was determined by collecting and drying fruit in an oven at 60°C for 24 hours. Then, the material was weighed on a precision scale to an accuracy of 0.01g. Pepper yield (kg ha^{-1}) was obtained according to fruit production per plant and the area occupied by each plant.

Data were submitted to ANOVA analysis of variance, and means were compared by Tukey's test. Regression equations were generated for the parameters fruit weight and yield. Statistical analyses were performed using the statistical software SISVAR 5.6 (7).

Results and discussion

ANOVA showed that all irrigation regimes had significant effects on the studied parameters at 1% significance by the F-test (table 1). The sewage concentrations had no significant effect on the evaluated parameters, but their interaction with irrigation regimes had a significant effect on plant height and fruit length.

Table 1. ANOVA for plant height (cm), fruit length (cm), and stem diameter (cm) in response to irrigation regimes and treated domestic sewage concentrations.

Tabla 1. Resumen del ANOVA para altura de la planta (cm), longitud del fruto (cm) y diámetro del tallo (cm) en respuesta a los niveles de reposición de agua y las concentraciones de reúso de aguas domésticas tratadas.

		Average square		
Variation Source	**DF**	**Plant height**	**Fruit length**	**Stem diameter**
Irrigation level (L)	3	117.69**	0.22287 **	4.55608 **
Sewage concentration (C)	2	24.00 ns	0.08100 ns	1.37479 ns
Interaction (L x C)	6	51.65 *	0.25642 **	1.25004 ns
Treatment	11	64.63 **	0.21538 **	2.17437 *
Block	3	42.07 ns	0.00266 *	0.55655 ns
Residue	33	20.47	0.03859	0.77309

(**) *p-value* ≤ 0.01; (*) p-value ≤0.05; (ns) non-significant at 5% probability level by the F-test.

(**) Efecto significativo al nivel de probabilidad del 1%; (*) significativo al 5% de probabilidad; (s) no significativo al nivel de probabilidad del 5% por la prueba F.

Tavares *et al.* (2019) found no significant effect of treated wastewater irrigation on pepper height, but on stem diameter and leaf number. Faccioli *et al.* (2017) also found no significant effect of treated wastewater on cowpea bean height, thus differing our study (table 1). Silva *et al.* (2019) verified no significant effect of different wastewater irrigation depths on chilli pepper fruit length, unlike our results. Tukey's test ($p \leq 0.05$) showed that sewage concentrations significantly affected plant height for L_1 at C_3 (table 2, page 179).

Table 2. Tukey's test for average plant height and fruit length in response to irrigation regimes and concentrations of treated domestic sewage.

Tabla 2. Prueba de Tukey para la altura media de la planta y la longitud media del fruto en respuesta a los niveles de reposición de agua y las concentraciones de reúso de aguas domésticas tratadas.

Plant height (cm)			
Sewage concentration			
Irrigation regime (mm)	**C_1**	**C_2**	**C_3**
L_1	31.00 aA	35.21 aA	24.96 bB
L_2	33.00 aA	31.58 aA	29.12 bA
L_3	32.17 aA	36.12 aA	39.62 aA
L_4	35.67 aA	36.71 aA	36.87 aA
Fruit length (cm)			
Sewage concentration			
Irrigation regime (mm)	**C_1**	**C_2**	**C_3**
L_1	1.64 bA	1.87 aA	1.64 bA
L_2	1.75 bB	2.15 aA	2.15 aA
L_3	2.12 aA	1.88 aA	1.92 aA
L_4	2.19 aA	2.06 aA	1.86 aB

L_1: 75 %, L_2: 100 %, L_3: 125 %, and L_4: 150 % of the crop evapotranspiration (ETc). C_1: 0% of treated domestic sewage + 100% water supply, C_2: 50% treated domestic sewage + 50% of water supply, C_3: 100% treated domestic sewage. Means followed by the same letter, lower-case in the column and upper-case in the row, did not differ statistically.

L_1: 75%, L_2: 100%, L_3: 125% y L_4: 150% de la evapotranspiración del cultivo (ETc). C_1: 0% de reúso de aguas domésticas tratadas y 100% de agua del sistema de suministro, C_2: 50% de reúso de aguas domésticas tratadas + 50% de agua del sistema de suministro, C_3: 100% de reúso de aguas domésticas tratadas. Los promedios seguidos de las mismas letras no difieren estadísticamente. En columna letras minúsculas y en la línea letras mayúsculas.

L_3 and L_4 irrigation regimes had effects on plant height only in plants receiving C_1, thus increasing plant heights. When analysing different irrigation depths for biquinho pepper cultivation on different substrates, Silva *et al.* (2016) found that an irrigation depth of 100% of the ETc produced higher plants (23 cm), which is significantly lower than our result (39.62 cm) at L_3. However, opposite to our study, these authors found higher plants when 100% water was applied (table 2). Nascimento *et al.* (2021) observed that water stress caused by deficit irrigation reduced plant height, seed mass, and pod yield of peanut, while full irrigation (100% of crop evapotranspiration replacement) led to yields of 4,141 to 5,102 kg ha^{-1}, approximately three times higher than those obtained with the lowest irrigation level (8% replenishment of crop evapotranspiration).

In our study, the longest fruits (2.19 cm) were obtained at L_4, with C_1, thus longer than that of C_3. Conversely, the smallest fruits (1.64 cm) were produced when the plants were subjected to L_1 and at C_3.

The irrigation regime effect was significant at a 5% probability for average fruit weight and yield. No significant effect was observed for concentrations of treated domestic sewage or their interactions with irrigation regimes for the studied parameters (table 3).

Table 3. Summary of ANOVA for data on average fruit weight and yield in response to irrigation regimes and concentrations of treated domestic sewage.

Tabla 3. Resumen del ANOVA de los datos del peso promedio de la fruta y la productividad en respuesta a los niveles de reposición de agua y las concentraciones de reúso de aguas domésticas tratadas.

		Mean square	
Variation Source	**DF**	**Fruit weight**	**Fruit yield**
Irrigation regime (L)	3	1035.68369 *	161825.57892 *
Sewage concentration (C)	2	411.52465 ns	64300.73272 ns
Interaction (L x C)	6	439.82768 ns	68723.06810 ns
Treatment	11	597.18786 ns	93310.60098 ns
Block	3	465.54059 ns	72740.71853 ns
Residue	33	356.50274	55703.55354

(**) *p-value* ≤ 0.01; (*) *p-value* ≤0.05; (ns) non-significant at 5% probability level by the F-test

(**) Efecto significativo al nivel de probabilidad del 1%; (*) significativo al 5% de probabilidad; (s) no significativo al nivel de probabilidad del 5% por la prueba F.

Regression equations were adjusted to express changes in average fruit weight (g) and yield (t ha^{-1}) as a function of the irrigation depths, respectively (figure 1).

Maximum yield of biquinho pepper was 14.3 t ha^{-1}, achieved by applying 143.4% ETc irrigation (figure 1). This result is lower than that found by Barroca *et al.* (2015), who obtained a maximum production of 43.6 t ha^{-1} for the variety 'Pimenta-de-Cheiro' when applying 119.6% ETo. However, the same authors found a lower value for the variety 'Dedo-de-Moça', which was 15.8 t ha^{-1} when applying 113.6% ETo. In our study, maximum average fruit weight was 99.5 g for a water supply of 142.5% ETc (figure 1).

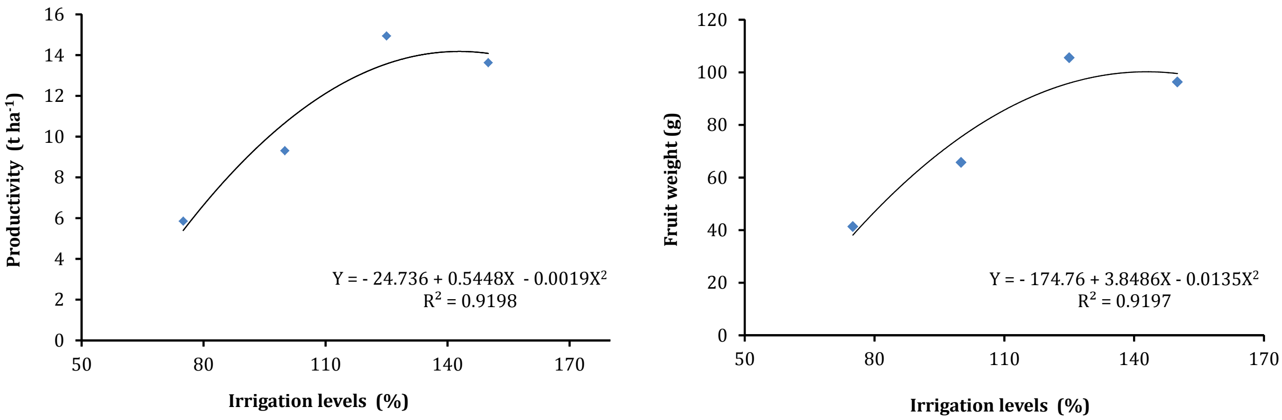

Figure 1. Regression analysis for average yield (a) and average fruit weight (b) of biquinho pepper plants as a function of irrigation regimes.

Figura 1. Análisis de regresión para productividad (a) y peso medio de la fruta (b), en función de los niveles de reposición de agua para el pimiento de pico.

Conclusion

Growth of biquinho pepper with treated domestic sewage can bring benefits in terms of crop nutrient supply, while environmental impact can be reduced by replacing chemical fertilisers. Treated domestic sewage reuse can constitute an alternative both for quality water saving and for chemical fertilization of biquinho pepper crops.

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CO_2 emission allowances and their interacion with economic and energy factors in the European Union

Los derechos de emisión de CO_2 y su interacción con los factores económicos y energéticos en la Unión Europea

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Abstract

Analysis of emission allowances prices has important environmental and political connotations. This article aimed to identifying the possible variables that may influence their behaviour and studied their relationship with fundamental factors: energy (brent petroleum, gas, coal) and economy (industrial production index, baltic dry index, purchasing managers index). With the objective of analyzing possible mutual interactions, Multivariate VAR or Error Correction Models (VECM), were applied. The information analysed derived from different sources (World Bank, Sendeco2 and various financial websites). The results obtained showed, not only the influence of past prices on the emission allowances actual price, but also the interaction with energetic and economic variables.

Keywords
VAR & VECM model • cointegration • allowances emission • environment • fundamental factors

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Resumen

El análisis del precio de los derechos de emisión resulta de gran interés por sus connotaciones medioambientales y políticas. Por ello, este artículo se centra en identificar las posibles variables que pueden influir en su comportamiento y estudiar su relación con factores fundamentales: energéticos (petróleo brent, gas, carbón) y económicos (índice de producción industrial, índice báltico seco, índice de gestión de compras). La técnica multivariante aplicada corresponde a modelos VAR o en su caso de corrección de errores (VECM). La información utilizada procede de diversas fuentes (Banco Mundial, Sendeco2 y de diversas web de carácter financiero). Los resultados obtenidos manifiestan no solo la influencia de los precios pasados en el precio de los derechos de emisión sino también la interacción de las variables energéticas y económicas sobre aquellos.

Palabras clave

modelo VAR/VECM • cointegración • derechos de emisión • medioambiente • factores fundamentales

Introduction

Climate change has become one of the most complex challenges of this century. No country is exempt, nor can face this global problem alone. As a current priority for all societies, international cooperation needs to address this environmental challenge with coordinated policies aiming at stabilizing or reducing greenhouse gas levels.

In this context, economic growth and reducing emissions constitute priority objectives. Making them compatible is essential in order to sustainably grow while fighting against climate change (17, 18, 20). For many years now, the fact that economic growth has increased greenhouse gas emissions causing intensified energy absorptions and aggravating the greenhouse effect, has produced widespread awareness.

Concerns regarding increasing greenhouse gas emissions have led to certain international consensus about fighting climate change. A series of legal regulations intended to deal with this problem, have also been issued. Thus, two international treaties were drafted: the United Nations Framework Convention on Climate Change (UNFCCC) and the Kyoto Protocol.

In 1994, the Framework Convention on Climate Change (United Nations, 1992) came into force in numerous countries. In the case of the EU, it was ratified by 94/69/EC: Council Decision, 15 December 1993. This Convention recognized an increase in greenhouse gas concentration both due to human causes and natural factors (Article 1). In view of this, the Convention sought to achieve stabilization of atmospheric CO_2 while pursuing sustainable economic development. However, this Framework Convention neither imposed restrictions on the emission of greenhouse gases, nor established mechanisms for their reduction.

To extend that treaty and proceed in the fight against climate change, in 1997 the famous 'Kyoto Protocol' established emission limits for developed countries. Subsequently, meetings called "Conference of the Parties" (COP) aimed to develop the actions to be consequently followed. Among the 25 conferences held to date, in the 'Paris Agreement' adopted in 2015, countries promised to limit global warming to 1.5 °C above pre-industrial levels.

In this context, reduced emissions have constituted a global and climatic priority. However, the way to achieve this goal, has been neglected. A number of instruments, known as flexibility mechanisms, were introduced with the intention of upholding the commitments undertaken by the Parties. Thus, these "Joint Implementation" mechanisms regulated the investment resulting from projects aiming at limiting anthropogenic emissions in industrialized countries. Another mechanism was "Clean Development", which consisted of an industrialized country investing on a developing country. Another way of upholding the commitments in the Protocol was the possibility of negotiating emission allowances. This is currently the most widely used procedure and has led to the creation of a financial market for these assets (European Emission Allowances, EUA).

With focus on economic theory, some researchers consider that Greenhouse Gas emissions are negative externalities associated with climate change, considered a "public bad" complying with the conditions of non-rivalry and non-exclusion (16, 21). Furthermore, given this condition of "public bad", it turns evident that a solution to the problem requires global commitment (5). Therefore, estimating a proper carbon price turns indispensable for an emissions reduction strategy (19). Economic literature describes the calculation of social prices and the CO_2 shadow price. Market price, social cost of carbon and marginal cost of reducing emissions were identified as evaluation methods (4). Additionally, carbon pricing can be implemented through carbon credits price as a proxy variable of society's willingness to pay for reducing GHG emissions. However, this does not seem to reflect the true social value (5).

Considering this background, this article investigated the relationship between emission allowances pricing and energy and economic variables. This interrelation is considered a starting hypothesis, to be contrasted through its analysis. Consequently, the objective of this work focused on modelling emission allowances prices along with energy and economic factors, during the 2008-2019 period. This analysis intended to identify certain variables that may influence the emission allowances prices, and if the latter could affect the former. To this end, their relationships were studied through autoregressive vector models (VAR) and error correction (VECM). These models are useful when evidence of a certain temporal relationship between variables, is available (12, 13, 22).

Material and methods

Studies on emission allowances have focused on two different approaches, one regulatory and another related to the analysis of emission allowances prices. When considering this analysis, and given the existing knowledge on the market and its possible interrelations, to reference both approaches seems necessary.

Regulatory framework of emission allowances

In relation to the regulatory context, a wide range of international regulations have been issued. In Europe, Decision 2002/358/EC, ratified the Kyoto Protocol, and the EU committed to reduce its greenhouse gas emissions, however, with a different distribution among the member states.

In order to meet the objective set by the Kyoto Protocol, the EU issued Directive 2003/87/EC, amended by Directive 2004/101/EC and Directive 2009/29/EC, among others, establishing a regime for the EU Emission Trading Systems (EU-ETS). These directives constituted complementary instruments for cost reduction and efficiency improvement of emissions. This trading scheme established a market mechanism called "cap and trade", setting a total limit on annual greenhouse gas emissions and a trading system for these allowances. It also established allowance equivalence, an instrument which allowed emitting a ton of carbon dioxide equivalent, within a certain period of time. Furthermore, this allowance was negotiable and transferable.

Different phases for developing this emission allowances trading regime, were established. Pilot phase I, lasted three years (2005-2007). The EU Emission Trading System (EU-ETS) was launched in January 2005 and each State defined its own emission ceiling with a decentralized allowance apportionment structure. Overall, allowance allocation was free of charge while facilities' historical activity constituted the reference point determining the quantity of allowances. This led to emission overestimation and resulted in market allowances excess.

Phase II lasted five years (2008-2012) and free allowance allocation was the chosen procedure meeting undertaken commitments undertaken by each Member State. Additionally, a series of instruments relaxing these commitments, were introduced: The "Banking" instrument allowed acquired emission rights to be used in a subsequent period, while "borrowing", meant that emissions and the corresponding emission allowances from a preceding period, could be fulfilled with allowances issued in subsequent periods.

Phase III (2013-2020) introduced big changes and carried out an important revision of the EU-ETS regulated by Directive 2003/87/EC, intended to promote the efficient reduction of greenhouse gas emission. Thus, the EU issued Directive 2009/29/EC, which partially amended Directive 2003/87/EC improving and expanding the EU-ETS, and committing to limit global greenhouse gas emission on at least 20% of 1990 levels, by 2020. This set an emission ceiling in Europe, with a 1.74% annual linear decrease of allowances, up to 2020.

This phase also eliminated free allotment of emission allowances for power generators. In the industry, free allotment is temporary, and based on EU benchmarks rather than on facilities' historic indicators. This temporary allotment is to be gradually reduced over time, going from an initial 80% in 2013 to 30% in 2020 and 0% in 2027. However, exceptions are established for those facilities exposed to "risks of carbon leakage", getting 100% of their allowance free of charge (relocation of industries in countries with no legislation equivalent to EU-ETS).

Another relevant element of Directive 2009/29/EC was the inclusion of a harmonized method of allowances allocation at EU level, being auction the main procedure for allocation. They were controlled through various regulations (Regulation 1031/2010, amended by Regulation 1210/2011 and Regulation 1143/2013).

For its part, Regulation 176/2014 amended Regulation 1031/2010, establishing the volume of gas emission allowances that would be auctioned in 2013-2020. Thus, in view of an excess of allowances in the market by the end of 2013 (the end of Phase II and beginning of Phase III), the European Commission proposed measures to avoid market imbalances. One of these measures was "backloading", a mechanism involving withdraw of about 900 million EUA from available volumes within the 2014-2016 period, reintroducing it in instalments in 2019 and 2020. However, the planned reintroduction –300 million EUA in 2019 and 600 million EUA in 2020 - as stated in Regulation 176/2014, was then thought to cause structural supply-demand unbalances. Therefore, those 900 million EUA were not to be auctioned in 2019 or 2020 but added to a market stability reserve (Decision 2015/1814).

In addition, other regulations aimed at intensifying emission reductions in the EU, have raised. For instance, the 2050 Low-Carbon Economy Roadmap towards a competitive low-carbon economy, the Climate & Energy Package (2013-2020), Directive 2018/410, issued to, once more, amend Directive 2003/87/EC, and Decision (EU) 2015/1814 with the purpose of enhancing cost-effective emission reductions.

Background in the research on emission allowances

Prior research lines analyzing emission allowances price, have focused on applying univariate or multivariate procedures. Univariate models, mainly apply ARIMA models and, in some cases, volatility models (8). Multivariate methods intend to justify the behaviour of emission allowances price through different variables, (2, 11, 12, 15). In this multivariate context, economic, energy and climatic variables are chosen for analysis.

Some economic variables focus on stock indices (11), macroeconomic and financial indicators (6) or business indices such as the Industrial production index (1, 7, 8). Their positive influence on allowances prices has been widely demonstrated, although depending on the variable itself and the study period.

In relation to energy factors, prices depending on different energy sources (renewable, hydroelectric, fossil), and fossil fuels (oil, coal and gas) are widely used. In general, research using energy prices study their positive relationship with emission allowances prices, while fossil fuel prices impacts are not as evident as the former (1, 7, 15, 19). Additionally, among climatic variables, temperature is mostly studied (1, 15).

In this context, numerous empirical studies have analyzed the behaviour of emission allowances prices in phases I and II. Applied research dealing with phase III, or all periods simultaneously, is scarce (22). In addition, many of these studies have focused on Europe and, in a smaller number, on other markets, such as the Nordic (19), Chinese (12) or American (21).

Methodology

In this work, multivariate models (VAR / VECM) were applied, considering, a priori, all variables as endogenous and each one affecting the others. Therefore, a VAR model (p) (Y_t) is defined according to its p lags (Y_{t-i}) weighted by the coefficient matrix A_i, by exogenous variables (X_t) affected by coefficient matrix B, and by error terms (e_t), i.i.d N(0, Ω).

$$Y_t = A_1 Y_{t-1} + \cdots + A_p Y_{t-p} + BX_t + e_t$$

Thus, the VAR (p) model for the two studied variables is:

$$Y_{1t} = \beta_{10} + \beta_{11} Y_{1t-1} + \cdots + \beta_{1k} Y_{1t-p} + \alpha_{11} Y_{2t-1} + \cdots + \alpha_{1k} Y_{2t-p} + e_{1t}$$

$$Y_{2t} = \beta_{20} + \beta_{21} Y_{2t-1} + \cdots + \beta_{2k} Y_{2t-p} + \alpha_{21} Y_{1t-1} + \cdots + \alpha_{2k} Y_{1t-p} + e_{2t}$$

The VECM can be inferred based on this VAR model, with level and differences variables, in the following terms:

$$\Delta Y_t = \pi Y_{t-1} + \sum_{i=1}^{p-1} \pi_i \Delta Y_{t-i} + B X_t + e_t$$

where:
ΔY_t = the differential operator (Y_t - Y_{t-1})
$\pi = -(I - \sum_{i=1}^{p} A_i)$ = a matrix with rank r containing the cointegration relations existing

between the k variables
$\pi_i = - \sum_{j=i+1}^{p} A_j$ = the coefficient matrix for ΔY_{t-i}

Also, if the π matrix rank is r, being r < k, this matrix can be broken down into the product of two matrices (π **= β'**)

where:
α = a k x r matrix whose coefficients correspond to speed adjustment
β' = a r x k matrix, collecting the cointegration relations coefficients.
Therefore, in order to define the VAR or the VECM model, stationarity of each series in the first place, and their potential cointegration, had to be analyzed.

Variables and information sources

The information analyzed corresponds to monthly series, from January 2008 to July 2019 (139 months) obtained from various institutions. For emission allowances prices, the European trading platform for emission allowances (Sendeco2), was referenced. Regarding the economic factors, the EU-28 Industrial Production Index (IPI), The Baltic Dry Index (BDI) and the Purchasing Managers Index (PMI), were analyzed.

All historical data series cover the 139 months. The EU-28 Industrial Production Index, was calculated after information from Eurostat. The PMI and BDI indices, created by IHS Markit Economics and The Baltic Exchange respectively, were obtained through the financial web.

The European Industrial Production Index (IPI) was selected for being a relevant indicator of evolution undergone by the industrial productive activity. It eliminates price influence, allowing the macroeconomic situation to be characterized. BDI and PMI, novel variables in the analysis context of emission allowances price, also tightly related to the economic situation, have gained significant international impact. The BDI index, created in 1985, is related to ocean freight contracting for raw materials and bulk goods. Since economic evolution affects fright contracting, this index can be considered a world demand proxy variable. The European PMI constitutes an important indicator of business sentiment. Contrasting with the industrial production index, it considers future prospects contemplating preceding periods. The IPI takes a neutral value of 50, with higher levels implying activity progress and lower levels marking slowdowns. It constitutes the most widely used indicator –along with the Economic Sentiment Indicator (ESI)– for economic assessment (European Commission, European Business Cycle Indicators, 2nd Quarter 2017). Consequently, these variables and their evolution may affect the allowances price, since economic growth, related to these variables, influences greenhouse gases emissions.

Data on monthly prices of energy factors, was obtained from the World Bank information. Oil (Brent type), gas (European type) and coal, (average price in Australia and South Africa, main world coal markets) were considered. As these factors are priced in dollars for different energy units, they were converted to the same monetary and energy unit (€/Mwh), according to euro-dollar exchange rate, and the conversion of the International Energy Agency.

A priori, the price of fossil fuels is expected to influence the emission allowances prices, since fuel type and its level of use, could influence allowances demand. Thus, the cheaper but more polluting coal would cause greater demand for allowances, (Megawatt-hour). The use of gas, on the contrary, means lower emissions, but higher costs. Finally, brent oil produces higher emissions than gas but lower than coal, while being the most expensive per unit of produced energy.

Results

The results obtained were structured in different stages. Firstly, a descriptive analysis addressed behaviour and time relationship of emission allowances prices, fossil fuels prices and economic indices. Then, the VAR/VECM was estimated, contrasting and validating the model.

Descriptive analysis of fundamental factors

Emission allowances prices (EUA) have experienced two major trends, one decreasing trend from the beginning of 2008 to mid-2017 and, a rising trend from mid-2017, onwards. In this initial decreasing trend, monthly prices ranged between 27 and 3 €, reflecting non-stationary behaviour. After the sharp fall in 2008, these prices have maintained certain levels through different periods: 10-15 € in 2008-2011, 3-10 € in 2013-2016, and around 5 € in 2016-2017. Some institutions and researchers justified the EUA price drop in terms of a supply *vs.* demand imbalance caused by different factors: lower energy consumption as a result of the economic crisis and the collapse of industrial activity and consequently, of CO_2 emissions. However, an increasing trend has been observed since mid-2017 in terms of the EUA price, achieving almost 27 € (figure 1).

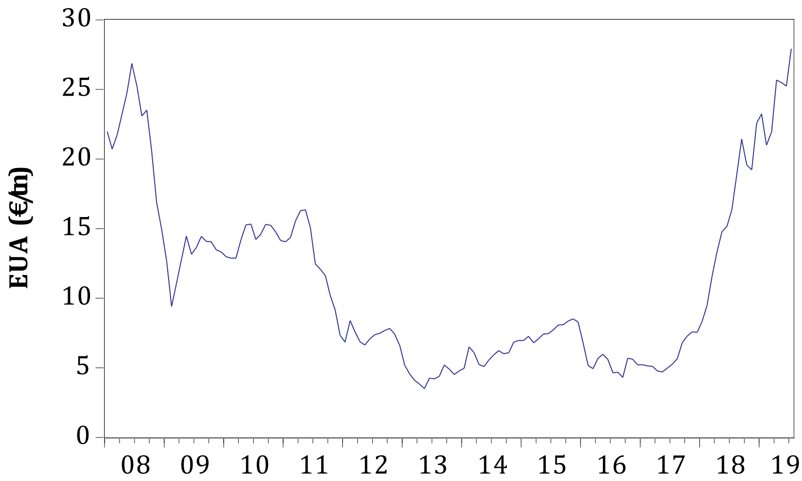


Figure 1. Monthly allowances emission prices (EUA) (2008-2019).

Figura 1. Precios mensuales derechos emisión (EUA) (2008-2019).

This situation brought a significant emission rights surplus in the system, which in turn caused prices to fall, meaning that these prices did not have a disincentive effect in relation to the use of less contaminating energies. Generous national free emission allowances in previous negotiation periods, as well as the use of cheap international emissions reduction credits or policies, oriented to energy efficiency and renewable energies sources (25), had significant effects.

Many factors may have influenced this change in trend. The recovery in industrial activity has meant higher emissions and has therefore caused a higher industrial demand of CO_2 emission allowances. Approved measures by the end of 2016, regulating the market and addressing emission rights surplus with the Market Stability Reserve, is another reason worth noting. Furthermore, increasing energy factor prices have also affected the EUA price.

Finally, the application of the new legal framework since January 2018 (Directive 2014/65/EU), with new financial agents to operate and additional legal requirements, is likely to result in an increasing demand for emission allowances.

Therefore, emission allowances prices present an important variation (3-27 €) reflecting non-stationarity in the considered period. The same behaviour is shown by the series corresponding to energy factors, oil (BRENT), coal (COAL) and gas (GAS), and by those related to the economic indexes, IPI, PMI and BDI. However, the IPI and PMI variables showed to be somewhat more stable than the BDI index (figure 2).

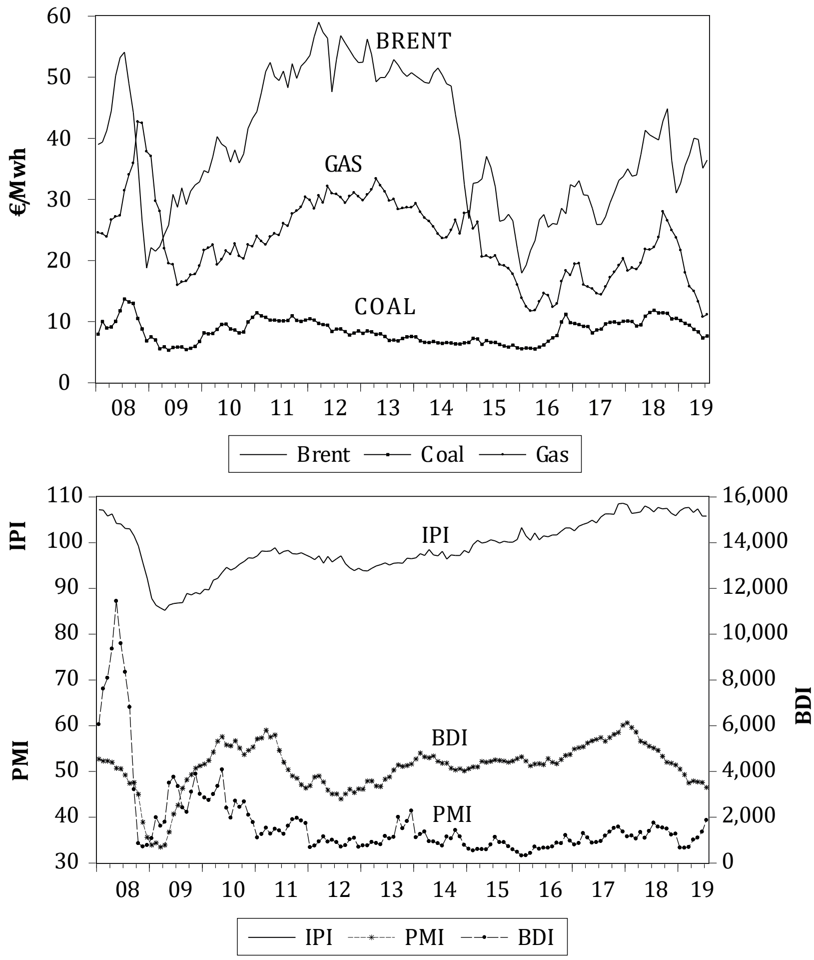


Figure 2. Energy and economic factors evolution (2008-2019).

Figura 2. Evolución de los factores energéticos y económicos (2008-2019).

VAR/VECM Model estimation

Endogenous variables are constituted by emission allowances prices (EUA), energy variables prices (BRENT, COAL, GAS) and economic indices (IPI, PMI, BDI). All variables were transformed into logarithms, reducing their variability (variables with initial "L"). The VAR/VECM model estimation made it necessary to study stationarity and the possibility of cointegration of these series. In summary, if the series resulted to be stationary, a level VAR model should be chosen, but if the series were integrated but not cointegrated, a difference VAR model had to be applied. Finally, if the series were integrated and cointegrated, a VEC model (Vector error correction) ought to be used.

The stationarity analysis was performed by contrasting the existence of unit roots, for series in level and in first differences. The Dickey-Fuller Augmented (ADF) and the Philips-Perron (PP) tests, with a constant term, were used. In general, the “level series” concluded that the null hypothesis of the existence of a unitary root, was not rejected, reflecting lack of stationarity, while for the “series in first differences” the null hypothesis was rejected, so the series were integrable of order 1, I (1) (table 1).

Table 1. Unit root tests, in levels and differences (Δ), (t-statistic and p-value in parentheses).

Tabla 1. Test raíces unitarias, en niveles y diferencias (Δ), (t-estadístico y p-value en paréntesis).

Energy Variables	Series	Dickey-Fuller Augmented	Philips-Perron	Economic Variables	Series	Dickey-Fuller Augmented	Philips-Perron
LBRENT	Level	**-2.42**	**-1.80**	LBDI	Level	**-2.58**	**-2.42**
		(0.13)	(0.37)			(0.09)	(0.13)
	Δ	**-8.80**	**-8.61**		Δ	**-11.25**	**-12.54**
		(0.00)	(0.00)			(0.00)	(0.00)
LCOAL	Level	**-2.54**	**-2.49**	LIPI	Level	**-1.26**	**-1.73**
		(0.10)	(0.12)			(0.64)	(0.40)
	Δ	**-9.84**	**-9.89**		Δ	**-9.46**	**-10.35**
		(0.00)	(0.00)			(0.00)	(0.00)
LGAS	Level	**-1.52**	**-1.69**	LPMI	Level	**-3.09**	**-2.82**
		(0.52)	(0.43)			(0.04)	(0.06)
	Δ	**-9.18**	**-9.39**		Δ	**-5.53**	**-5.67**
		(0.00)	(0.00)			(0.00)	(0.00)
LEUA	Level	**-1.13**	**-1.14**				
		(0.69)	(0.69)				
	Δ	**-8.28**	**-8.24**				
		(0.00)	(0.00)				

For cointegration series analyses, a regression model with the considered variables (9) and the existence of unit roots in the contrasted residues, was constructed. The Augmented Dickey-Fuller test (ADF) did not reject the null hypothesis of the existence of a unit root and, therefore, showed lack of stationarity and non-cointegration, resulting in integrable series of order 1, but not I (0) (table 2).

Table 2. Regression model and residuals test.

Tabla 2. Modelo de regresión y contraste de residuos.

Dependent Variable: LEUA					**RESIDUALS** (Critical value, 4.48 (5%) (variables=7, sample=137)			
Variable	Coefficient	Std. Error	t-Statistic	Prob.	Null Hypothesis: unit root		t-Statistic	Prob.
LBRENT	-0.61	0.18	-3.30	0.00	Augmented Dickey-Fuller test		-1.886	0.337
LCOAL	0.94	0.17	5.70	0.00	Test, critical values:	1% level	-3.479	
LGAS	0.19	0.20	0.93	0.35		5% level	-2.883	
LIPI	0.22	0.43	0.50	0.62		10% level	-2.578	
LPMI	-0.57	0.48	-1.17	0.24	Engle-Granger test (D(Residuals)=β*Residuals(-1)), (β= -0.073)			0.06
LBDI	0.43	0.05	8.80	0.00				

This non-cointegration led to a VAR model, although after converting the original series to stationary, differentiating previously logarithm transformed variables (variables with initial “D”).

Optimum lag lengths estimation in the VAR model, was also necessary. Excessive lag lengths would unnecessarily reduce degrees of freedom, but insufficient lag lengths would result in a lack of specification, probably affecting residual autocorrelation. Therefore, various criteria to select lag number for the variables analyzed, were applied: one lag for criteria like Akaike (AIC), Schwarz (SC) and Hannan-Quinn (HQ), or three lags for the LR sequential test. Considering that the choice must be consistent with the non-autocorrelation residuals, property verification with both alternatives, was also necessary. Consequently, considering the Akaike and Schwarz criteria and the goodness level, the VAR model with three lags, was finally chosen (table 3).

Table 3. VAR Lag Order Selection (* lag order selected by the criterion, 5% level).

Tabla 3. Selección de retardos modelo VAR (* orden retardo seleccionado por criterio, 5% nivel).

Criteria (LR: sequential modified test; AIC: Akaike; SC: Schwarz; HQ: Hannan-Quinn).

Lag	LogL	LR	AIC	SC	HQ
1	1,252.05	NA	-20.56*	-19.40*	-20.09*
2	1,287.15	61.79	-20.32	-18.01	-19.38
3	1,326.62	64.76*	-20.16	-16.69	-18.75
4	1,352.70	39.69	-19.77	-15.14	-17.89

Next, VAR model stationarity, a condition analyzed through characteristic polynomial roots, was contrasted. Given that these values were lower than one and included within the unit circle, their stability was verified. In addition, as in the VAR model the variables may have been related, contrasting their causality in order to find their interaction level (Annex A1, Table A1_1), was important. Therefore, the Granger test was applied, variable pairwise, using lags order (3) in the explanatory variable (table 4).

Table 4. Causality Granger, variables pairwise (Lags: 3).

Tabla 4. Causalidad de Granger entre variables (retardos: 3).

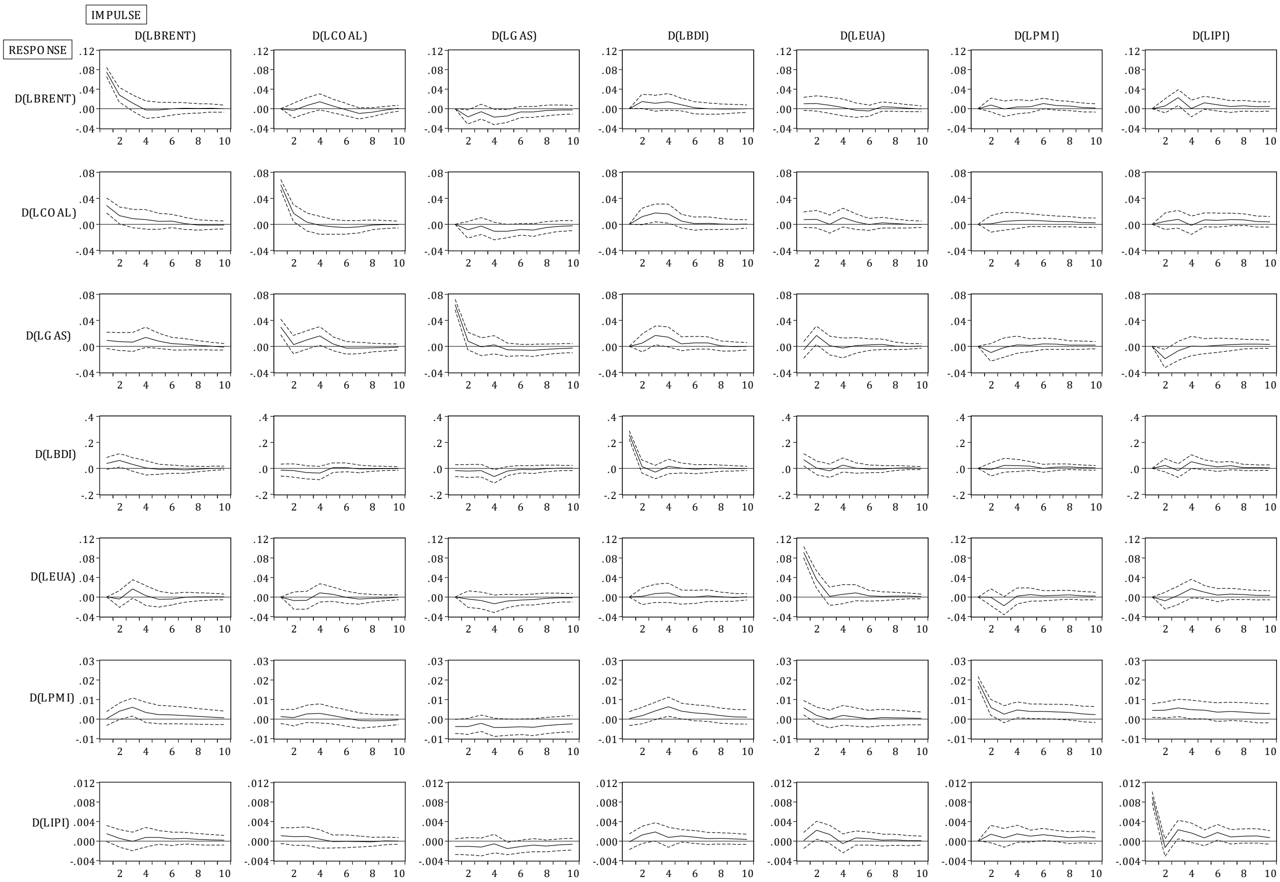
	Null Hypothesis (doesn’t Cause)	Prob.		Null Hypothesis (doesn´t Cause)	Prob.
D(LEUA)	**D(LBRENT) on D(LEUA)**	**0.005**	D(LGAS)	**D(LCOAL) on D(LGAS)**	**0.009**
	D(LEUA) on D(LBRENT)	0.362		D(LGAS) on D(LCOAL)	0.917
	D(LGAS) on D(LEUA)	0.283		**D(LIPI) on D(LGAS)**	0.489
	D(LEUA) on D(LGAS)	0.140		D(LGAS) on D(LIPI)	0.569
	D(LCOAL) on D(LEUA)	0.538		**D(LPMI) on D(LGAS)**	0.902
	D(LEUA) on D(LCOAL)	**0.058**		D(LGAS) on D(LPMI)	0.576
	D(LIPI) on D(LEUA)	0.421		**D(LBDI) on D(LGAS)**	0.157
	D(LEUA) on D(LIPI)	0.074		D(LGAS) on D(LBDI)	**0.029**
	D(LPMI) on D(LEUA)	0.339	D(LCOAL)	**D(LIPI) on D(LCOAL)**	0.094
	D(LEUA) on D(LPMI)	0.764		D(LCOAL) on D(LIPI)	**0.033**
	D(LBDI) on D(LEUA)	0.167		**D(LPMI) on D(LCOAL)**	0.388
	D(LEUA) on D(LBDI)	0.894		D(LCOAL) on D(LPMI)	0.062
D(LBRENT)	**D(LGAS) on D(LBRENT)**	0.092		**D(LBDI) on D(LCOAL)**	**0.028**
	D(LBRENT) on D(LGAS)	0.204		D(LCOAL) on D(LBDI)	**0.054**
	D(LCOAL) on D(LBRENT)	0.438	D(LIPI)	**D(LPMI) on D(LIPI)**	**0.005**
	D(LBRENT) on D(LCOAL)	0.239		D(LIPI) on D(LPMI)	**0.003**
	D(LIPI) on D(LBRENT)	0.061		**D(LBDI) on D(LIPI)**	**0.004**
	D(LBRENT) on D(LIPI)	0.124		D(LIPI) on D(LBDI)	0.411
	D(LPMI) on D(LBRENT)	0.090	D(LPMI)	**D(LBDI) on D(LPMI)**	**0.039**
	D(LBRENT) on D(LPMI)	**0.034**		D(LPMI) on D(LBDI)	0.810
	D(LBDI) on D(LBRENT)	**0.030**			
	D(LBRENT) on D(LBDI)	0.061			

Therefore, the past value D(LBRENT),influenced D(LEUA) and D(LPMI), while in turn, the variable D(LEUA) affected D(LCOAL). Likewise, a relation between the energy variable D(LCOAL) with D(LGAS) and with D(LIPI), was found.

Furthermore, the variables (D(LCOAL), D(LGAS) and D(LBRENT)) influenced the D(LBDI) index and, by contrast, D(LBDI) had an impact on (D(LBRENT) and D(LCOAL)) and on the economic factors D(LPMI)) and D(LIPI)). Finally, (D(LPMI)) and D(LIPI)) related to each other.

The Impulse-Response Function is a procedure based on VAR model reformulation, as moving averages. It analyzes the time effect that an impulse or alteration in each variable would produce on the remaining variables, given the dynamic interrelation among all variables. This type of analyses offered by the VAR model, constitute statistical interpretations of the responses of one variable after the impact of another.

In general, the response of each variable to impacts on its own innovations, is positive and decreasing over time, while the response of each variable to impacts coming from other variables, reflects different behaviours (figure 3).



Cholesky Ordering: D(LBRENT), D(LCOAL), D(LGAS), D(LBDI), D(LEUA), D(LPMI), D(LIPI).

Figure 3. Response to Cholesky (One S.D. Innovations ± 2 S.E.).

Figura 3. Función impulso-respuesta, Cholesky (Una S.D. Innovaciones ± 2 S.E.).

Thus, variables such as D(LBRENT), D(LEUA) and D(LBDI) usually cause a positive response in the rest of the variables, unlike the impact of D(LGAS), usually negative. Also, the impact of D(LIPI) or D(LPMI) positively affected the remaining variables, except for D(LGAS) and D(LEUA) which had an initial negative behavior, but a final positive performance.

D(LCOAL) impacts had different responses depending on the variable. It negatively impacted D(LBDI), while), it showed positive impacts on variables like D(LGAS), D(LIPI) and D(LPMI or temporal opposite effects (initially negative and then positive), on D(LEUA) and D(LBRENT).

The model was validated by means of residual analysis, contrasting the existence of non-autocorrelation, normality and homoscedasticity. To study autocorrelation, the Portmanteau and Lagrange tests were applied, not rejecting the null hypothesis of correlation absence.

Residuals normality was assessed using Cholesky Orthogonalization and the Jarque-Bera test for skewness and kurtosis. The null hypothesis was rejected and, therefore, residuals resulted not normally distributed. However, lack of normality in VAR models does not affect their validity (14). For homoscedasticity analysis, the White test without cross-terms was applied, and the null hypothesis of homoscedasticity was not rejected.

Discussion

Estimating carbon price constitutes an efficient strategy for reducing emissions and their negative impact (10). Even though some methods have attempted to estimate this price, the creation of an emissions market has allowed to precisely know this value, facilitating negotiations with emission allowances. Therefore, with a determined market price, relationships between emission allowances pricing with other variables (economic and energy) can be estimated. This approach identifies variables with major influence, their variations, and forecasts emission allowances prices.

In this context, approaches within the multivariate context of time series, should be considered. Some authors apply error correction models (VECM), considering the existence of cointegration variables (11, 19), while others prefer VAR models with non-cointegration variables (Chevalier, 2011b; Zeng *et al.*, 2017; Jiang *et al.*, 2018, among others).

Many significant factors like the procedure, the geographic context, or the model estimation period conditioned the obtained results. For this particular study case, the analyzed information comprised from 2008, period in which the emission allowances negotiations actually began in the EU (phase II), to 2019.

Regarding the interaction among emission allowances prices with the rest of the variables, causalities of D(LBRENT) on D(LEUA), and of D(LEUA) on D(LCOAL), were verified.

In particular, for D(LEUA), the impulse-response function analysis showed dependency on the impacting variable, while responses to D(LGAS) and D(LBDI),were negative and positive, respectively. For the remaining variables, D(LEUA) response was practically similar, but of opposite signs. These results are in accordance with those obtained by other authors, also stating the relationship among emission allowances prices and their past values (11, 12). In addition, Hu *et al.* (2018) showed, for the European market, the relationship with Brent oil and stock indices in the period 2014-2015, while Jiang *et al.* (2018) did not consider the influence of oil in the 2013-2017 period for the Chinese market.

Another research on the European market during the 2008-2009 period divided into subperiods, showed that the significance of brent, gas and coal prices on emission allowances prices, depended on the subperiod considered (7). Later, for the European market during 2005-2016, and three sub-periods, another study showed how fossil fuels (oil, gas and coal) and several financial market indexes influenced emission allowances prices, according to the subperiod analysed (23).

Likewise, Alexeeva-Talebi (2011) applied a VECM model in different European countries in the period 2005-2007 and observed that Brent price only influenced emission allowances prices in some countries. Finally, Koch *et al.* (2014) concluded that for the European market in the 2008-2013 period, emission allowances prices were only influenced by the economic satisfaction index and the price of gas and electricity provided by renewable sources.

Considering energy variables, certain literature discrepancy about their influence on emission allowances price is evident, but, in general, they coincide in the relationship with some fuels.

Finally, some authors state that economic variables like stock market indices, industrial production, economic satisfaction, futures and financial asset returns, affect emission allowances prices. In this work, the industrial production index and two novel variables, the European purchasing management index and the Dry Baltic index have been used, being this last variable, more significant regarding the emission allowances price.

Conclusions

This study investigated the relationship between emission allowances prices and energy and economic variables, by modelling their behaviour.

The analysis was approached under a multivariate basis, considering energy and economic variables. It was concluded that energy variables partly explain the behaviour of those prices, and their inclusion in any study, is recommended. Regarding economic variables, the Dry Baltic index (BDI) resulted a significant variable, related to energy factors. The Industrial Production Index (IPI) and the European Purchasing Management Index (PMI), resulted to be less important.

These conclusions confirm the studied energy variables and the BDI to be significant for explaining variations in the emission allowances prices. These conclusions may lead future research, not only regarding analysis procedures, but also confirming interrelation between some variables.

In order to achieve stationarity, the original variables required transformation, and the lack of cointegration led to estimating a VAR model, including temporal variable interrelation. Several conclusions related to causality and impulse-response functions were obtained. One conclusion stated that variations in emission allowances price are mainly conditioned by prior values and by some variables, mainly brent, gas and BDI.

Finally, emission allowances prices resulted to be sensitive to other variables impact, although in a heterogeneous fashion; Gas impacted negatively and BDI, positively. The remaining variables, such as brent petroleum and coal, and the economic factors (IPI and PMI) had somewhat similar behaviour, with changing sign, firstly negative and subsequently becoming positive). This sign change in the emission allowances price variation, may be due to an initial demand contraction. However, when price growth consolidates over time, the consequent demand turns greater, finally causing increasing price variations.

Supplementary tables

https://drive.google.com/file/d/1qfhWCF56M2GfHtx6Bua4igHFKFTW_Jef/view?usp=sharing

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Annex A1

Tabla A1_1. Estimación modelo VAR (3) (estadístico t-en paréntesis).
Table A1_1. Estimation VAR (3) model (t-statistic in parenthesis).

Variables	D(LEUA)	D(LBRENT)	D(LCOAL)	D(LGAS)	D(LBDI)	D(LIPI)	D(LPMI)
D(LEUA(-1))	0.392	-0.005	0.012	0.191	-0.079	0.014	-0.012
	(3.886)	(-0.062)	(0.159)	(2.423)	(-0.265)	(1.325)	(-0.521)
D(LEUA(-2))	-0.102	0.076	-0.061	-0.109	-0.202	0.008	-0.019
	(-0.950)	(0.854)	(-0.771)	(-1.299)	(-0.641)	(0.704)	(-0.775)
D(LEUA(-3))	0.046	-0.113	0.070	-0.034	0.189	-0.011	-0.014
	(0.449)	(-1.343)	(0.928)	(-0.426)	(0.636)	(-1.118)	(-0.601)
D(LBRENT(-1))	-0.006	0.343	0.031	0.094	0.818	0.000	0.039
	(-0.048)	(3.370)	(0.346)	(0.979)	(2.270)	(0.022)	(1.373)
D(LBRENT(-2))	0.232	-0.112	-0.003	-0.038	0.412	-0.021	0.019
	(1.819)	(-1.055)	(-0.027)	(-0.382)	(1.097)	(-1.650)	(0.636)
D(LBRENT(-3))	-0.231	-0.208	-0.028	-0.067	0.123	-0.008	-0.024
	(-1.836)	(-1.993)	(-0.295)	(-0.682)	(0.332)	(-0.632)	(-0.830)
D(LCOAL(-1))	-0.066	0.044	0.331	0.060	-0.168	0.025	0.017
	(-0.422)	(0.341)	(2.879)	(0.497)	(-0.368)	(1.589)	(0.461)
D(LCOAL(-2))	0.044	0.064	-0.056	0.240	-0.412	0.016	0.022
	(0.288)	(0.503)	(-0.489)	(1.992)	(-0.911)	(0.992)	(0.617)
D(LCOAL(-3))	0.218	0.316	0.074	0.230	-0.183	0.001	0.026
	(1.471)	(2.563)	(0.672)	(1.981)	(-0.418)	(0.071)	(0.758)
D(LGAS(-1))	-0.085	-0.220	-0.113	0.064	-0.303	-0.014	-0.033
	(-0.636)	(-1.990)	(-1.146)	(0.614)	(-0.776)	(-1.060)	(-1.076)
D(LGAS(-2))	-0.132	0.089	0.075	-0.015	-0.095	-0.005	0.016
	(-1.050)	(0.851)	(0.801)	(-0.149)	(-0.257)	(-0.380)	(0.565)
D(LGAS(-3))	-0.074	-0.207	-0.114	0.091	-0.797	0.004	-0.017
	(-0.591)	(-1.983)	(-1.227)	(0.925)	(-2.162)	(0.336)	(-0.603)
D(LBDI(-1))	0.005	0.057	0.046	0.018	0.047	0.005	0.007
	(0.139)	(2.079)	(1.854)	(0.698)	(0.479)	(1.390)	(0.881)
D(LBDI(-2))	0.035	0.019	0.048	0.065	-0.163	0.007	0.010
	(1.047)	(0.690)	(1.904)	(2.441)	(-1.636)	(1.906)	(1.312)
D(LBDI(-3))	0.027	0.042	0.043	0.052	0.062	0.002	0.013
	(0.740)	(1.391)	(1.621)	(1.832)	(0.583)	(0.539)	(1.557)
D(LIPI(-1))	-0.850	0.426	0.485	-1.876	2.706	-0.182	0.357
	(-0.866)	(0.523)	(0.667)	(-2.446)	(0.939)	(-1.828)	(1.582)
D(LIPI(-2))	0.853	1.733	0.290	-0.746	-2.975	0.144	0.408
	(0.821)	(2.011)	(0.376)	(-0.919)	(-0.975)	(1.365)	(1.706)
D(LIPI(-3))	1.677	-0.662	-0.769	-0.251	2.121	0.125	0.099
	(1.610)	(-0.765)	(-0.998)	(-0.308)	(0.693)	(1.181)	(0.411)
D(LPMI(-1))	-0.033	0.393	0.025	-0.493	-0.522	0.073	0.306
	(-0.073)	(1.063)	(0.074)	(-1.414)	(-0.398)	(1.614)	(2.984)
D(LPMI(-2))	-0.884	-0.392	0.125	0.184	0.603	0.020	-0.021
	(-1.834)	(-0.980)	(0.350)	(0.487)	(0.426)	(0.403)	(-0.186)
D(LPMI(-3))	0.562	0.125	0.149	0.426	0.698	0.066	0.146
	(1.316)	(0.352)	(0.472)	(1.276)	(0.556)	(1.532)	(1.485)
R-squared	0.274	0.376	0.288	0.298	0.203	0.324	0.420
Akaike AIC	-1.795	-2.167	-2.398	-2.288	0.360	-6.371	-4.732
Schwarz SC	-1.325	-1.697	-1.927	-1.817	0.830	-5.900	-4.262

Protected Designation of Origin as driver of change in goat production systems: Beyond added value

La Denominación de Origen protegida como impulsor de la transformación de los sistemas de producción caprina: más allá del valor añadido

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Abstract

Protected Designation of Origin (PDO) is one of the EU tools for rural development. Most of the literature on this subject is focused on premium prices and consumers' willingness to pay for local products, since PDO and other labels aim to provide premium incomes for farmers. Our hypothesis states that PDO may also drive unexpected changes in farming styles, not only related to processing or market strategies, but also related to local resources usage, establishing different approaches to agriculture and food production. The PDO of *Queso Palmero* (La Palma cheese) was analyzed as a dual label system case (brand–certification and common label), thus enabling comparisons between farmers involved in a PDO scheme with farmers who work outside the systems. It was concluded that, for price formation, private brands are more important than common label certification, but complementary to each other, since PDO reinforces farmers' efforts to improve quality. Beyond premium price, PDO also drives a radical change in farm structures, since it reconnects products to local resources (grazing *vs* intensification) and redesigns relationships with markets (shortening and diversifying chains and widening product offer). This change is characterized by implementation of new farming strategies in the context of a PDO structure that coexists with classical farming strategies closer to intensification, not only in terms of productivity, but also in terms of decoupling from local resources and productive and market specialization. Therefore, PDO is a powerful tool for rural development in a wider sense (resilience, empowerment, local capacity and network formation, among others) far beyond its narrow remit of promoting economic growth (local or regional). Therefore, coupling with local resources and strengthening local networks and relationships as sources of resilience, knowledge and capabilities improvement, have to be included in performance assessment of GIs (Geographical indications) in order to broaden the appraisal of their role in regional development.

Keywords
Quality label • farming style • rural development • farmers' perception • innovation

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Resumen

Las denominaciones de origen protegida (DOP) es una de las herramientas de la Unión Europea para el desarrollo rural. La mayor parte de la literatura en relación con las DOP, está centrada en el valor añadido y en la disposición a pagar por los consumidores por productos locales, ya que las DOPs y otras etiquetas tienen como objetivo, entre otras cosas, incrementar los ingresos de los productores. Nuestra hipótesis es que las DOPs y otras etiquetas de calidad impulsan cambios inesperados en los sistemas y estilos de agricultura, no solo relacionados con las estrategias de comercialización o los procesos industriales sino también con el uso de los recursos locales y el establecimiento de diferentes estrategias de producción agraria y alimentaria. En este trabajo analizamos la DOP Queso Palmero (Islas Canarias, España) como un estudio de un sistema dual de etiquetado (marca individual – etiqueta común) ya que nos permite comparar productores implicados en el esquema de la DOP con ganaderos, que cumpliendo los requerimientos, no forman parte de la DOP. Concluimos que las marcas privadas individuales son más importantes que la etiqueta común (DOP) en la formación del precio, pero ambas son complementarias, ya que la DOP impulsa el esfuerzo de los ganaderos por mejorar la calidad. Más allá del incremento de precio, la DOP impulsa cambios radicales en los sistemas de producción al reconectar las explotaciones con los recursos locales (pastoreo frente la tendencia a la intensificación productiva) y reconfigura las relaciones con el mercado (acortando y diversificando los canales de comercialización y ampliando la oferta de productos). Estas nuevas estrategias desarrolladas en el contexto de la DOP, coexisten con estrategias clásicas más cercanas a la intensificación, no solo en términos de la importancia de la productividad en la toma de decisiones sino en términos de desacoplamiento respecto de los recursos locales y de especialización productiva y en los canales de comercialización. Por tanto, las DOPs son una potente herramienta de desarrollo rural en un sentido amplio (resiliencia, empoderamiento, formación de redes y capacidad locales entre otros), más allá del objetivo de crecimiento económico (local y regional). El reacoplamiento con los recursos locales y el fortalecimiento de las redes y relaciones locales como fuente de resiliencia, así como la mejora del conocimiento y las capacidades, deben ser incluidos en la evaluación de las indicaciones geográficas y otras etiquetas de calidad, para ampliar el análisis de su rol en el desarrollo regional.

Palabras clave

Etiquetas de calidad • estilos de agricultura • desarrollo rural • percepción de los productores • innovación

Introduction

Protection of local food products (Geographical indications) is an EU tool for rural development included as a measure within the second CAP (Common Agricultural Policy) pillar, and a priority related to improving agriculture competitiveness by increasing farmers' incomes through adding value to products (46). Several studies show that certain groups of consumers are willing to pay higher prices for local products, since they are associated with benefits such as health, freshness, reduced carbon footprint, local culture, different tastes and traditions (4, 35, 37). These local products respond to postindustrial society's demands, as these new attributes play an important role in nutrition (5), price and income (48) choices. Therefore, late modern (postmodern) consumers are willing to pay for these (new and rediscovered) attributes of food products. In this context, EU labeling protects these products against fraudulent marketing, providing information to ensure higher prices of labeled products, thus improving farmers' income (46) as driver of rural development. Ten percent of over 3,300 European geographical indications are in Spain, where labels have increased more than 5% between 2012 and 2017 (32).

However, there are two important factors related to the implementation of quality certification systems. On the one hand, some authors have found that labeling systems entail higher costs and lower efficiency that is not always offset by higher incomes from added value (7, 10, 27). Therefore, the viability for these farms in comparison with more industrialized ones can be undermined. In addition, premium prices are often low in products with high processing levels, such as cheeses, wines, or olive oil (13). This is due to the longer crafting process used to imbue these products with distinct and specific traits in order to differentiate them, even from official protection labels. Moreover, for some products, brands are preferred to protected labels by consumers, as they also guarantee quality and origin (6). Indeed, in many cases, personal relationships with farmers, proximity (5) and contribution to local economy (35) play a more important role in purchasing behavior than certification. Finally, depending on the market structure and organization, premium pricing does not always make an impact on rural development (8). However, quality labels can play a prominent role in the protection of ecosystem services, such as cultural heritage (19, 28) and local genetic animal resources (49). In this context, systems and institutions for local product valorization can facilitate local breed protection and rural development (21, 22) since many Geographical Indication (GI) regulations require farms to produce on the basis of local breeds. Therefore, the results of PDO initiatives are conditioned by local socio–economic conditions (9), both in terms of not reaching expected objectives as well as in producing unexpected and indirect effects such as changes in land use (20, 40).

Literature on premium prices and the effect of GIs on rural development at regional scale is extensive, particularly as the main aims of European quality labels are related to these issues (8, 9, 47). However, the role of GIs as unexpected drivers of farm transformation has been less addressed (20), not only from the point of view of productive structure changes, but also from the point of view of farmers' characteristics (value, goals and emotions) and decision–making (22). Benefits of GIs beyond economic dimensions are not sufficiently understood (31) and not frequently included in decision–making and assessment tools (18).

This research attempts to answer some key questions. First, do GIs provide premium prices for goats' cheese? Could there be other reasons for farmers and the public administration's involvement in GIs besides premium price? Second, to what extent do GIs drive changes in production strategies and farming styles (different strategies to obtain similar income levels and ensure system reproduction (38))? Finally, do GIs lead to innovation in agriculture beyond the required rules?

A PDO for goats' cheese, *Queso Palmero*, was used as a case study for three reasons: 1) As a processed product, its premium price is probably low (PDO price similar to non-PDO prices); 2) Brands are always present among PDO cheeses; 3) It involves a *supply chain strategy,* since there is a focus on managing production levels, improving product quality and implementing effective marketing (47). Therefore, these circumstances highlight motivations other than the expected advantages (46) for farmers' involvement in PDO structures. PDO *Queso Palmero* is managed by a regulating council under the Canary Island Regional Government's authority. The regulating council is composed of technical experts, farmers and cheese makers.

From an empirical perspective, this case allows us to compare the two types of farmers (PDO and non-PDO farmers) in a shared socio-economic and cultural context and in a limited and isolated geographical area. Therefore, study factors have been effectively isolated in the statistical models. Hence, it can be assumed farmers have similar production costs (ultraperipheral islands are closed systems, input market options are reduced), and they all know and are aware of the market costs of different channels. Therefore, information is symmetrically available, and farmers' decisions are made based on equal access to information regardless of their relationship with the PDO institution or socioeconomic traits (age, previous occupation, and education level among others). Thus, farm and market structure between PDO and non-PDO farms can be compared. In addition, farmers' perceptions of farm activities and PDO functions for both groups of farmers can be explored.

Material and methods

The study was performed on the island of La Palma (Canary Islands, Spain), where traditional *Queso Palmero* (La Palma cheese) is produced and PDO *Queso Palmero* has an important market share. *Queso Palmero* is mainly consumed in the Canary Islands. The study was based on interviews with 68 farmers (30 of them participants in the PDO and 38 not) who fulfill the required conditions to access PDO labelling (such as raising the *Palmera* goat breed), and produce and sell their own cheese. These sampling conditions allow us to isolate changes in farms not produced by the effect of PDO. Moreover, over 90% of goat farms in the studied area were included in the sample. The interviews included open-ended questions sorted by importance–relevance and sense (negative or positive) and close-ended questions to determine quantitative data. The analysis of open-ended answers was based on coding, identifying different sections of responses with different emerging concepts or ideas. The coding of responses allowed us to establish the frequency of core ideas and to classify the responses related to each emerging concept (44).

We performed distance-based permutational ANOVA (PERMANOVA) to analyze the effects of PDO (PDO label – non-PDO label), type of cheese (fresh, soft, semi-cured or cured) and market channel (middlemen, supermarket or direct to consumers) as fixed effects and private brands (farmers) as nested random factors of PDO label effect on price. PERMANOVA analyzes dependent variables as a multidimensional matrix based on distance between samples, which avoids the effects of lack of normality, homoscedasticity and independency of residuals (2). When there were significant differences, pair-wise comparisons with t-statistics were applied (2). Farmers' participation in the PDO scheme was evaluated with logit models (36), where the dependent variable takes a value of 0 if farmers do not participate in the protected label scheme and value 1 if they do. Categorical explanatory variables are age (indicator of generational replacement), educational level (as measure of innovation capacity (14)), former occupation (indicator of linkage to rural sector), length of activity (indicator of linkage to goat sector), proportion of family in workforce (measure of internal resources use, level of commoditization as indicator of autonomy (41)), farm size (measure of farm structure), grazing hours (measure of linkage to land resources), sales channels and cheese types (measure of diversification of products and channels). Conditional backward method was used to establish the best model.

Correspondence analysis (CA) was used to evaluate the frequency of ideas about cheese production and PDO performance. The effect of each group of farmers (PDO (0) and non-PDO (2) farmers) on ideas about farmers' activities was evaluated using logistic regression with CA scores on the two first axes as independent variables. PERMANOVA was implemented using PRIMER 6.0 and PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK). CA and logistic regression were run with SPSS statistical package (43).

Results

Protected Designation of Origin *Queso Palmero* has protected traditional goat farmers of La Palma (Canary Islands, Spain) since 2001. The most important requirements are related to the protection of the local goat breed (other breeds are not permitted) and traditional characteristics of cheese (shape, weight, size, color, protein and fat content among others). Although dairy industries can produce PDO cheese, most of the farmers prefer to make their own cheese on their family farms. Therefore, PDO protects the current production systems, and its rules and requirements do not entail extra production costs.

The results are organized in three parts. Firstly, the effects of a common label (PDO) or private brand on cheese price are described. Secondly, goat production systems as a measure of the effect of a PDO on farm style are characterized. Finally, farm perception analysis completes the description of farm styles.

PDO and price

Farmers participating in PDO schemes received significantly higher prices than non-PDO farmers (pseudo-F = 37.72; p level < 0.000), but this premium price is more related to the type of cheese they produce, as the interaction between type and PDO label shows (pseudo-F = 20.91; p level < 0.000) (table 1, page 200). Furthermore, the effect of a private brand (pseudo-F = 2.34; p level = 0.002; η^2 = 0.207) is stronger than the PDO label effect (η^2 = 0.177) on cheese price. Additionally, the overall effect of market channel, brand and type of cheese jointly explain over 60% of total variance (η^2 = 0.615).

Different lower-case letters represent significant differences among types of cheese and different capital letters represent significant differences between PDO and non-PDO farms ($p < 0.05$).

Diferentes letras minúsculas indican diferencias significativas entre tipos de quesos y diferentes letras mayúsculas indican diferencias significativas entre quesos por etiquetado ($p < 0,05$).

Table 1. Average and standard error of prices received by farmers based on PDO and type of cheese. All types of cheese, except fresh, are included in the PDO label.

Tabla 1. Media y error estándar de los precios percibidos por los productores en relación con la pertenencia a la DOP y al tipo de queso. Todos los tipos de quesos, excepto el queso fresco, son etiquetados por la DOP.

	PDO	Non-PDO	Total
Fresh	8.39 (0.41)^{Aa}	7.82 (0.17)^{Aa}	8.01 (0.18)^{a}
Soft	8.74 (0.36)^{Aab}	6,71 (0.36)^{Bb}	8.06 (0.34)^{a}
Semi-cured	9.70 (0.45)^{Ab}	7.73 (0.26)^{Ba}	8.98 (0.33)^{b}
Cured	13.31 (0.54)^{Ac}	8.17 (0.60)^{Bab}	12.34 (0.68)^{c}
Total	9.99 (0.32)^{A}	7.64 (0.14)^{B}	

Market channels also affect cheese prices (pseudo-F = 30.92; p level < 0.000). For example, farmers receive significantly lower income from middlemen (traditional channel) compared with more modern market chains (supermarket, grocery stores, hotels and direct to consumers). Therefore, shorter and more modern sales channels tend to provide higher prices for farmers. Farmers that use direct channels tend to be involved in PDO schemes more frequently in comparison with non-PDO farmers (B = 3.70; Wald = 2.71; p – level < 0.001), who prefer hotels, grocery stores, supermarkets and restaurants (Log likelihood = 53.13; Nagelkerke R^2 = 0.46). However, a traditional middleman channel is excluded from the logit model as both (PDO and non-PDO farmers) use this channel, even though it offers lower prices.

Cheese prices also increase with curing processes, as expected, since consumers are often willing to pay more for cured cheese. However, these increases occur only in the case of cheese produced by PDO farmers, whereas non-PDO farmers are not able to get added value for more cured cheese, and they receive significantly lower prices except in the case of fresh cheese. It seems that PDO farmers produce specific products for the market, but in the case of non-PDO farmers, curing processes are probably more related to a strategy for fresh cheese surplus management rather than product diversification.

Characterization and farmers' perceptions

A Logit model based on backward method procedure establishes education level, grazing, types of cheese and market channel diversification as independent variables (Log likelihood = 46.95; Nagelkerke R^2 = 0.66). Results show that PDO farmers tend to have significantly higher educational levels than non–PDO farmers (B = 2.89; Wald = 5.81; p – level = 0.016). They are also likely to manage goats in grazing systems (B = 4.60; Wald = 10.62; p – level = 0.001) and produce more than three different types of cheese (B = 3.69; Wald = 6.32; p – level = 0.012) that they sell to several customers through different market channels (B = 1.58; Wald = 4.06; p – level = 0.044). Farmers' age, previous jobs, labor force structure, farm size or activity length do not characterize PDO farmers or non-PDO farmers.

Farmers' ideas and perceptions significantly affect the likelihood of their being involved in a protection structure (figure 1, page 201), as can be seen by the logit model between CA axis 1 scores and farmer group (B = 3.06; Wald = 9.55; p level < 0.05; Log likelihood = 75.66; Nagelkerke R^2 = 0.33). However, ideas related to CA axis 2 do not explain farmers' behavior regarding PDO labels (B = 0.41; Wald = 1.73; p level > 0.05; Log likelihood = 93.06; Nagelkerke R^2 = 0.04). Therefore, PDO farmers often highlight that two of the most positive features of goats' cheese production is the recognition of product quality ("I like my customers' recognition"; "Customers like my cheese and they admit it"; "prizes in cheese competitions") and the fact that they are the sole owners of their farms ("I'm my own boss"; "I don't have a boss"). Therefore, they manage their farms according to their own criteria and ideas, and they control the production process ("I control my daily schedule"). Negative ideas about cheese prices and consequences to farm economy are less frequent in the PDO group. Therefore, this group of farmers is more concerned about their relationship with customers in terms of quality, since recognition is an important part of their activity, and they focus on the control of cheese production processes and quality (brands explain more than 20% of price variance). Therefore, their satisfaction and motivation are more outward dependent and a PDO structure provides a frame that enhances relations with consumers through quality.

Only variables with 1 or 2 dimension scores above 1 were included. △ positive ideas related to work and control of their own farms. ∗ positive ideas related to social recognition of product. ◊ negative ideas about the lack of support and strict requirements of public administration. X positive ideas about pleasure to work with goat management and cheese production + negative ideas about production costs and cheese prices.

Solo las variables con puntuaciones superiores a 1 en la dimensión 1 o 2 fueron incluidas. △ ideas positivas relacionadas con el trabajo y el control de sus propias explotaciones. ∗ ideas positivas relacionadas con el reconocimiento social del producto. ◊ ideas negativas relacionadas con la falta de apoyo y los requerimientos de la administración pública. X ideas positivas sobre el placer de trabajar con ganado caprino y producir queso. + Ideas negativas sobre los costes de producción y los precios del queso.

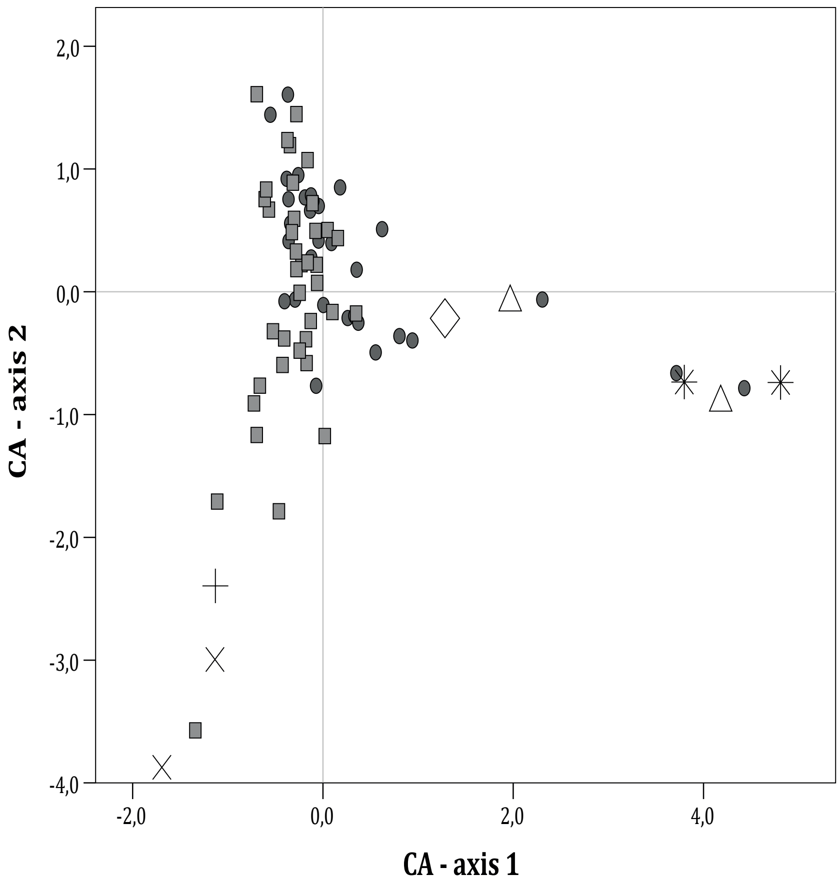

Figure 1. CA plot of PDO (●) and non-PDO (■) farmers according to frequency of positive and negative ideas about goats' cheese production activity. Eigenvalue of axes 1 and 2 are 0.525 and 0.465, respectively (16.0% the total inertia).

Figura 1. Resultados del análisis de correspondencia (CA) de los productores pertenecientes a la DOP (●) y no pertenecientes a la DOP (■) de acuerdo con la frecuencia de ideas positivas y negativas sobre la actividad de la producción de queso de cabra. Autovalores de los ejes 1 y 2 son 0,525 y 0,465, respectivamente (16,0% de la inercia total).

By contrast, non-PDO farmers tend to have positive ideas about the pleasure of goat farming and cheese production ("I like the animals and this job"; "We like goats and the job of cheese making") and negative ideas about cheese prices and production costs ("Fodder is so expensive"; "Fodder is expensive and cheese prices don't increase"). Positive ideas about recognition, about the advantages of farm ownership and the lack of administration support ("The institution's requirements are too tough"; "too much bureaucracy"; "We are the forgotten sector") are less frequent (figure 1). Non–PDO farmers' satisfaction and motivation are more related to the activity itself regardless of market and customer recognition, and therefore is inward dependent. Thus, their main concerns are more focused on the production system and its problems (costs, cheese prices and others). In this context, a PDO is not a source of potential interests for this group of farmers.

Correspondence Analysis of frequency of ideas regarding PDO organization activities shows different perceptions and views among PDO farmers (35.6% the total inertia explained by axis 1 and 2). Low scores of some variables in CA axes 1 and 2 show a similar frequency of groups of ideas among farmers. There is a general agreement about cheese and milk quality control ("They [PDO] analyze milk and cheese"; "They control milk and cheese quality") and about the importance of *Queso Palmero* PDO label ("My cheese is differentiated by a quality label"; "The label certifies the cheese's quality and helps us to export it"). These results highlight quality enhancement as farmers' main motivation to join a PDO scheme.

Discussion

Several authors have found that protected label schemes provide added value and premium prices for farmers (10, 15). It has also been found that cheese labels under PDO systems achieve significantly higher prices than those that do not have labels. However, the importance of brand on price formation is more relevant than protected labels as some researchers have suggested (3). Indeed, in a dual context such as the PDO *Queso Palmero* case (private and common labels), cheese price depends on brand reputation and common label reputation (10), both are extrinsic attributes of products that affect consumers' quality perceptions and willingness to pay for these products (1, 24, 33). Therefore, premium prices for PDO farmers could be the result of the combined effect of private brand and common label, since the PDO or the brand itself would be not enough to achieve these premium prices. This is related to the combination of quality–geography nexus and consumers' willingness to pay for products related to 'localness and *terroir'* (25, 29, 35). The PDO effect is more effective for those farmers who are also involved in building private reputations (30) mainly through differentiated quality. In the case of the PDO for *Queso Palmero*, private reputation is also enhanced through the production of more processed cheese (cured and semi-cured) based on local resources (grazing), which provides specific traits and identities (private and common), and through product diversification (different types of cheeses). These characteristics are more frequent in PDO farmers, who state their concerns about consumer satisfaction and acknowledge the role of the PDO in quality control and promotion, as our results have shown. Therefore, PDO structures reinforce previous private efforts for quality and reputation rather than providing an overall reputation for cheese production.

The PDO label explains less than 20% of price variability, but it provides other benefits and structural changes that encourage some farmers to adopt the protected label (15, 26). Apart from brand, price is related to two other factors. On the one hand, the type of cheese produces greater price differences than any other factor in the model but differentially affects PDO and non–PDO farmers. In the context of PDO, results show how price responds to quality, since cheese is more expensive as it cures only under the common label (PDO). On the other hand, market channel provides higher prices if the chain is shorter probably because, among other factors, consumers appreciate the contact with farmers, as some authors have found (5). It also appears that PDO systems interest farmers who like to have a closer relationship with consumers and who produce more cured cheese to achieve better prices. Both factors are related to differentiated quality and reputation and confirm the assumption of complementary roles of private brands and common labels (11, 30).

Results suggest that PDO and non-PDO farmers represent two different farming styles that entail different conceptions of agriculture and food production (table 2, page 203). One is a more *production-oriented* style (non–PDO farmers) that responds to a productivism agrarian model mainly focused on productivity and agricultural models (17, 45). The other is more *market oriented* (PDO farmers) that matches a neo–endogenous rural model, since it represents the combination of local (natural resources) and external (network and connections with other activities and initiatives) forces and contributes to local institutional capacity (16, 17, 41). According to our results, both models coexist, though they are not stages of rural development, as the weak effect of age on type of farmers shows. However, education level does differentiate between PDO and non–PDO farmers, which suggests education is driving changes in rural perceptions toward diversification and multifunctionality.

Production–oriented farmers (non–PDO farmers) focus on productivity and cost management and, therefore they perceive that their farms depend on production and economic efficiency that is mainly related to inward structures and strategies. Probably, these farmers produce for specific consumers who are more concerned with price-quality balance and could be classified as pragmatic consumers (5). As quantity is one of their most important concerns, farms are mainly intensive with industrial structures and with management that aims to maximize productivity. However, differentiated quality is not as important, since these farmers do not implement strategies for differentiation, such as local resource usage (intensive systems) or more processed cheese production (curing highlights specific traits). Their production is more concentrated on less processed cheese as curing processes do not provide added value (but rather increase loss risks) and is probably a strategy for surplus management. Moreover, their market structure is based on traditional channels, such as middlemen. These factors prioritize short-term revenues and investment in quality improvement is not encouraged (30). These systems are closer to *classical farming strategies* such as labor-driven intensification (concern about productivity) and high–tech intensification (less importance of grazing) (38).

Table 2. Main traits of two farming styles as a result of the effect of PDO implementation.

Tabla 2. Rasgos principales de los estilos de agricultura como resultado del efecto de la implementación de la Denominación de Origen Protegido.

Characteristic	**Non–PDO**	**PDO**
Farm style	Production – oriented	Market – oriented
Land use – local resources	Low	High
Price	Medium	Medium – high
Production - market diversification	Low	High
Main market chain	Supermarket and groceries	Direct market and other short chains
Drivers of decision-making	Farm and production system Inward dependent	Consumer relationship Outward dependent
Main concerns	Productivity Relation costs – incomes	Quality Relation with consumers
Main strategies	*Classical* Labor – driven intensification High – tech intensification	*New* Creation of new micro – enterprises Multi-functionality in agriculture

However, farmers in PDO schemes have a *market-oriented style* and are concerned about differentiated quality, since their farms are based on market relationships instead of production system efficiency. Therefore, they aim to establish a fluent and effective dialogue with consumers. This allows them to adapt production and improve quality as a path to increasing income. Production is based on grazing systems and focuses on more processed cheese as a way of providing specific traits that would entail premium prices. Furthermore, most of their production is sold direct to consumers and, therefore, their interactions with the market are more intense and productive in terms of feedback. PDO producers are more centered on quality seekers for whom certifications (common labels) are quality guarantees (5). These farms are implementing *new farming strategies* (38), such as creating new micro enterprises and multifunctionality in agriculture (market and product diversification) and agroecology (relevance of grazing).

Therefore, a PDO is a powerful development tool that drives changes in the livestock sector beyond just adding value (27). These changes are mainly related to reattachment with local resources, as other authors have found (40), shortening and diversification of market channels and focusing on quality instead of quantity. All these changes not only provide novel attributes for which consumers are willing to pay more for (4, 39) but also strengthen farms through market and production diversification that provide higher resilience against market fluctuations thanks to the intensification of networks (12). Although the effect of GIs on farm transformation have been little studied, some examples have been analyzed in the case of Roquefort cheese (40), GIs of French Alps (20, 21) as well as for some local breeds in Mediterranean areas (23). These authors found a significant effect of GIs on farm transformation, mainly in terms of unexpected results. Beside farm–scale transformation and benefits, PDOs can contribute to protecting and developing other resources on a wider scale, such as native breeds and local culture heritage protection (26).

PDO assessment based on *supply chain strategy* or of *extended territorial strategy* (increasing employment and revenues within supply chain, and/or local economy) (47) could be complemented by measurements related to intensity of local resource use (grazing and local forage) or to endogenous or neo-endogenous models and increasing local relationships as resilience drivers (12). Although these are key elements of current models of development in Europe (34, 45), our results suggest there is a coexistence of two different farming strategies in the same geographical and socio-economic context. Indeed, they are not stages of linear evolution but rather two paths of development, therefore they must not be approached in terms of backward or advanced systems. Both require different supporting strategies that are hidden behind aggregated statistics based on common variables (38).

Conclusions

PDO schemes work as change drivers for farms that go beyond adding value through premium prices and increasing income. Indeed, the PDO effect entails radical change in farms and farmers involving coupling (or recoupling) with local resources and establishing new relationships and networks, among others. However, it should be noted that PDO schemes do not create production systems in terms of substitution of classical ones, what it does is create novel spaces of development that coexist with other farm styles. Therefore, our analysis suggests that rural development initiatives such as PDOs and other GIs would function as socio–economic spaces of creation and entrepreneurship in a horizontal relationship with others. These would entail that production models could be approached like a patchwork of different farm styles (horizontal) instead of different stages of evolution (vertical).

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Waste assessment in distribution and marketing logistics of horticultural products: evidence from Brazil

Evaluación de residuos en logística de distribución y comercialización de productos hortícolas: evidencias en Brasil

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Abstract

Worldwide, one-third of the food produced is inconsumable due to marketable quality losses. Parallelly, and given the growing world population, levels of waste have become unacceptable. Supply channels play a fundamental role in establishing strategies for food waste mitigation. The objective of this research was to analyze if good operational practices with different dimensions of distribution and commercialization logistics may contribute to reduce fruit and vegetable waste. The studied dimensions were: logistics, operations, technology, trade and management. Several questionnaires were administered to 83 specialized wholesale merchants from a large supply center in Brazil, for lettuce, potato, orange, papaya and tomato. The results indicated that for papaya and potato, low and high waste generation are associated with a greater number of anti-waste actions (p = 0.0071 and p = 0.0469 respectively). For tomato, lettuce and orange, no significant differences for high and low waste in relation to the number of actions undertaken in these chains, was found. These results represent a first approach to understanding the reasons for food waste at wholesale centers.

Keywords

food waste • supply chain management • waste minimization • sustainable marketing • fruit surplus • waste prevention

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Resumen

A nivel mundial, un tercio de los alimentos producidos no se consume debido a la pérdida de calidad para la comercialización, y con la creciente población mundial, los niveles de desperdicio son inaceptables. Los canales de suministro juegan un papel fundamental en la mitigación del desperdicio de alimentos y en el establecimiento de estrategias para reducirlo. El objetivo de esta investigación fue analizar si las buenas prácticas operativas de diferentes dimensiones de la logística de distribución y comercialización contribuyen a reducir los niveles de desperdicio de frutas y hortalizas. Las dimensiones fueron: logística, operaciones, tecnología, comercio y gestión. Los cuestionarios fueron administrados a 83 comerciantes mayoristas especializados de un gran centro de suministro en Brasil para lechuga, papa, naranja, papaya y tomate. Los resultados indican que, para papaya y papa, bajo y alto desperdicio están asociados con el mayor número de acciones realizadas por los comerciantes (p = 0,0071 y p = 0,0469 respectivamente). Para tomate, lechuga y naranja, no hay evidencia significativa de diferencias para alto y bajo desperdicio y el número de acciones tomadas en estas cadenas. Los resultados obtenidos en esta investigación representan un primer acercamiento para comprender las razones del desperdicio de alimentos en los centros mayoristas.

Palabras clave

desperdicio de alimentos • gestión de la cadena de suministro • minimización de desperdicios • comercialización sostenible • excedente de frutas • prevención de desperdicios

Introduction

Food loss and waste generation persist as world essential challenges. Approximately one-third of globally produced food, is not consumed. This is largely due to loss of quality for marketing and/or consumption. Given the relentless growth of the world population, these unacceptable levels cause financial and nutritional losses worldwide (5, 19, 40, 41).

Research on causal agents like management mechanisms and quantification of food loss/ waste levels, as well as on efficient actions promoting food loss/waste reduction, should be considered and approached (2). According to Abiad and Meho (2018), information on food loss and waste is still limited to specific investigations, and in many cases, sampling design does not allow results generalization or even comparisons among studies.

Reducing food loss and waste contributes to increasing food supply by reducing the need to complement its availability through public policies or even through commercial imports and donations. Higher food availability contributes to lowering prices, implying, under normal market conditions, lower prices for final consumers (14, 36, 42).

Conceptually, food loss refers to the reduction in the amount of available edible food throughout its production chain. Waste, in turn, constitutes losses in retail and/or caused by the final consumer. Only products intended for human consumption are considered waste, excluding inedible parts and animal feed. Food intended for human consumption but used, for instance, in bioenergy, still constitutes a loss (19). The causes of food loss and waste generation are mainly related to financial, managerial, and technological limitations during harvest, postharvest and storage, packaging systems and marketing infrastructure (22). In the distribution and commercialization logistics, food degradation affects all stages, from production, harvest and postharvest, to processing, distribution and sales (3, 5, 26, 31, 37, 45). According to Cunha (2015), loss is also associated with pathogen attack in cooling chambers and packages, budding in roots and tubers, and advanced maturation. Postharvest losses and quality deterioration of fruit and vegetable crops are mainly caused by pests, microbial infection, natural ripening, and environmental conditions such as heat, drought, moisture, and improper postharvest management (35, 45). Marketing channels, especially short channels, hold importance since they reduce transaction costs by mediating contact between growers and final consumers (24). However, these channels are inherent to intermediary agents and actors involved in product sales (9). A marketing channel allows growers to reach various distribution centers, such as fairs, industries, industrial centers, cooperatives, events and even to export their products overseas, increasing marketing reach (43). Identifying the distribution/commercialization stages with the highest levels of waste generation, allows a more efficient management of the supply chain, with lower levels of associated loss (5, 34).

In Europe, initiatives for accurately quantifying waste in the main economic sectors, are increasing. In 2012, the European Union, estimated food retail waste at 5 million tons (5, 38). In these countries, the food retail sector accounts for approximately 5% of total food waste in the entire food chain. This sector was responsible for wasting 2% of food in the United Kingdom and about 2 to 4% in Poland, Germany and Sweden (18, 20). In Latin America, initiatives regarding food waste are still modest, failing to quantify food waste among the different food systems. According to FAO (2016), Latin America and the Caribbean are responsible for 6% of global food losses. In these regions, 15% of food for human consumption is wasted each year (21). According to Parfitt *et al.* (2010), small retailers, especially those not connected to other chains, are more likely to generate higher volumes of food waste given their limited resources and inadequate customer demand forecasting. Therefore, management and maintenance of supplier-customer relationships cannot guarantee food delivery and reduced waste in food retailing.

Kummu *et al.* (2012), after assessing waste generation at different stages through the chain for different countries, classified them into two groups: middle- to high-income countries, with more than 50% of waste associated with distribution and consumption, and low-income countries, where the most waste is associated with production and postharvest. These same authors indicated that cereals account for 57% of total losses in the food supply chain, fruit and vegetables, for 39%, and roots and tubers, for 33%. The highest levels of food loss and waste in absolute terms are associated with industrialized Asia and South and Southeast Asia (29). In this sense, Affognon *et al*. (2015), when assessing food waste through meta-analysis, indicated that waste of roots and tubers, and fruit and vegetables in sub-Saharan Africa were estimated at 33 to 60% and 37 to 55%, respectively. In the same sense, Stensgard *et al.* (2016), when assessing food waste during 2010-2015 in Norway, indicated that, considering the volume of wholesale sales, percentages of food waste decreased as a result of increased sales. Among the evaluated groups, fruit and vegetables showed the highest waste generation, resulting, for the analyzed period, in between 1.0 and 1.03% of wholesale sales.

Nonetheless, controlling key physiological aspects that contribute to the loss of quality of fruit and vegetables in the distribution and marketing chains contributes to reducing commercial loss, increasing food supply for human consumption. Thus, the objective of this study was to evaluate how the logistics, operations, technology, trade, and management dimensions may interrelate with each other affecting waste production, given that the analysis of only one dimension cannot properly evaluate food waste in its entirety.

Materials and methods

The chosen methodology was based on a semi-structured questionnaire administrated to wholesale merchants of the Campinas Supply Center - CEASA Campinas (2018). This methodological approach, known as *rapid assessment* or *quick appraisal* (17), Kumar (1993), Dunn (1994) and Beebe (1995) is founded on data obtained from secondary sources along with semi-structured interviews in which more detailed data and/or information is required to understand the dynamics of the sector assessed.

The five chosen products (orange, papaya, lettuce, tomato and potato) and their respective categories, namely (1) fruit, (2) leaf, flower and stem vegetables, and (3) root, bulb, tuber and rhizome, are among the most consumed by the Brazilian population, according to the latest Family Budget Survey - POF 2008 - 2009 (13, 27). According to the POF 2008-2009, orange and papaya consumption is 2.807 and 2.045 kg/capita/year, respectively, second only to watermelon (3.368 kg/per capita/year) (27). Tomato consumption is 4.916 kg/capita/year, first in the fruit group. In the leaf group, lettuce consumption is 0.91 kg/capita/year, behind cabbage with 1.032 kg/capita/year. Finally, ranking first in the tuber vegetables group, consumption of potato is around 4.037 kg/capita/year (27). Twelve wholesale merchants were interviewed for lettuce, 15 for potato, 14 for orange, 19 for papaya and 23 for tomato, comprising a total of 83 wholesale merchants. To maintain anonymity, merchants were coded by sequential numbers from 1 to 83.

The administrated questionnaires referring to business practices, storage, transport conditions, postharvest treatment and management, provision and delivery of services, marketing and after-sales strategies, were administered during the second half of 2018. During data collection, each wholesale merchant was asked to report the amount of waste associated with each product. Thus, the declared waste depended on each wholesale merchant, functioning as waste estimator during trade and distribution logistics.

Seventeen variables related to marketing and distribution were obtained from the questionnaire, providing a positive (+) or negative (-) relationship with food waste depending on their association with the distribution and commercialization logistics chain (table 1). Values of 1 (yes) or 0 (no) were associated to each variable indicating if wholesale traders carried out such procedure in the logistics chain of the analyzed product.

Table 1. Description of variables collected from CEASA-Campinas wholesale merchants and used for analysis.

Tabla 1. Descripción de las variables recopiladas de los comerciantes mayoristas CEASA-Campinas y utilizadas para el análisis.

Variable	Description	Contribution to or loss
Handling during trade	To identify procedures assessing product quality status. Represented by “tipman”.	-
Transshipment operations	To identify transshipment operations. Represented by "oprtrans".	+
Package trading	To identify packaging in product marketing . Represented by “comtpemb”.	-
Product quality control	To identify product quality control . Represented by “tipcotr”.	-
Supplying package	To identify packaging supply for the producer or buyer. Represented by “fornemb”.	-
Classification and reclassification operations of sold products	To identify product classification and/or reclassification procedures. Represented by "clarecla".	-
Cooling chamber	To identify the use of a cold chamber. Represented by "camfria".	-
Postharvest treatment	To identify postharvest treatments such as wax, application of fungicides, or drying, among others. Represented by “tratpos”.	-
Transport type	To identify chosen transport Represented by “tiptrns”.	-
Provision of service	To identify customer services (consignment of products, exhibitors). Represented by “tipreserv”.	-
Promotional pricing	To identify promotional pricing. Represented by “prcprom”.	-
Price tracking	To identify if the wholesaler follows price movements. Represented by “acmovpr”.	-
Purchase by contract	To identify the sale, such as a contract and contractual requirement. Represented by "comp".	-
Purchase by opportunity	To identify purchase opportunity. Represented by “compop”.	+
Alternate destinations	To identify whether unsold products are discarded or donated to institutions such as food bank and charities. Represented by “destcomr”.	-
Waste control	To identify waste control. Represented by “cntrdesp”.	-
Fixed producer/supplier	To identify whether the products sold are supplied by fixed producers or suppliers. Represented by “pffixo”.	-

When defining analyses dimensions such as the representation of a group, to assume that causal relationships for waste analysis are multi-factorial and dynamic, is possible. Thus, in order to evaluate the relationship between variables and practices/conducts on the evaluated fruit and vegetable waste generation, five dimensions were defined (33, 46).

Given this context, these dimensions constituted a form of representation capable of sorting various elements for analysis, considering a complex and multi-factorial intersection between waste, established variables, chain actors, their practices and procedures, and the fruit and vegetable analyzed. The five defined dimensions were: logistics, operations, technology, trade and management (figure 1). Based on a self-declared value of waste and an average value (obtained by the arithmetic mean of all values reported by wholesale merchants), wholesale merchants were grouped into "high" or "low" level of food waste for each product. The database was created considering response frequency and leading to proportions. Descriptive analyses of variables and dimensions were performed for each of the five products evaluated.

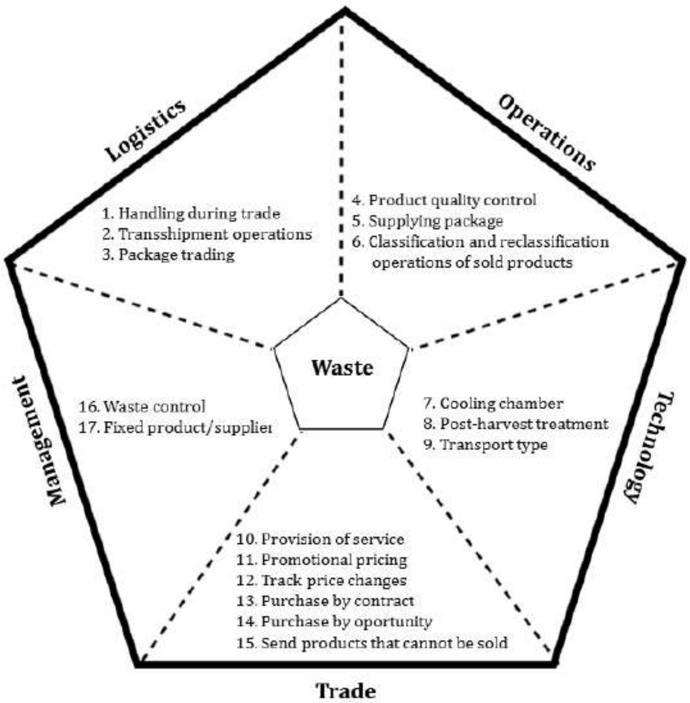


Figure 1. Dimensions and associated variables.

Figura 1. Dimensiones y variables asociadas.

Statistical analysis

To test the hypothesis stating that "the distribution of the number of waste preventive actions is the same for groups with low and high waste", the Wilcoxon-Mann-Whitney non-parametric test was performed for each product.

The analyses were performed with R software version 4.0.2, aided by the coin package (23, 39, 47). A significance level of 5% was considered for all tests.

Results

For high waste and for all evaluated products, the highest averages for declared waste were observed with the smallest number of preventive or anti-waste actions. Among all evaluated products, potato stood as the one with the least actions (49 shares) (table 2, page 212).

Table 2. Frequency for the group of high waste.
Tabla 2. Frecuencia para el grupo de residuos altos.

Dimension/Variables	Papaya	Potato	Lettuce	Orange	Tomato
Logistics	**18**	**12**	**14**	**9**	**21**
Tipman	6	1	5	2	5
Oprtrans	5	4	5	2	6
Comtpemb	7	7	4	5	10
Operations	**16**	**3**	**13**	**9**	**18**
tipcotr	5	2	5	3	3
fornemb	2	0	4	3	9
clarecla	9	1	4	3	6
Technology	**19**	**6**	**14**	**9**	**13**
camfria	6	0	3	1	1
tratpos	6	6	6	4	6
tiptrns	7	0	5	4	6
Trade	**40**	**22**	**26**	**23**	**43**
tipreserv	4	3	1	4	6
prcprom	6	4	6	3	10
acmovpr	8	4	5	4	10
comp	7	3	6	4	5
compop	6	3	3	3	2
destcomr	9	5	5	5	10
Management	**13**	**6**	**9**	**7**	**14**
cntrdesp	7	4	4	4	7
pffixo	6	2	5	3	7
Average waste	**8.4**	**9.8**	**27.4**	**3.3**	**6.5**
Total shares	**106**	**49**	**76**	**57**	**109**
Minimum number of shares	9	5	10	9	7
Maximum number of shares	14	11	15	15	13

For low waste and all evaluated products, the highest averages for declared waste were observed when the largest number of anti-waste actions were performed. Tomato and papaya resulted to have the highest number of shares, with 109 and 106 shares, respectively (table 3, page 213).

Orange and papaya wholesalers carried out the largest number of anti-waste actions (80%), with reported low levels of residues of 1.3% and 3.5%, respectively (figure 2, page 213-214). The high waste generation rates for orange (3.3%) and papaya (8.4%) were observed for the fewest waste avoiding actions (60% for orange and 67% for papaya).

Low levels of waste for tomato (0.8%) and lettuce (16.6%) were observed when wholesale traders carried out the most anti-waste actions, with 67% for tomato and 72% for lettuce (figure 2, page 213-214). Wholesale traders with the highest waste of tomato (6.5%) and lettuce (27.4%) were the ones that carried out the fewest anti-waste actions (64%) (figure 2, page 213-214).

Wholesale potato traders with low declared waste (2.4%) and high declared waste (9.8%) carried out 60% and 41% of anti-waste actions, respectively. Among all the considered products, wholesale potato traders were the ones performing the fewest preventive actions (figure 2, page 213-214).

The highest waste generation rate reported by wholesale traders was observed for lettuce (average 22.5%). The lowest level of waste was observed for orange (average of 2.2%). Papaya and potato resulted to have an average level of waste of 5.8%, while tomato showed 3.3% (figure 3, page 214).

For low papaya waste, the lowest frequencies were observed for the variables purchase opportunity and transshipment operation, with 30% and 40%, respectively (figure 4, page 214).

The highest frequencies of the management and trade variables were observed for low lettuce waste, resulting in 100 and 80%, respectively (figure 4, page 214).

Table 3. Frequency for the group of low waste.
Tabla 3. Frecuencia para el grupo de bajo residuo.

Dimension/Variables	Papaya	Potato	Lettuce	Orange	Tomato
Logistics	**20**	**11**	**11**	**23**	**27**
tipman	7	3	3	8	12
oprtrans	3	0	2	6	2
comtpemb	10	8	6	9	13
Operations	**24**	**8**	**8**	**24**	**26**
tipcotr	10	4	4	9	5
fornemb	6	1	4	6	9
clarecla	8	3	0	9	12
Technology	**25**	**16**	**15**	**18**	**20**
camfria	10	1	4	6	3
tratpos	7	8	6	9	10
tiptrns	8	7	5	3	7
Trade	**50**	**34**	**30**	**41**	**53**
tipreserv	8	4	4	2	8
prcprom	10	7	6	8	13
acmovpr	9	7	6	8	13
comp	9	8	5	6	6
compop	4	0	3	8	1
destcomr	10	8	6	9	12
Management	**18**	**12**	**12**	**15**	**21**
cntrdesp	10	0	6	6	10
pffixo	8	8	6	9	11
Average waste	**3.5**	**2.4**	**16.6**	**1.3**	**0.7**
Total shares	**137**	**81**	**106**	**121**	**147**
Minimum number of shares	12	9	11	11	7
Maximum number of shares	15	13	15	14	14

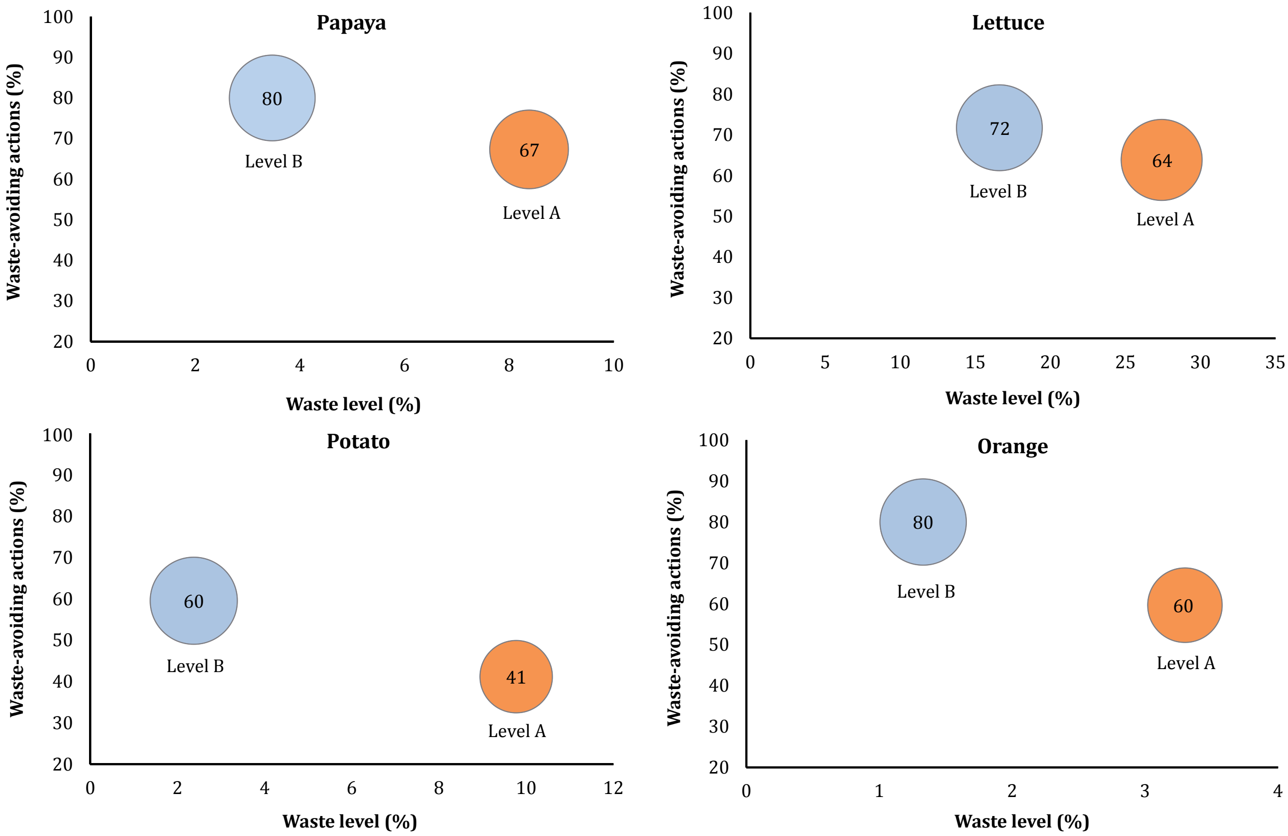


Figure 2. Levels of declared waste and number of anti-waste actions.
Figura 2. Niveles de residuos declarados y número de acciones que evitan los residuos.

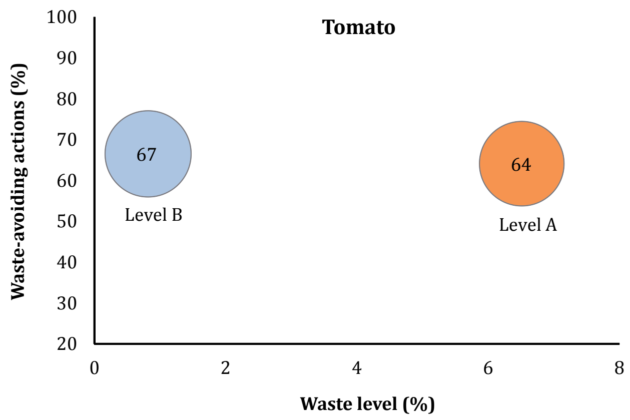


Figure 2 (cont.). Levels of declared waste and number of anti-waste actions.

Figura 2 (cont.). Niveles de residuos declarados y número de acciones que evitan los residuos.

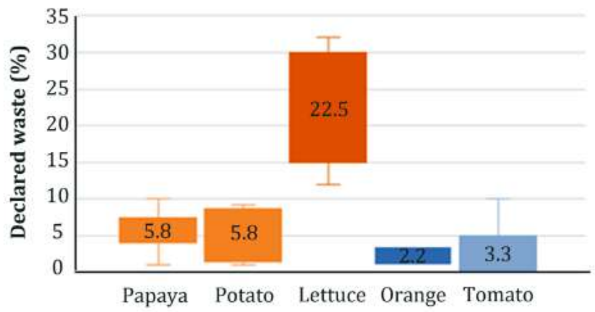
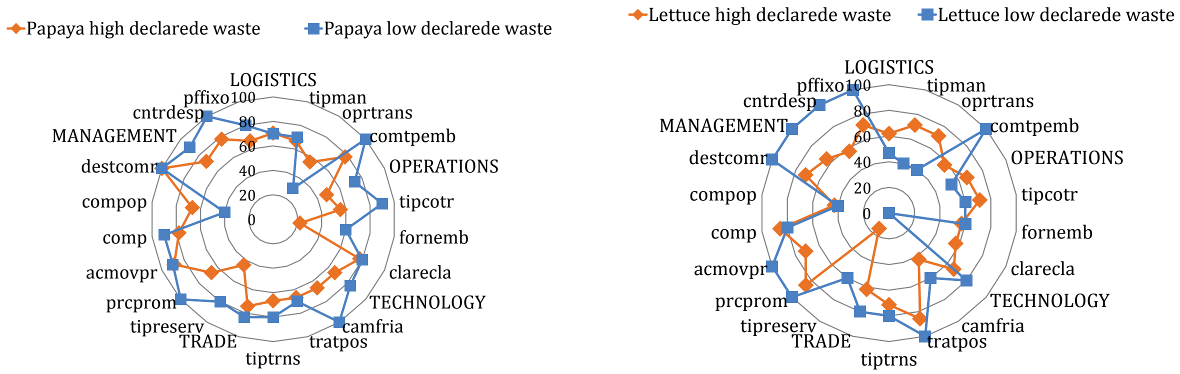


Figure 3. Declared waste of evaluated fruit and vegetables.

Figura 3. Desecho declarado de las frutas y verduras evaluadas.



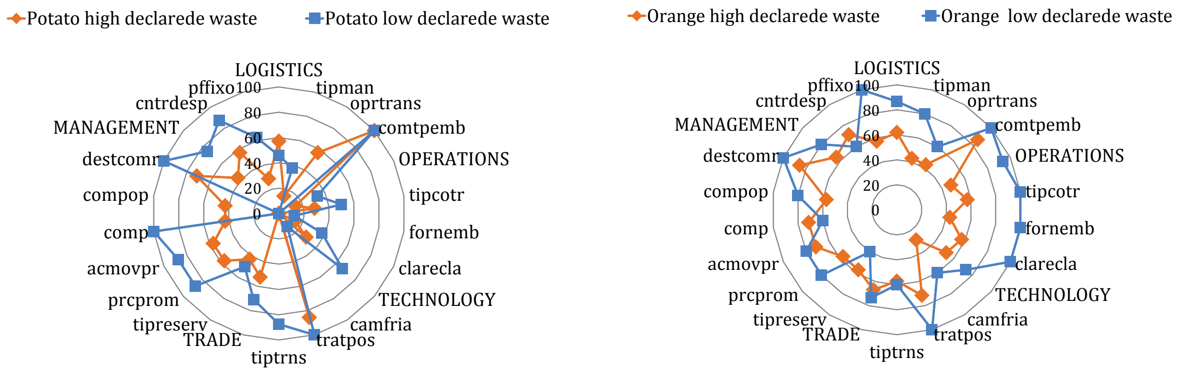
Legend: tipman: trade handling operations, oprtrans: transshipment operations, comtpemb: package trading, tipcotr: type of control for traded products, fornemb: supplying package, clarec: classification and reclassification operations of sold products, camfria: cooling chamber, tratpos: postharvest treatment, tiptrns: transport type, tipreserv: provision of services, precprom: promotional pricing, acmovpr: track price changes, comp: purchase by contract, compop: purchase by opportunity, destcomer: unsoldable sent products, contrdesp: waste control, pffixo: fixed producer or supplier.

Leyenda: tipman: operaciones de manipulación comercial, oprtrans: operaciones de transbordo, comtpemb: comercio de paquetes, tipcotr: tipo de control para productos comercializados, fornemb: paquete de suministro, clarec: operaciones de clasificación y reclasificación de productos vendidos, camfria: cámara de enfriamiento, tratpos: poscosecha tratamiento, tiptrns: tipo de transporte, tipreserv: prestación de servicios, precprom: precios promocionales, acmovpr: seguimiento de cambios de precio, comp: compra por contrato, comp: compra por oportunidad, destcomer: productos enviados no soldables, contrdesp: control de residuos, pffixo: fijo productor o proveedor.

Figure 4. Percentage contributions of dimensions and indicators to papaya and lettuce waste levels.

Figura 4. Contribuciones porcentuales de dimensiones e indicadores a los niveles de residuos de papaya y lechuga.

For low potato waste, the highest frequencies were observed for management and trade, 80 and 70%, respectively (figure 5).



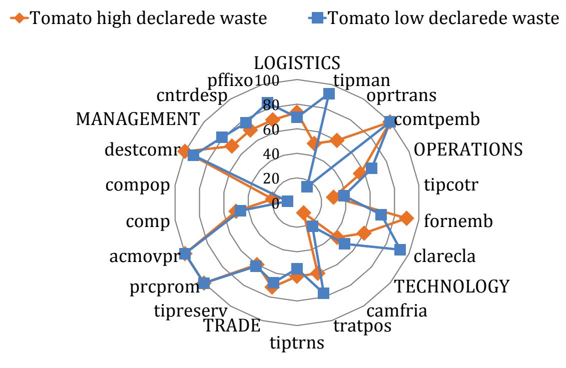
Legend: tipman: trade handling operations, oprtrans: transshipment operations, comtpemb: package trading, tipcotr: type of control for traded products, fornemb: supplying package, clarec: classification and reclassification operations of sold products, camfria: cooling chamber, tratpos: postharvest treatment, tiptrns: transport type, tipreserv: provision of services, precprom: promotional pricing, acmovpr: track price changes, comp: purchase by contract, compop: purchase by opportunity, destcomer: unsoldable sent products, contrdesp: waste control, pffixo: fixed producer or supplier.

Leyenda: tipman: operaciones de manipulación comercial, oprtrans: operaciones de transbordo, comtpemb: comercio de paquetes, tipcotr: tipo de control para productos comercializados, fornemb: paquete de suministro, clarec: operaciones de clasificación y reclasificación de productos vendidos, camfria: cámara de enfriamiento, tratpos: poscosecha tratamiento, tiptrns: tipo de transporte, tipreserv: prestación de servicios, precprom: precios promocionales, acmovpr: seguimiento de cambios de precio, comp: compra por contrato, comp: compra por oportunidad, destcomer: productos enviados no soldables, contrdesp: control de residuos, pffixo: fijo productor o proveedor.

Figure 5. Percentage contributions of dimensions and indicators to potato and orange waste levels.

Figura 5. Porcentaje de contribuciones de dimensiones e indicadores a los niveles de desechos de papa y naranja.

The highest frequencies of the operations dimension (93.3%) and logistics dimension (86.7%), were observed for orange low waste (figure 5). The highest frequencies in the management dimension were 80.8% for low tomato waste and 70.0% for high tomato waste (figure 6).



Legend: tipman: trade handling operations, oprtrans: transshipment operations, comtpemb: package trading, tipcotr: type of control for traded products, fornemb: supplying package, clarec: classification and reclassification operations of sold products, camfria: cooling chamber, tratpos: postharvest treatment, tiptrns: transport type, tipreserv: provision of services, precprom: promotional pricing, acmovpr: track price changes, comp: purchase by contract, compop: purchase by opportunity, destcomer: unsoldable sent products, contrdesp: waste control, pffixo: fixed producer or supplier.

Leyenda: tipman: operaciones de manipulación comercial, oprtrans: operaciones de transbordo, comtpemb: comercio de paquetes, tipcotr: tipo de control para productos comercializados, fornemb: paquete de suministro, clarec: operaciones de clasificación y reclasificación de productos vendidos, camfria: cámara de enfriamiento, tratpos: poscosecha tratamiento, tiptrns: tipo de transporte, tipreserv: prestación de servicios, precprom: precios promocionales, acmovpr: seguimiento de cambios de precio, comp: compra por contrato, comp: compra por oportunidad, destcomer: productos enviados no soldables, contrdesp: control de residuos, pffixo: fijo productor o proveedor.

Figure 6. Percentage contributions of dimensions and indicators to tomato waste levels.

Figura 6. Contribuciones porcentuales de dimensiones e indicadores a los niveles de residuos de tomate.

For the supply and marketing logistics of papaya and potato, low and high waste were associated with the greater number of actions taken by wholesalers (figure 7). For the distribution and marketing logistics of tomato, lettuce and orange, no significant differences among high and low waste generation and number of anti-waste actions, could be found (figure 7).

Blue asterisks represent mean values, *p-values* were obtained by the Wilcoxon-Mann-Whitney test.

Asteriscos azules representan el valor medio. Los valores p son resultado de la prueba de Wilcoxon-Mann-Whitney.

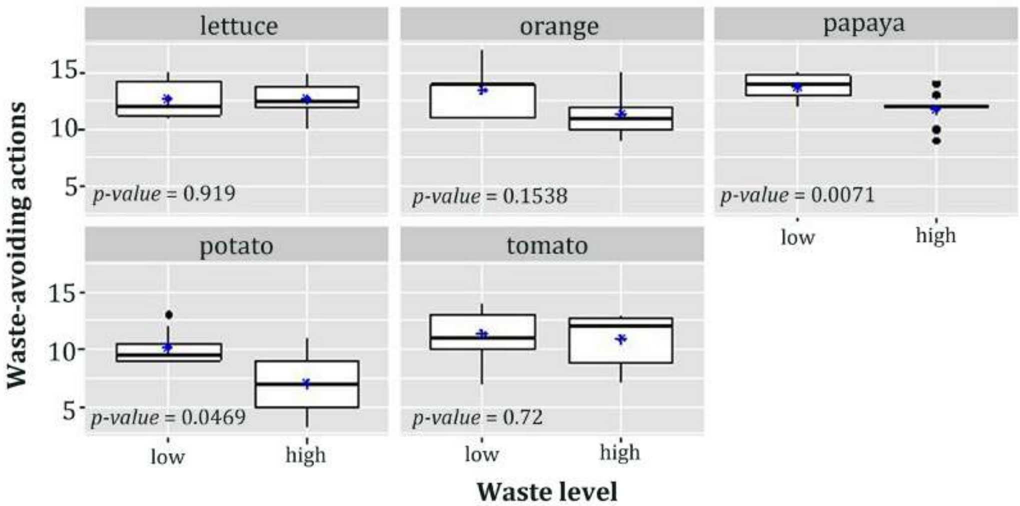


Figure 7. Boxplot of the number of anti-waste actions by level of waste, for each product.
Figura 7. Boxplot del número de acciones anti-desperdicio por nivel de desperdicio, por producto. Asteriscos azules representan el valor medio.

Discussion

For papaya, the low content of declared waste resulted to be associated with the use of cardboard boxes, plastics and other types of packaging, as well as the use of cooling chambers that contribute to a slower cellular metabolism and longer storage. On the other hand, some practices such as transshipment operations, open truck transport (often covered with canvas) and the use of "K" type packaging (wood), were associated with higher waste levels.

Potato, with the same declared waste level as papaya, also shares inadequate storage and commercialization. Load stacking and the volume of potato packages (20 kg) contribute to accelerating tuber metabolism and its consequent deterioration.

The low frequency of operation and management variables contributed to higher waste production for all the evaluated products. Levels of waste in the distribution and supply chains varied according to the product considered and were related to procedures in the distribution and marketing logistics chains. A complex intersection between declared waste, defined variables, wholesalers, and established procedures in the distribution and marketing logistics determine that the highest levels of waste are related to inadequate commercial and logistics practices, technical limitations and inappropriate storage, commerce and transportation infrastructure (11, 19). According to Abiad and Meho (2018), equipment, low quality techniques and inadequate handling in the fruit and vegetable chain cause mechanical, physiological and pathological damage. Fruit and vegetable waste is associated, to a greater or lesser extent, with specific conditions of the distribution and marketing logistics, such as: storage, processing, packaging, transportation and marketing (8, 23, 32). According to Kasso and Bekele (2018), maintaining relative humidity provides longer shelf-life and quality, reducing postharvest losses and deterioration. Other factors such as the use of cooling chambers, plastic or cardboard packaging, storage, adequate transport and shorter distances are also associated with lower levels of waste. The main factors causing postharvest loss and quality deterioration are also related to improper handling, cargo handling operations, inadequate packaging, inappropriate transport, storage, processing and display for sale (28, 38). In addition, shared fruit and vegetable transport, or combining different stages of ripeness, may negatively impact quality, accelerating maturation (6).

Conclusion

The larger the expanded area of the radar chart formed (figure 4, page 214; figure 5, page 215 and figure 6, page 216) the lower the level of waste declared for papaya and potato. For these products, high and low frequencies of all dimensions are associated with high and low waste, respectively.

For the distribution and marketing logistics of tomato, lettuce and orange, no significant differences for high and low waste in relation to the number of anti-waste actions was found.

Papaya and potato waste generation at CEASA Campinas involve multiple factors and depend on the practices established in the distribution and marketing chains.

Due to the physiology of the fruits and vegetables analyzed, and given post-harvest conditions, some level of food waste is expected. However, to establish good practices aimed at minimizing waste in these chains, is necessary.

Considering the characteristics of each product and the practices established in each distribution and marketing logistics, to compare different products does not lead to an assertive assessment of facts. Since each product demands different and specific conditions of transport, packaging, cooling and storage, a careful analysis of the various dimensions and variables, must be approached.

This work does not represent a definite assessment of waste from the perspective of the CEASA Campinas distribution and marketing logistics. However, it stresses the need to create instruments capable of reliably quantifying food waste, with demands to be met by local public authorities and the industry, in order to minimize fruit and vegetable losses in wholesale markets.

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Influence of sugarcane *(Saccharum officinarum)* straw on weed germination control

La influencia del manejo de residuo agrícola de cosecha en el control de los flujos de germinación de malezas en caña de azúcar *(Saccharum officinarum)*

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Abstract

This research studied the effectiveness of herbicide treatments in weed control and during different periods of emergence, when applied to varying amounts of straw. The experiment was conducted in a greenhouse with pre-emergent herbicides: amicarbazone, metribuzin, indaziflam, isoxaflutole, amicarbazone + indaziflam, metribuzin + indaziflam, and isoxaflutole + indaziflam, against three species (*Sorghum halepense*, *Rottboellia exaltata,* and *Mucuna aterrima*). The experimental design was completely randomized in a 5x4 factorial scheme, with five amounts of straw (0, 1, 2, 3, and 5 t/ha) and four periods of weed emergence (0, 30, 60, and 90 days after treatments). The residual control of indaziflam was influenced by the amount of straw. Metribuzin presented a low residual control, while isoxaflutole was not affected by the amount of straw. Amicarbazone offered residual control for *Mucuna aterrima*. The association between indaziflam + isoxaflutole displayed a suitable residual control against *Sorghum halepense* and *Rottboellia exaltata*. The association of indaziflam + metribuzin adequately controlled *Mucuna aterrima* and *Sorghum halepense*. herbicide physical-chemical characteristics can influence their performance, and the association of products may increase residual and weed spectrum control.

Keywords
Leaching • germination-asynchronous • residual • wet season

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Resumen

El objetivo de este trabajo fue estudiar la efectividad de los tratamientos con herbicidas en el control de malezas en distintos periodos de emergencia aplicados en diferentes cantidades de residuo agrícola de cosecha. El experimento fue conducido en invernadero con los herbicidas pre-emergentes: amicarbazone, metribuzin, indaziflam, isoxaflutole, amicarbazone + indaziflam metribuzin + indaziflam) y isoxaflutole + indaziflam, en el control de tres especies (*Sorghum halepense*, *Rottboellia exaltata* y *Mucuna aterrima)*. El diseño experimental fue completamente aleatorio en esquema factorial 5x4, con cinco cantidades de paja (0, 1, 2, 3 y 5 t / ha) y cuatro periodos de emergencia de malas hierbas (0; 30; 60 y 90 días después de la aplicación de los tratamientos). El indaziflam tuvo su residual influenciado por la cantidad de residuo agrícola de cosecha. El metribuzin presentó un residuo bajo, mientras que el Isoxaflutole no fue afectado por la cantidad de paja. Amicarbazone ofreció control residual para *Mucuna aterrima*. La asociación indaziflam + isoxaflutole presentó residual adecuado para el control de *Sorghum halepense* y *Rottboellia exaltata*. La asociación de indaziflam + metribuzin presentó un control adecuado de *Mucuna aterrima* y *Sorghum halepense*. Las características físico-químicas de los herbicidas pueden influir en su desempeño, y la asociación de productos puede aumentar el espectro de control de malas hierbas y el residual.

Palabras claves

Lixiviación • germinación asincrónica • residual • estación lluviosa

Introduction

In Brazil, cane fields harvested without earlier burning, were found to yield about 10 to 30 t/ha of straw, usable for electricity cogeneration, adding value in the sugar and alcohol agroindustry (11). Faced with this possibility, several mills have started to collect and exploit this material resulting in about 0 to 10 t/ha of remaining straw on the soil surface (11).

This partial or total removal of remaining sugarcane straw from the soil surface alters weeds dynamics in sugarcane fields. Concenço *et al.* (2017) observed that, in Brazil, areas cultivated with sugarcane where cane straw is removed from the crop lines and in-between lines, were considerably more infested with weeds than areas not harvested in the second year of cultivation. Weed species composition also changed, with eudicot weeds such as *Euphorbia heterophylla* in the line-up and standard areas. Straw removal caused the emergence of *Commelina benghalensis*, *Brachiaria plantaginea*, *Digitaria insularis,* and *Digitaria Horizontalis*, species, until then, absent in most areas. In other words, straw line-up did not eliminate eudicots infestation and even caused an increase in the outbreak of monocot plants, allowing the composition of a mixed flora.

Therefore, areas that have recently started to completely remove sugarcane straw after harvesting, now present an infesting flora, with hard to control monocotyledonous specieslike *Sorghum halepense* and *Rottboellia exaltata,* (7, 14). These weed species have small seeds, with rapid germination and quick field colonization (13). These monocot weeds were found in addition to other often reported species in mechanically harvested cane fields, such as *Mucuna aterrima* (9).

This heterogeneous composition of the infesting flora follows three main causes: (1) The uneven distribution of straw on the soil surface; (2) Years of cultivation in a system without straw burning or its removal, favouring the establishment and high density of weed seeds like *Mucuna aterrima* in the soil seed bank (8, 17). Sugarcane straw on the soil surface may constitute a physical barrier to the application of herbicides with exclusive or preferential soil action. The straw intercepts and retains the herbicides, that become more susceptible to volatilization and/or photolysis before reaching the soil where they are expected to have an effective action (6).

Therefore, finding efficient herbicides against monocot and eudicot weeds, while selective for sugarcane has become necessary. Among these species, some plants are even more difficult to control, such as *Sorghum halepense*, *Rottboellia exaltata*, and *Mucuna aterrima* (7, 9, 13, 14).

Among available options for weed control in sugarcane crops, amicarbazone (photosystem II), metribuzin (photosystem II), isoxaflutole (IFT), and indaziflam can be used isolated and/or associated (19). This experiment had the objective of studying the effectiveness of herbicide treatments in weed control, during different periods of emergence and when applied to different amounts of straw.

Materials and methods

The experiment was conducted in a greenhouse in 2016 and repeated in 2017. The experimental design was completely randomized, in a 5x4 factorial scheme, with four replications, five amounts of straw (0, 1, 2, 3, and 5 t/ha) and four periods of weed emergence (0, 30, 60, and 90 days after treatments (DAT)), along with a control without herbicide application.

This factorial scheme was adopted in isolation for each of the seven herbicide treatments (amicarbazone, metribuzin, indaziflam, isoxaflutole, amicarbazone + indaziflam, metribuzin + indaziflam, and isoxaflutole + indaziflam) and three weed species (*Sorghum halepense*, *Rottboellia exaltata*, and *Mucuna aterrima*), meaning that herbicides did not take part of the statistical factors. The experimental units were composed of 20 L polyethylene pots.

Each weed emergence period represented a *flush of seedlings* of the germinating species, after? one single sowing, in each period. That is, for each pot, weeds were sown at a single point in time, provoking one germination *flush* (0, 30, 60, and 90 DAT) causing minimum soil rotation and avoiding moving the herbicide out of its application range and/or the location where the weed seeds were positioned.

The pots were filled with a dystrophic Red Latosol from the previously sifted arable layer. This soil was collected from an area with no former use of herbicide, and previously characterized in relation to its chemical and physical properties in the ESALQ / USP soil laboratory (table 1).

Unit: Al, H+Al, K, Ca, Mg, SB and CTC ($mmol_c$ dm^{-3}); P (resina) (mg dm^{-3}); V, clay, silt, sand (%).

Unidad: Al, H + Al, K, Ca, Mg, SB y CTC (mmolc dm^{-3}); P (resina) (mg dm^{-3}); V, arcilla, limo, arena (%).

Table 1. Result of soil chemical analysis (0 to 20 cm). Piracicaba - SP.

Tabla 1. Resultado del análisis químico del suelo (0 a 20 cm). Piracicaba - SP.

pH ($CaCl_2$)	Al	H+Al	P (resina)	K	Ca	Mg	SB	CTC	V	Clay	Silt	Sand
5.3	< 1.0	25.0	10.0	2.8	26.0	13	41.8	66.8	63	41.0	5.0	54.0

Sugarcane straw was removed from a sugarcane field, shortly after harvesting and before herbicide application, avoiding, therefore, field decomposition or herbicide contamination. Afterwards, this straw was air-dried, manually chopped with scissors, and distributed on the pot's surface establishing proportional straw quantities, in tons per hectare, for each treatment (0, 1, 2, 3, and 5 t ha^{-1}, respectively).

Following this step, herbicide treatments, amicarbazone (1,400 g ha^{-1}), metribuzin (1,920 g ha^{-1}), indaziflam (100 g ha^{-1}), isoxaflutole (105 g ha^{-1}), amicarbazone + indaziflam (1,005 + 75 ha^{-1}), indaziflam (75 g ha^{-1}), metribuzin + indaziflam (960 + 75 g ha^{-1}), and isoxaflutole + indaziflam (75 + 75 g ha^{-1}) were applied against pre-emergence weeds using a CO_2 pressurized spool sprayer carrying a spray bar containing two Teejet 110.02 fan nozzles, with an application volume of 200 L ha^{-1}. During herbicides application, relative air humidity was 65%, temperature was 26.6 °C (10), and a wind speed was 2.3 km / h.

After 24 hours of herbicide application, 30 mm of water were irrigated with a sprinkler irrigation system (1 L / min) . Then, the straw was let to dry for 48 hours. Subsequently, the pots were re-watered in a daily basis, with 10 mm, through a sprinkler irrigation system. After 0, 30, 60, and 90 days after applying the treatments, all straw was carefully removed, and weeds were sown.

Following the periods of 0, 30, 60, and 90 DAT (Weeds germination *flushes*), *Sorghum halepense*, *Rottboellia exaltata*, and *Mucuna aterrima* seeds were individually and carefully sown in the pots, intending a minimum soil turnover. The amount of seed used was sufficient to obtain five plants per pot. For the *Mucuna aterrima* species, dormancy breakage was achieved through mechanical seed scarification.

At 7, 14, 21, 28, and 35 days after weed emergence (DAE), visual phytotoxicity evaluations were performed based on the Asociación Latino Americana de Las Malezas (1) criteria, and according to a percentage scale of grades, where 0 is absence of damage and 100% is plant death. At 35 DAE, the plants were cut close to the soil level, packed in paper bags, and oven dried at 60 °C for 24 hours, until constant weight.

Each treatment dry mass was interpreted as a reduction percentage in relation to the control treatment. All data were submitted to ANOVA, and means were compared by Tukey test using the AgroEstat computational statistical software (4). When significant, variable effects were analyzed by non-linear regressions, with SIGMAPLOT computational program. Data analysis was individually conducted for each herbicide.

Results

Weed control is generally considered "very good" when herbicide application achieves 81-90% control of weeds, and "excellent" with control percentages between 91 and 100% (1). For highly invasive weeds like *Mucuna aterrima*, *Sorghum halepense,* and *Rottboellia exaltata,* control effectiveness must exceed 90% (7, 14, 17).

Indaziflam resulted not effective against *Mucuna aterrima.* Only during the first emergence period, at 0 DAT, with the application on 0 t/ha, control achieved 80%. For all other treatments, control was considered ineffective, with values under 80%. During the emergence period at 90 DAT, and regardless of the amount of straw, control percentages for *Mucuna aterrima* were under 40%, (figure 1-A).

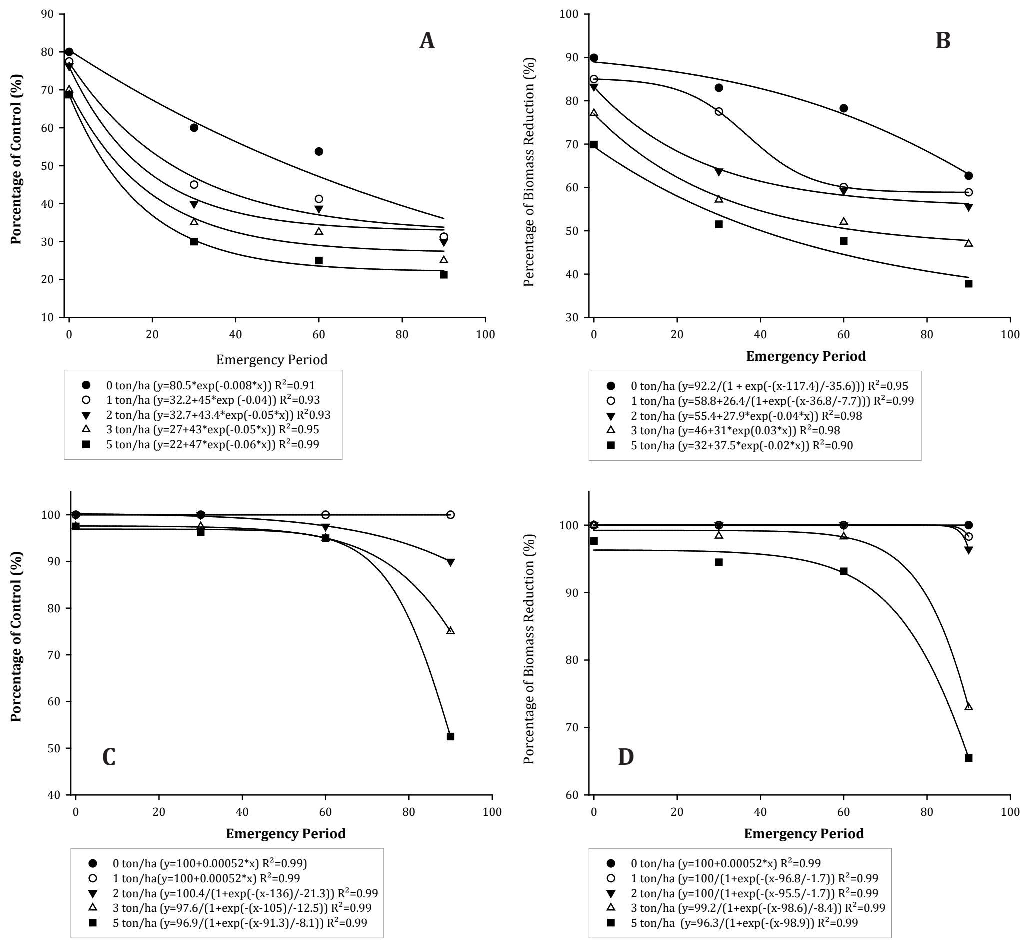

Figure 1. Biomass Control and reduction at 35 DAE with indaziflam: *Mucuna aterrima* (A- B) and *Rottboellia exaltata* (C-D).

Figura 1. Control y reducción de la biomasa a 35 DAE a través del herbicida indaziflam: *Mucuna aterrima* (A-B) y *Rottboellia exaltata* (C-D).

Control of *Rottboellia exaltata* by indaziflam, in the emergence periods at 0, 30, and 60 DAT, was over 80% for all straw amounts. In the emergence *flush* at 90 DAT, only for 3 and 5 t/ha of straw, control was ineffective, reaching 75% and 52.5%, respectively (figure 1-C, page 223). For *Sorghum halepense*, no statistical difference was observed for biomass reduction and control, however, the latter was considered to be "excellent", with percentages over 90% in all treatments, (data not shown). Regarding metribuzin, excellent control of *Mucuna aterrima* was observed in the first emergence period at 0 DAT, where control percentages exceeded 98%, regardless of straw amount. In the emergence period at 30, 60, and 90 DAT, regardless straw amount, control was inadequate (figure 2-A).

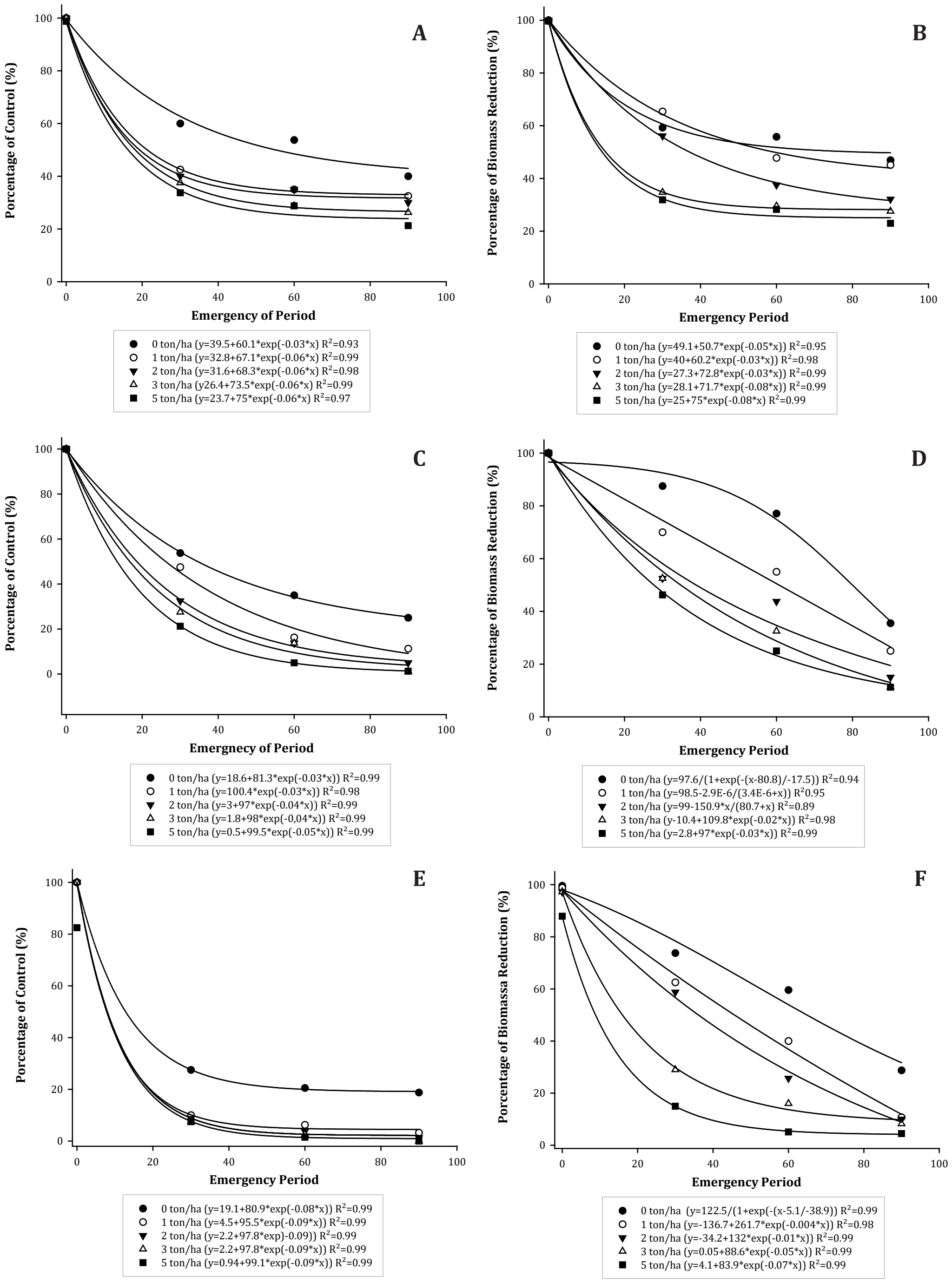

Figure 2. Biomass control and reduction at 35 DAE by metribuzin: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D); and *Rottboellia exaltata* (E-F).

Figura 2. Control y reducción de la biomasa a 35 DAE a través del herbicida metribuzim: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D) y *Rottboellia exaltata* (E-F).

Control of *Sorghum halepense* and *Rottboellia exaltata* by metribuzin, in the emergence period at 0 DAT, achieved 100% independently from straw amounts. However, in the sowing periods of 30, 60, and 90 DAT, this herbicide was not effective against these species, regardless of straw amount (figure 2-C-E, page 224). For isoxaflutole, in the emergence period at 0 DAT, control percentages of *Mucuna aterrima* were over 80% when herbicide applications occurred on 0, 1, and 2 t/ha. For 3 and 5 t/ha, control resulted ineffective. In the emergence flushes of *Mucuna aterrima* at 30, 60, and 90 DAT, isoxaflutole was not effective, regardless of straw amount (figure 3-A).

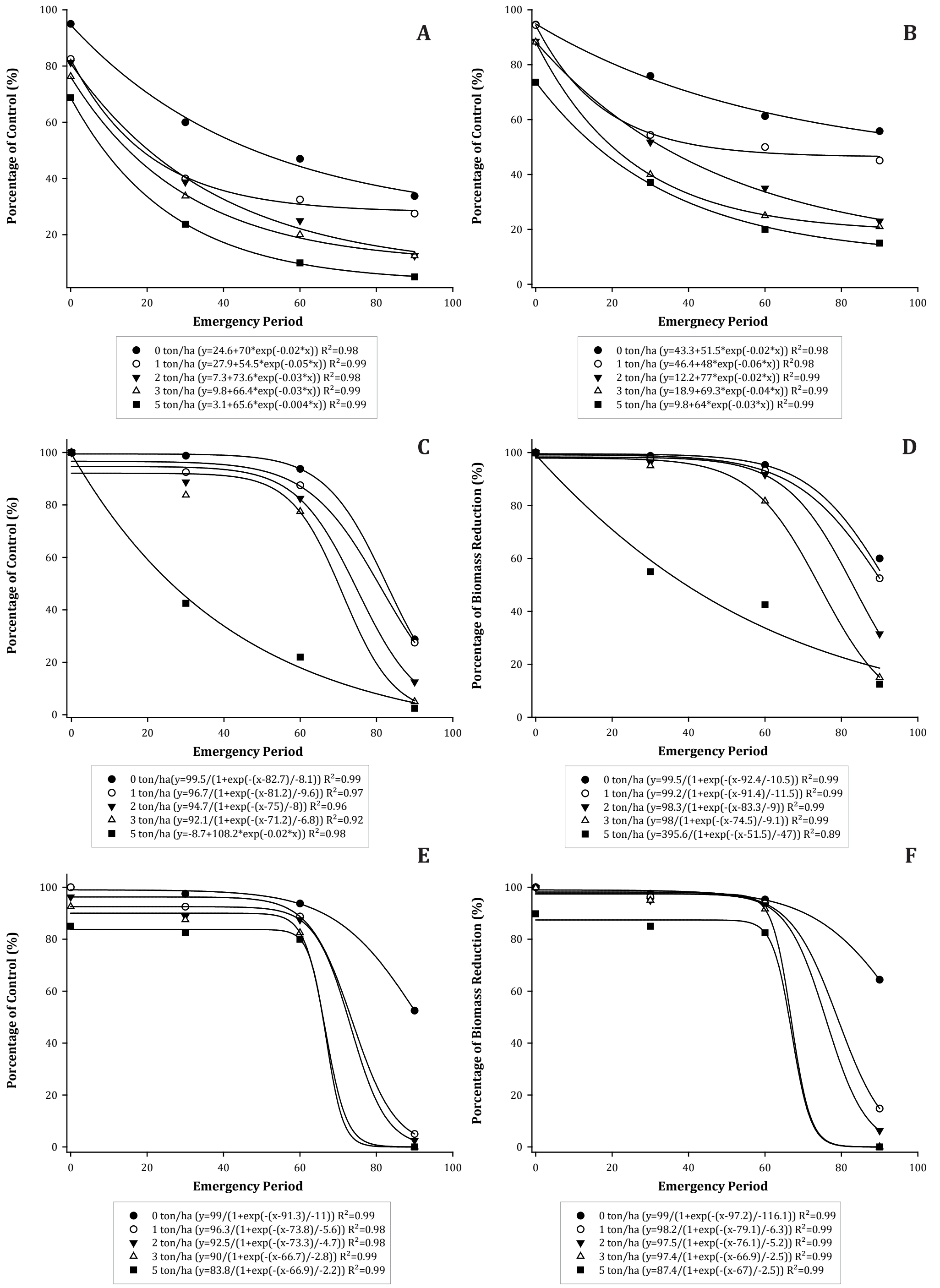

Figure 3. Biomass Control and reduction of at 35 DAE by isoxaflutole: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D); and *Rottboellia exaltata* (E-F).

Figura 3. Control y reducción de la biomasa a 35 DAE a través del herbicida isoxaflutole: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D) y *Rottboellia exaltata* (E-F).

For *Sorghum halepense*, control through isoxaflutole was considered excellent in the emergence period at 0 DAT, reaching 100% for all straw amounts. In the emergence period at 30 DAT, control exceeded 80% for all straw quantities. At 60 DAT, control resulted ineffective for 3 and 5 t/ha of straw and at 90 DAT, regardless of straw amount, control was ineffective (figure 3-C, page 225). Isoxaflutole adequately controlled *Rottboellia exaltata,* (above 80%) at 0, 30, and 60 DAT for all straw amounts, while at 90 DAT, control was ineffective, regardless of straw quantities (figure 3-E, page 225).

Amicarbazone, in the emergence period at 0 DAT, provided excellent control of *Mucuna aterrima*, with percentages over 93% for all amounts of straw. At 30 DAT, for 0, 1, and 3 t/ha, control was excellent, over 95%. For 5 t/ha, control was adequate. For the 60 DAT emergence period, and 0, 1, and 2 t/ha, control exceeded 90% but resulted inefficient for the applications on 3 and 5 t/ha of straw. At 90 DAT, control was effective only in the absence of straw, exceeding 90% (figure 4-A, page 227). Regarding *Sorghum halepense* and *Rottboellia exaltata*, when amicarbazone was applied at 0 DAT, and for all amounts of straw, control exceeded 85%. At 30, 60, and 90 DAT, control of these species was considered ineffective, regardless of straw quantities (figure 4-C-E, page 227).

At 0 DAT emergence period, the association between indaziflam and isoxaflutole resulted effective against *Mucuna aterrima* for all amounts of straw, exceeding 80%. At the 30, 60, and 90 DAT emergence periods, control was considered ineffective, regardless of straw amount (figure 5-A, page 228). Control of *Sorghum halepense* by the association of indaziflam with isoxaflutole, at 0 DAT, was 100% for all amounts of straw. In the 30 DAT emergence period , regardless of straws amounts, control exceeded 90%. At 60 and 90 DAT, for 0, 1, 2, and 3 t/ha, control was over 90%; while in 5 t/ha, control was over 80% (figure 5-C, page 228). *Rottboellia exaltata* treated with indaziflam + isoxaflutole, at 0 DAT, exceeded 95% for all amounts of straw. At 30, 60, and 90 DAT, control of *Rottboellia exaltata* achieved over 85% for 0, 1, 2, and 3 t/ha. However, for 5 t/ha, control was below 75% at all emergence periods (figure 5-E, page 228).

Control of *Mucuna aterrima*, by application of indaziflam associated with amicarbazone, can be observed in figure 6-A (page 229). In the 0 DAT emergence period, control was ineffective only for 5 t/ha of straw. At 30 DAT, control was acceptable only for straw quantities of 0 and 1 t/ha, achieving control percentages of 87.5% and 83.75%, respectively. For the 60 DAT emergence period and 0 t/ha of straw, control was 81.25%. For the other straw quantities, weed control was below 80%. Regarding the emergence period at 90 DAT, for any amount of straw, control percentages were lower than 52.5%.

The application of amicarbazone + indaziflam resulted in excellent control of *Sorghum halepense* during the emergence period at 0 DAT, with control percentages higher than 98% for all straw amounts. At 30, 60, and 90 DAT, control was considered ineffective only for the amount of 5 t/ha, but exceeded 90 % for 0, 1, 2, and 3 t/ha (figure 6-C, page 229). Control of *Rottboellia exaltata* through the association between indaziflam and amicarbazone, achieved 100% at 0 and 30 DAT, for all straw amounts, while it also achieved 100% at 60 DAT with 0, 1, and 2 t/ha straw. However, with amounts of straw of 3 and 5 t/ha, control was 95 and 80%, respectively. In the emergence period at 90 DAT, control was considered effective only for straw quantities of 0 and 1 t/ha, with 96.25% and 92.50%, respectively (figure 6-E, page 229).

For *Mucuna aterrima*, results on the association of indaziflam with metribuzin can be observed in Figure 7 (page 230). In the emergency periods at 0 DAT, control was above 80%, regardless of straw amounts. At 30 DAT, only for 5 t/ha, control was considered ineffective, with percentages below 80%, while at 60 DAT, only in the absence of straw, control was effective. At 90 DAT, and for all amounts of straw, control was under 80% (figure 7-A, page 230).

Control of *Sorghum halepense* using indaziflam associated with metribuzin, at 0 DAT, was above 90%, regardless of straw amounts. In the 30 and 60 DAT emergence periods, control was only considered inadequate for 5 t/ha of straw. At 90 DAT, ineffective control was found only in applications of 3 and 5 t/ha, with 67.5 and 45% control, respectively (figure 7-C, page 230). For *Rottboellia exaltata,* application of indaziflam associated with metribuzin, for 0, 30, and 60 DAT emergence periods, achieved excellent control exceeding 87.5%, for all amounts of straw. However, at 90 DAT, no effective control of *Rottboellia exaltata* was observed for 3 and 5 t/ha (figure 7-E, page 230).

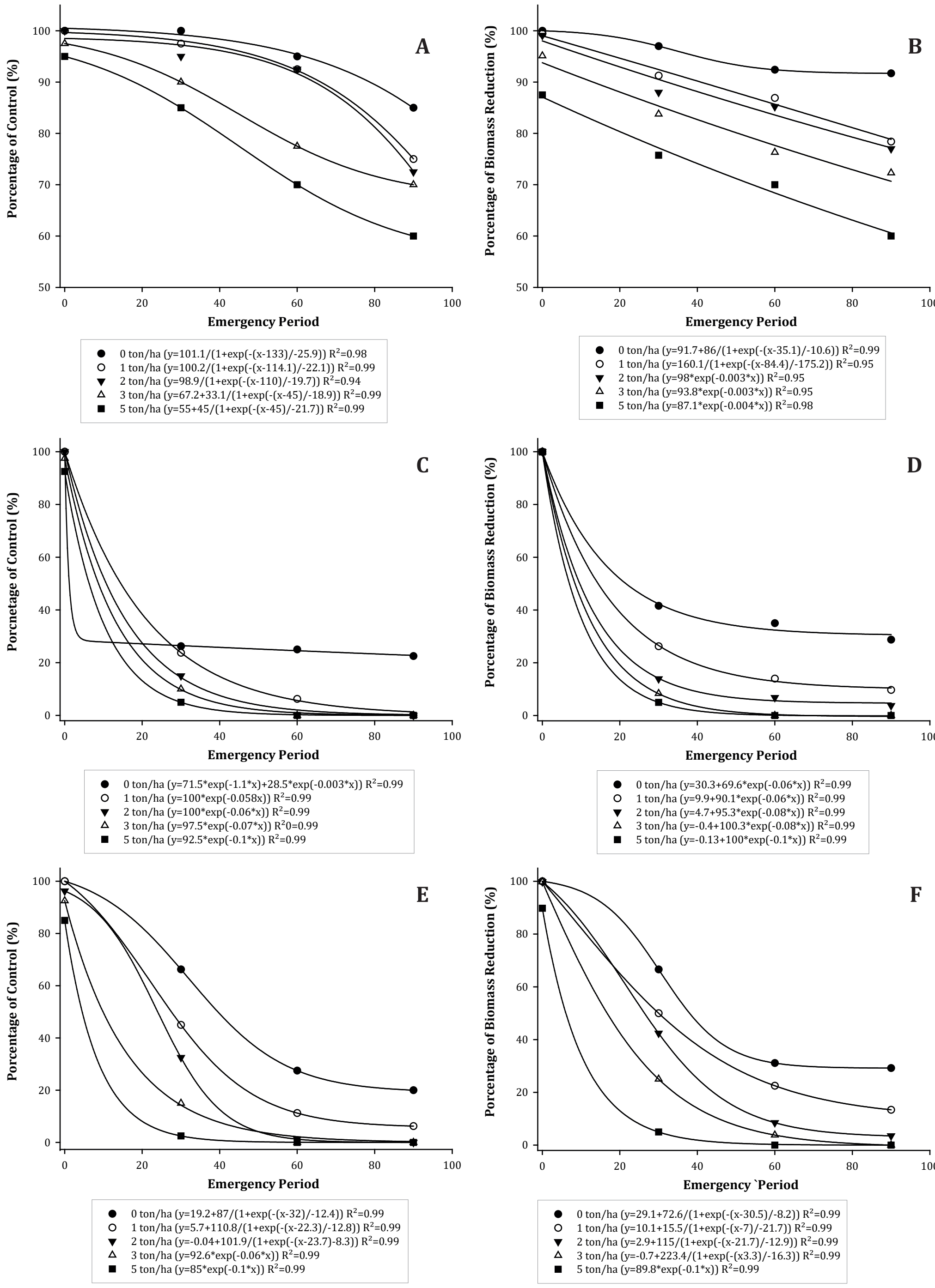

Figure 4. Biomass control and reduction at 35 DAE by amicarbazone: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D); and *Rottboellia exaltata* (E-F).

Figura 4. Control y reducción de la biomasa a 35 DAE a través del herbicida amicarbazona: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D) y *Rottboellia exaltata* (E-F).

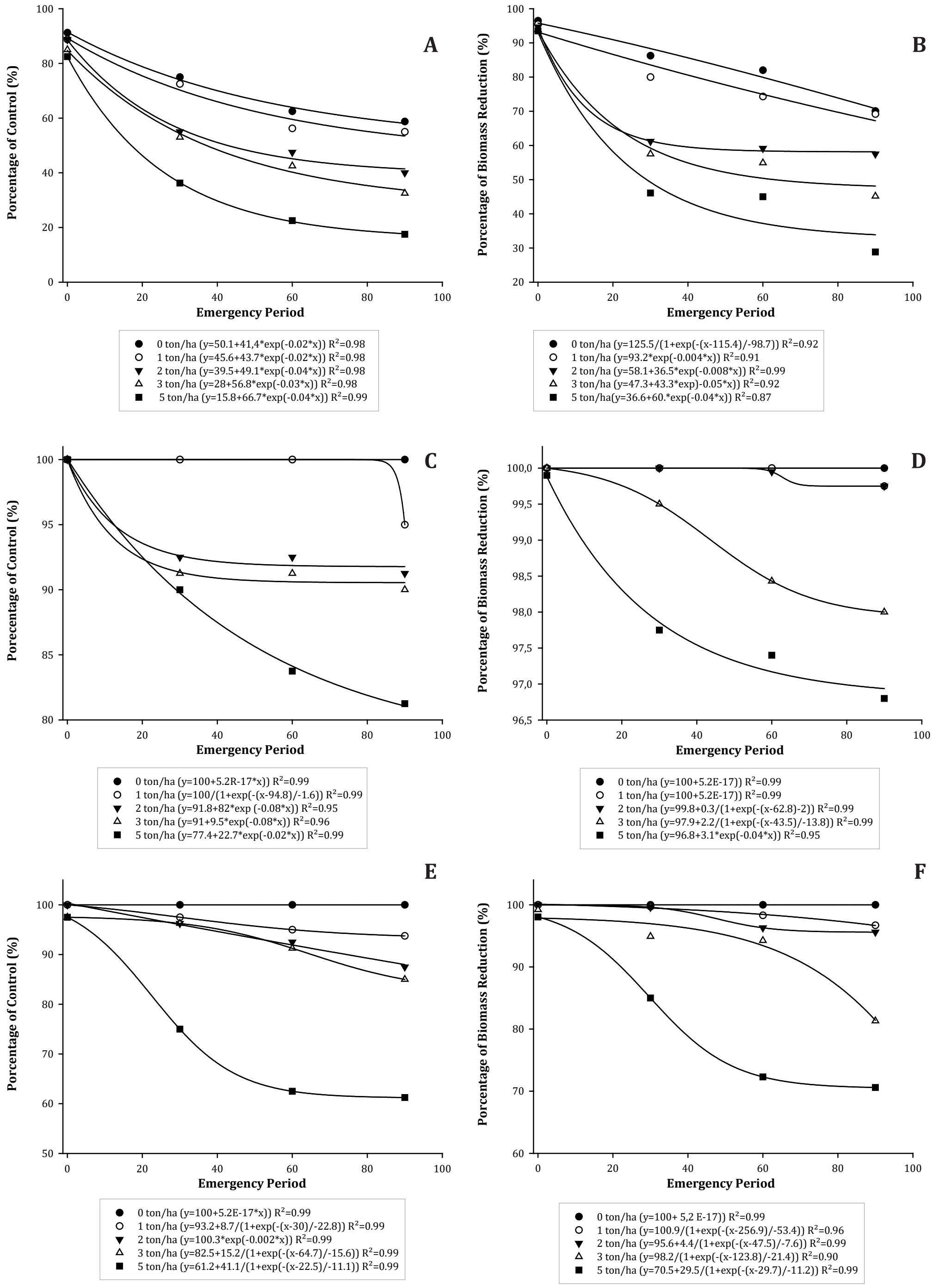

Figure 5. Biomass control and reduction at 35 DAE through indaziflam + isoxaflutole: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D); and *Rottboellia exaltata* (E-F).

Figura 5. Control y reducción de la biomasa a 35 DAE a través del herbicida indaziflam + isoxaflutole: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D) y *Rottboellia exaltata* (E-F).

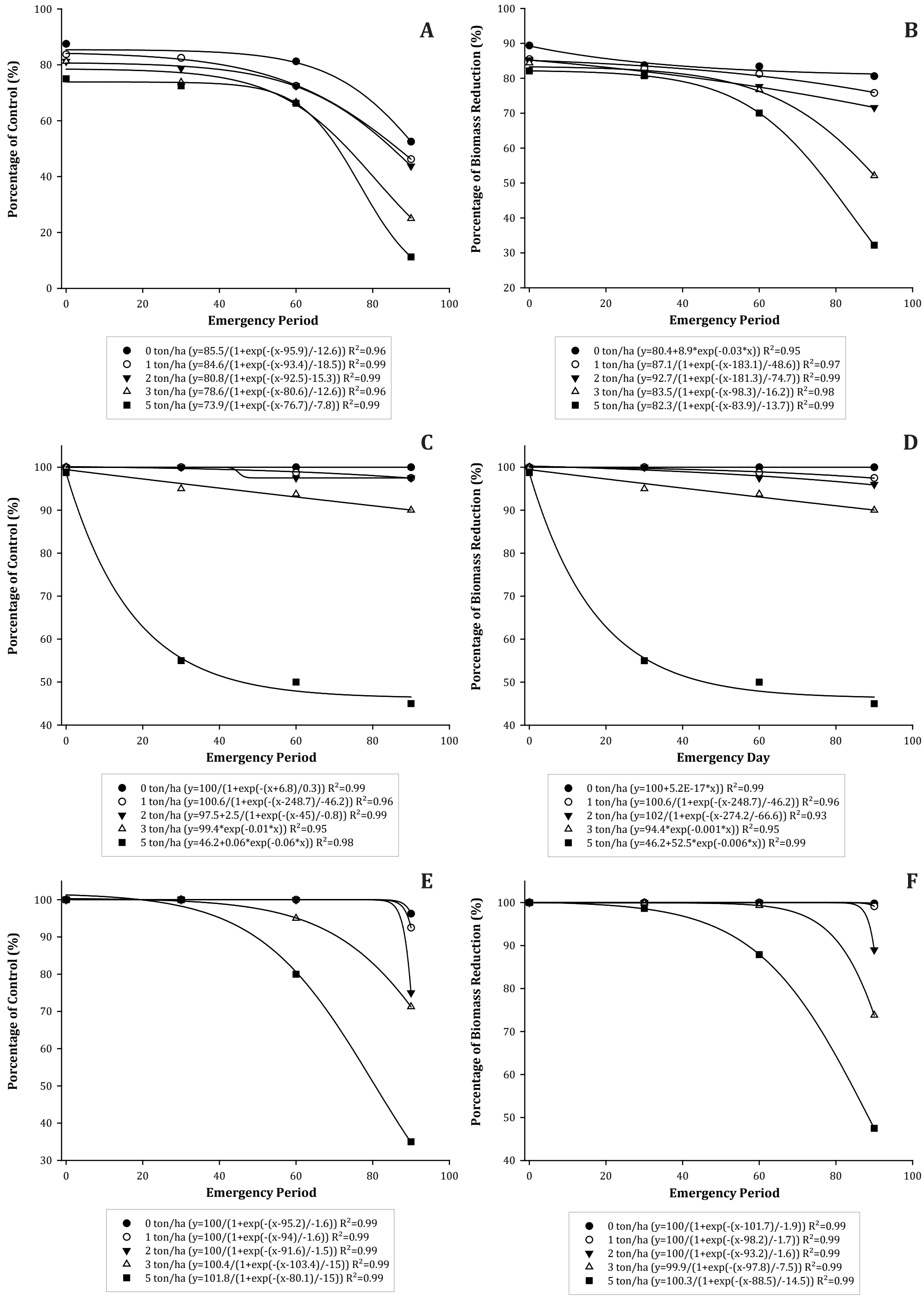

Figure 6. Biomass control and reduction at 35 DAE through indaziflam + amicarbazone: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D); y *Rottboellia exaltata* (E-F).

Figura 6. Control y reducción de la biomasa a 35 DAE a través del herbicida indaziflam + amicarbazone: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D) and *Rottboellia exaltata* (E-F).

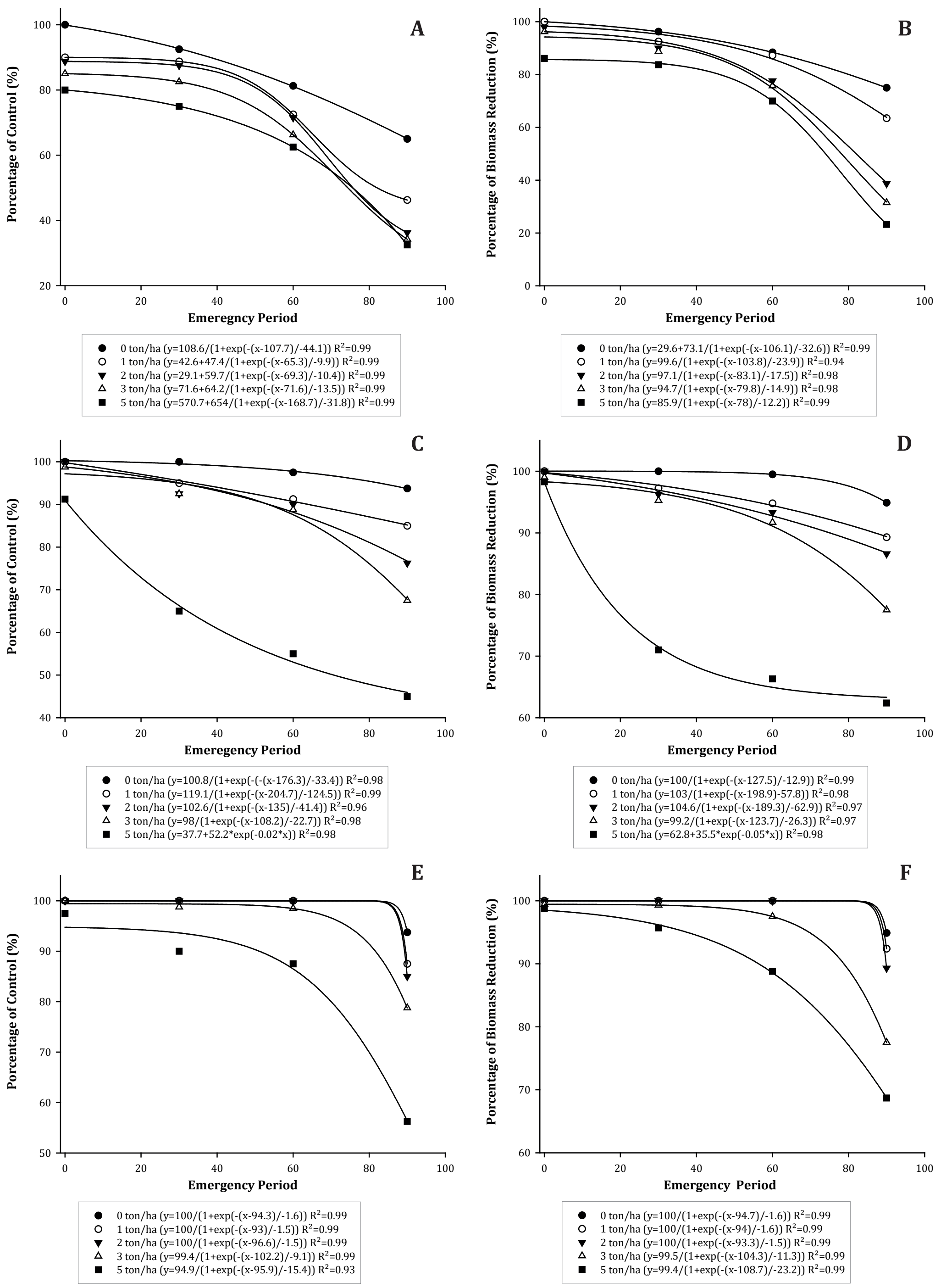

Figure 7. Control and reduction of biomass at 35 DAE through the indaziflam + metribuzin herbicide: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D); and *Rottboellia exaltata* (D-E).

Figura 7. Control y reducción de la biomasa a 35 DAE a través del herbicida indaziflam + metribuzin: *Mucuna aterrima* (A-B); *Sorghum halepense* (C-D) y *Rottboellia exaltata* (E-F).

Discussion

The results obtained for the isolated application of indaziflam indicated that this herbicide achieves better control over monocotyledonous species than over eudicot species. This can be explained by the long soil residual effect of indaziflam (more than 150 days). However, this residual effect might be lower in tropical regions, such as Brazil, with higher temperatures and abundant rainfall (2). Soil moisture is essential for the bioavailability of indaziflam in the soil solution, favouring its effectiveness. This factor turns crucial for sugarcane, where herbicide applications occur during wet, semi-humid, and dry conditions (6, 18). The results obtained in this experiment indicated that higher control efficiency with indaziflam is achieved when applied during rainy seasons, given its high Kow and Kd (18, 19). Sebastian *et al.* (2016), verified soil moisture influence over *Kochia scoparia* L control, confirming this behaviour. Water potential of -400 KPa resulted in a 100% dry mass reduction, while -100 KPa concluded in a dry mass decrease of almost 30%. That is, the higher the amount of soil water, the higher the effectiveness of indaziflam against *Kochia scoparia*.

The ineffective control of *R. exaltata* at 90 DAT with indaziflam and 3 and 5 t/ha straw, may have been conditioned by interception and/or adsorption. With Kow = 2.88, the amount of herbicide transported to the soil and its consequent availability in soil solution, is reduced (6).

In short, we consider that metribuzin did not present lasting soil residue effect. This herbicide presents high water solubility (1100 mg L^{-1}), medium Koc (60 mL g^{-1}), and a half-life of 30 to 60 days (12, 19). Given these physicochemical characteristics, for sugarcane, metribuzin is often applied during the dry season (3). Ben *et al.* (2015), performing leaching and persistence experiments of metribuzin, using bioindicators (*Cucumis sativus*), verified that increasing soil water blade resulted in a gradual increase in the control of *C. sativus*. With a water depth of 100 mm, satisfactory control (> 96%) was observed down to the layer of 20-25 cm depth, indicating high leaching potential of metribuzin, mainly for applications during rainy seasons.

For isoxaflutole (IFT), the results suggest a sort of degradation route or alternate transport, making it unavailable for the weed root system. IFT is considered a pro-herbicide that is later converted to diketonitrile (DKN), the active weed controlling molecule (19), depending on soil water availability. In the present experiment, IFT was applied during the wet season and maintained throughout the experiment with constant rainfall (10 mm daily). This application may have resulted in the fast conversion of IFT to DKN, making DKN available in the soil solution and susceptible to leaching and degradation phenomena (19). Another aspect that may explain the inadequate control achieved by IFT during the sowing period at 90 DAT, is the short, 56 days half-life of DKN, (19).

The application of amicarbazone, with constant rainfall after application, may have resulted in an intense leaching and a subsequent reduction of its weed control efficiency, due to its high solubility (4,600 mg / L in the range of pH 4-9) (19). Possamai *et al.* (2013) found high amicarbazone leach in the 60 mm blade of water in sandy soils, resulting in a lower residual effect for this herbicide. In clay soil, simulations of water depths of 20 and 80 mm after amicarbazone applications reduced plant biomass at 5-10 cm depths.

The indaziflam + isoxaflutole association can be considered a good option for monocotyledon weed control, since they have a wide control spectrum for monocots. This association was based on two products with very different physicochemical characteristics: indaziflam has low solubility and high Kow, while DKN (after conversion of IFT in water) has high solubility and low Kow. The combination of contrasting characteristics may have favoured an adequate amount of herbicide in the weed sowing range, achieving proper control of monocotyledon weeds. On the other hand, this association did not increase weed control spectrum, keeping effectiveness only upon monocots.

The association between indaziflam and amicarbazone was not a viable alternative to weed control in this experiment. Isolated applications achieved better results against *Mucuna aterrima* and *Rottboellia exaltata*, while *Sorghum halepense* could not be combated in applications on higher amounts of straw.

The association between indaziflam and metribuzin provided better control performance than their isolated applications. This association provided greater residual control for *Mucuna aterrima* and *Sorghum halepense* and *Rottboellia exaltata*, This improved control efficiency can be attributed to physicochemical characteristics and weed control spectrum of each herbicide. providing a potential option for sugarcane fields with small amount of straw on the soil surface, as occurs, for instance, in line up straw and/or plant-cane systems.

Conclusions

Indaziflam herbicide presented a better performance in controlling monocotyledonous plants than eudicot plants. Applications on more substantial amounts of straw showed to be less effective than applications on lower amounts of straw. The herbicides amicarbazone and metribuzin presented adequate control of *Mucuna aterrima* despite having lower residual control. Metribuzin presented some residual control at different times of emergence. On the other hand, isoxaflutole showed long residual control of *Sorghum halepense* and *Rottboellia exaltata,* regardless of straw amounts.

The association of indaziflam + metribuzin, resulted in satisfactory results for *Mucuna aterrima*, *Sorghum halepense,* and *Rottboellia exaltata*, even for applications on different amounts of straw, probably due to the various emergence times.

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Anti-infective properties of medicinal plants from the Baja California peninsula, Mexico for the treatment of *Fusarium oxysporum* f. sp. *basilici* in organic sweet basil *(Ocimum basilicum)*

Propiedades anti-infectivas de plantas medicinales de la península de Baja California, México para el tratamiento de *Fusarium oxysporum* f. sp. *basilici* en albahaca orgánica *(Ocimum basilicum)*

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Abstract

Certified-organic farming systems in Baja California Peninsula and Northwest Mexico are nationally and globally recognized, especially due to the production of vegetables and aromatic herbs under protected agriculture systems. Based on the background of some species of the flora of Baja California Sur (BCS) to inhibit a diversity of microorganisms, the effect of 22 medicinal plants of the region was explored to know the *in vitro* activity against the fungus *Fusarium oxysporum* f. sp. *basilici* isolated from basil (*Ocimum basilicum* L.). The plants processed as crude ethanolic and aqueous extracts were analyzed in duplicate (three replicates) evaluating the inhibition of mycelial growth and spore germination. In mycelial inhibition test, all plants extracts (1000 mg L^{-1}) showed an effectiveness of 11 to 40% to inhibit *F. oxysporum.* The most effective plant extracts according to 50% effective inhibition dose (ED_{50}), were *Larrea tridentata*, *Hymenoclea monogyra* and *Lippia palmeri* with an ED_{50} of 220, 303 and 3000 mg L^{-1}, respectively. Tukey's PostHoc tests indicated that *H. monogyra* and *L. tridentata* are ten times (ED_{50} <300 mg L^{-1}) more effective than *L. palmeri* (ED_{50} 3000 mg L^{-1}). In addition, the dose-response trend analyzes according to the logarithmic-logistic model (*drc* packages), showed the maximum slope values between 100 and 1000 mg L^{-1}. In the spore germination inhibition tests, most ethanolic extracts (5000 mg L^{-1}) showed an effectiveness between 21 and 80%. The results of this study demonstrated that the inhibitory potential of these plants used in BCS traditional medicine are a viable alternative for the control of *F. oxysporum* f. sp. *basilici* in organic basil production systems.

Keywords

organic agriculture • crown rot • fusariosis • Larrea • Lippia • crude extracts • wild oregano • BCS

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Resumen

Los sistemas de agricultura orgánica-certificada en la Península de Baja California y el noroeste de México son reconocidos a nivel nacional y mundial, por la producción de verduras y hierbas aromáticas bajo los sistemas de agricultura protegida. Con base en los antecedentes de algunas especies de la flora de Baja California Sur (BCS) para inhibir una diversidad de microrganismos, se exploró el efecto de 22 plantas medicinales de la región para conocer la actividad *in vitro* contra *Fusarium oxysporum* f. sp. *basilici* aislado de la albahaca (*Ocimum basilicum* L.). Los extractos etanólicos y acuosos crudos se analizaron por duplicado (tres réplicas). En las pruebas de inhibición del crecimiento micelial, todos los extractos (1000 mg L^{-1}) mostraron una efectividad entre el 11 al 40% para inhibir *F. oxysporum*. Los extractos más efectivos de acuerdo con la dosis efectiva de inhibición al 50% (ED_{50}) fueron *Larrea tridentata*, *Hymenoclea monogyra* y *Lippia palmeri* con una ED_{50} de 220, 303 y 3000 mg L^{-1}, respectivamente. Las pruebas PostHoc de Tukey indicaron que *H. monogyra* y *L. tridentata* son diez veces (ED_{50} <300 mg L^{-1}) más efectivas *que L. palmeri* (ED_{50} 3000 mg L^{-1}). Además, los análisis de tendencia dosis-respuesta de acuerdo con el modelo logarítmico-logístico (drc) mostraron los máximos valores de pendiente entre 100 y 1000 mg L^{-1}. En la inhibición de germinación de esporas, la mayoría de los extractos etanólicos (5000 mg L^{-1}) mostraron una efectividad entre 21 y 80%. Los resultados de este estudio demostraron que el potencial inhibitorio de estas plantas utilizadas en la medicina tradicional de BCS son una alternativa viable para el control de la fusariosis causada por *F. oxysporum* f. sp. *basilici* en los sistemas de producción orgánica de albahaca.

Palabras clave

agricultura orgánica • pudrición de la corona • fusariosis • Larrea • Lippia • extractos crudos • orégano • BCS

Introduction

Sweet basil (*Ocimum basilicum* L.) is a popular herb used both fresh and dry as flavoring and antioxidant in food and pharmaceutical industries (22). Fresh basil has been the most demanded herb in the United States of America (USA) markets for the gourmet cooking industry, and ranks first among aromatic herbs used by Californian restaurants (4). Most of the basil production takes place in the state of Baja California Sur (BCS) in Mexico, where approximately 700 t are produced by around 200 specialized farms under the certified-organic agriculture scheme (41).

Basil crop is susceptible to a number of fungal diseases caused mainly by two large groups; one related with leaf spots produced by the genera *Cercospora*, *Curvularia* and *Alternaria*, and the second one is related to soil-borne rot diseases caused by the genera *Phytophthora*, *Pythium*, *Rhizoctonia* and *Fusarium* (33). Thus, fusarium disease can produce wilt and crown rot symptoms, when caused by *F. oxysporum* f. sp. *radicis-lycopersici* (FORL) (28), or premature defoliation, vascular wilt and crown rot when associated to *F. oxysporum* f. sp. *basilici* (FOB) (20). It is known that the spread of *Fusarium* in the American continent is so wide. In Argentina, high infection values were observed in soybean seedlings when infected with *F. graminearum* isolated from maize crop residues (5). In Mexico, some variants of *F. oxysporum* have been found associated with corky-root in coffee (19) and several species of *Fusarium* associated with avocado diseases (31). FOB colonizes the xylem of the host plant, resulting in its blockage and decomposition, with the consequent appearance of the most common and recognizable symptoms, (36). Fusarium disease was widely detected and caused a significant problem in cultivated basil in the USA for two decades. This is currently reported to be one of the most persistent diseases of this crop worldwide (45). Essential oils and tincture preparation are commonly used in a traditional medicine to treat minor infections (10). In organic agriculture, the extracts are commonly utilized as repellants for a wide range of pests (21) and, to a lesser extent, as inhibitors of several pathogenic *Fusarium* species (1, 9).

In BCS, the most important production areas, such as San José del Cabo, Todos Santos and La Paz, different *Fusarium* species have become the predominant problem in shade mesh or open cultivation (38). This occurs, mainly because the only solvents allowed in organic agriculture are water and ethanol, which necessarily focus on the search for active molecules that may have activity with these solvents. In this sense, a variety of secondary metabolites and diverse compounds of aqueous and ethanolic fractions with antifungal activity have been described (18). However, despite the promising field of research on medicinal extracts with antimicrobial properties for agriculture (11, 24, 34), most of the ethnobotanical and biological heritage of BCS medicinal plants has focused on their potential use on bacteria and yeasts of clinical interest (12, 26).

Aware of the antifungal potential of rained plant resources in the southern part of the Baja California peninsula, the objective of this research was to explore the potential of 22 species of medicinal plants with known antimicrobial activity, as a source of antifungal compounds for the treatment of Fusarium wilt diseases in the production of organic basil in BCS, Mexico.

Materials and methods

Collection of plant material

Twenty-two plants used in traditional medicine with antimicrobial properties were selected and collected from different areas of the southern desert region of the Baja California in Mexico. They were grouped according to their traditional behavior and ethnobotanical uses. Table 1 (page 237) summarizes the species name, botanical identification and ethno-pharmaceutical data. The Voucher specimens were deposited in the Herbarium of the Centro de Investigaciones Biológicas del Noroeste (CIBNOR) in La Paz, BCS, México. All plant material was collected during the 2012 rainy season (August to October). The vegetative material was only from leaves that were transported and pressed with a botanical press and dried at room temperature.

Fungus isolation and molecular identification

Isolation was performed from plants collected in El Pescadero, BCS with symptoms associated with fusariosis disease according to Summerell *et al.* (2003). The presumptive *Fusarium* species were characterized based on colony growth on *Fusarium*-selective medium. Potato dextrose agar (PDA) medium was used to determine colony morphology, growth rates and pigmentation. For the production of chlamydospores, macro- and microconidia, the medium CLA (carnation leaf agar) was used (43, 47). For molecular identification, total DNA from the fungus recovery from agar medium was obtained as template in polymerase chain reaction (PCR). One microliter (50 ng µL) of the supernatant was amplified with the primer pair Bik 1 (5´-TTCAAGAGCTAAAGGTCC-3´) and Bik 4 (5´-TTTGACCAAGATAGATGCC-3'). PCR conditions were according to the standard procedures for specific detection of FOB (7).

Extracts preparation

Aqueous and ethanolic extracts were prepared. The aqueous extract was obtained by grinding 10 g of air-dried powder in 100 mL of sterile distilled water, which was boiled on slow heat for two hours. The macerate was sieved with a five-layer muslin cloth and centrifuged at 4000 g for 30 min. The collected supernatant was filtered with Whatman filter paper No. 2 (GE Healthcare, UK) and autoclaved at 121 °C and 15 lb pressure. The resulting solution of 100 g L^{-1} was considered as mother stock. For ethanol extracts, the leaves were dried at room temperature for two weeks and then ground into powder with a blender. Then, 50 g of the powdered plant material were soaked in 200 mL (95% ethanol) at room temperature for five days. Each of the solvent extracts was shaken daily with a shaker for regular infusion. The filtrates obtained were evaporated using a rotary vacuum vapor distillation (Buchi Rotavapor R-114, Labortechnik, AG) at 60 °C, re-suspended in 20 mL of ethanol and fixed at a concentration with 100 g L^{-1} as maximum in test concentration and stored in sterile glass bottles at 5 °C until use (12).

Preliminary inhibition screening

To investigate the inhibition potential of the selected medicinal plants, two different methods were used. The first was the sensi-disc diffusion method (SDD) selected to perform the general screening of the 22 selected plants at 1000 mg L^{-1}. This type of test provided a quick and inexpensive way to obtain reliable quantitative results. The three most active extracts in SDD were selected for screening in the agar-dilution method (ADL), which allowed the sample to dissolve the agar. Therefore, its concentration was constant throughout the plate and was used to evaluate the effective doses (ED_{50}) of mycelial growth inhibition in response to 50% of the expected effect (7). Inoculum concentrations were prepared and adjusted to 10^6 CFU mL^{-1}.

Table 1. Ethnobotanical data of plants collected from the Baja California peninsula in Mexico, according to the ethno-pharmacological use.

Tabla 1. Datos etnobotánicos de las plantas recolectadas de la península de Baja California de México, según el uso farmacológico.

[1] Key letters in voucher were according to the reference use; antibiotic (AB), antifungal (AF), antiseptic (AS), parasite prevention (AP), other traditional uses (TU).

[1] Las letras de las claves de la ficha botánica son de acuerdo a la referencia de uso; antibiótico (AB), anti fúngico (AF), antiséptico (AS), prevención de parásitos (AP), otros usos tradicionales (TU).

Family/plant species	Common name/ native name	Voucher[1]	Ethno pharmacological uses
Anacardiaceae *Cyrtocarpa edulis* (Brandegee) Standley	Plum tree/ciruelo del monte	AS-218	Disinfection of gums
Aristolochiaceae *Aristolachia montícola* Brandegee	Dutchman´s pipe/hierba del indio	AP-269	Intestinal parasites, amoeba and skin infections
Asteraceae *Ambrosia ambrosoides* (Cav.) Payne	Canyon ragweed/chicura	AB-111	Antiseptic and antibiotic
Ambrosia ludoviciana subsp mexicana	Triangle leaf bursage or donkeybush/estafiate	AP-21	Amoebae and parasites, insecticide
Anaphalis margaritacea (L.) Benth.	Western pearly everlasting/ gordolobo	AP-17	Stomach parasites, antiseptic
Artemisa absinthium L.	Master herb or ajenjo macho/ prodigiosa	AS-134	Antiseptic, parasites and anthelmintic
Baccharis glutinosa Pers.	Douglas' false willow/ guatamote	AS-271	Antiseptic for infections in sores and wounds
Perityle californica Benth	Rock daisy/manzanilla del monte	AB-250	Preventive of venereal diseases
Haplopappus sonorensis (A. Gray) S.F. Blake	Haplopappus/hierba del pasmo	AS-46	Preventive for minor infections, antiseptic
Trixis californica var. peninsularis S.F. Blake	California trixis/hierba de Santa Lucia	AP-185	Stomach parasites
Hymenoclea monogyra Torr. & A. Gray.	Singlewhorl or burrobrush/ romerillo	AB-47	Tetanus disease
Bignonaceae *Crescencia alata* Kunth	Calabash or cirian/ Tecomate	AP-131	Parasites
Boraginaceae *Borago officinalis* L.	Borege or cooltankard tailwort/borraja	AB-345	Measles and smallpox
Heliotropium curassavicun (L.) var. oculatum (Heller)	Wild heliotrope/hierba del sapo, berro, María Luisa	AS-23	Antiseptic and to wash bounds
Euphorbiaceae *Acalypha comonduana* Mill.	Cooper leaf/hierba sanalotodo	AP-150	Parasites and antiseptic
Ricinus communis L.	Castor beans/higuerilla	AS-179	Antiseptic, antibiotic, acaricidal, insecticidal
Euphorbia nutans (Lag.)	Nodding spurge/ hierba golondrina	AP-38	Parasites
Jatropha cinérea (Ortega) Muell.-Arg.	Arizona nettlespurge/ lomboy	AS-27	Antiseptic
Nyctaginaceae *Mirabilis jalapa* L.	Common four o´clock/ maravilla	AS-52	Infected wounds
Pedaliaceae *Proboscidea althaefolia* (Roxb) Benth	Desert unicorn/espuela del diablo,	AS-161	For the treatment of infected wounds
Verbenaceae *Lippia palmeri* S. Watson	Wild or Mexican oregano/ oregano del monte	AF-44	Antimicrobial
Zygophyllacceae *Larrea tridentata* (Seesé & Moc. Ex D.C.)	Creosote bush or governor/ gobernadora, oamis	AS-501	Parasites, antiseptic

Sensi-disc diffusion (SDD) method

After preparing the PDA medium, *F. oxysporum* strains were incubated at 25 °C to allow mycelial development from seven to 10 days. Consequently, six-millimeter cylindrical sections (explants) were cut from the fungal-medium, and used as inoculum for testing. The explant containing the test microorganism was inoculated in the center of the previously prepared sterile agar bed (15 mL) and contained in the Petri dishes. Radially, four diffusion paper discs (0.5 cm in diameter) were placed on each agar plate previously impregnated with 1000 mg L^{-1} of each extract. Subsequently, the diameters of the inhibition zones were measured, and the inhibition percentage of radial growth was calculated. Growth inhibition was established in ranges with values from 0.1 to 10% (-), 11 to 20% (±), 21 to 40% (+), 41 to 80% (++) and values ≥ 81% (+++).

Agar dilution method (ADL)

The best treatments for SDD with an activity between 21 and 40% (++) in all the tests evaluated were tested by the ADL method to obtain ED_{50} and $ED_{90.}$ The doses were determined logarithmically based on 2, 4 and 8 (20, 40, 80, 200, 400, 800, 2000, 4000, 8000 and 20000 mg L^{-1}). The plates were inoculated at the center with 5 mm of fungal culture disc and then incubated at 28 ºC for seven days. The effectivity of the extracts was calculated as mycelial growth inhibition using the formula inhibition percentage (%) = [(control growth-sample growth) / (control growth) x 100]. As controls, 0.1% benomyl (1000 mg L^{-1}) was used according to the *in vitro* doses of systemic (250 to 1000 mg L^{-1}) and non-systemic fungicides (1000 to 2000 mg L^{-1})(29). Treatments using only ethanol and water (1 mg mL^{-1} each) were included in the controls. The treatments had four repetitions and were carried out in duplicate.

Spore germination inhibition

Based on the literature, spore germination inhibition studies were carried out by the ADL method using only the ethanol plant extracts (29). The stock solutions (S) were prepared as ethanolic extracts at a concentration of 5 mg mL $^{-1}$. Fungal spores (~500) from one-week-growing cultures, together with one drop of each plant extract at each concentration, were placed on a glass slide and incubated (25 ±2 °C) for 24 h. The spores were stained with cotton blue and mounted in lactophenol; 100 µL were placed in a Neubauer chamber (Celeromics, Grenoble, FR) and observed under a light microscope. Inhibition percentage was calculated with the formula: percentage of spore germination inhibition (SGI) = [(A-B) / A) x 100, where A = Number of germinated spores in the control-number of germinated spores in the treatment, B = number of germinated spores in control (42).

Statistical analysis

The dose-response analysis was performed with a generalized log-logistic model using R Statistical Computing of *drc* packages (40). To fit the dose-response model, ED_{50} was determined according to the three-parameter log-logistic function (LL.3) where the lowest limit was zero. Data were expressed as the mean (± SE). The lack-of-fit was determined by one-way analysis of variance (ANOVA). Tukey HSD test was used for multiple comparisons between treatments (extracts) with a previous evaluation of normality (Shapiro–Wilks test) and homogeneity of variance (Levene's test). Statistical significance in spore germination was analyzed by Student's t-test. All experiments were conducted in duplicate.

Results and discussion

Fungus identification and disease determination

The fungus, consistently isolated from the vascular tissue, was preliminarily confirmed as *F. oxysporum* based on the morphological characteristics of the mycelia and conidia grown in CLA medium. The characteristics that describe the species were observed, from the pale pink pigmentation of the mycelium to the observation of microconidia, macroconidia and globose chlamydospores (47). The pathogenic fungus was identified as *Fusarium oxysporum* f. sp. *basilici* based on the specific amplification of the expected 943 bp with Bik 1 and Bik 4 primers. No amplification was obtained from DNA extracted from healthy seedlings. In addition, the symptoms of vascular wilt, crown and root rot observed in the field were similar to those reported in other basil production systems in the world (17, 36, 37, 46).

In vitro screening of mycelial growth activity

Most of the aqueous-ethanol extracts tested in initial screening by SDD method showed an inhibition effectiveness similar to those reported with other filamentous fungi worldwide (7, 8, 11, 15, 18, 28, 30) with inhibition ranges between 1000 mg L^{-1} for mycelial growth (29, 42). Table 2 shows that all selected plants (24) showed at least moderate activity (21 to 40%) in mycelial growth. Among the most promising crude extracts with potential to be used in organic agriculture against *F. oxysporum* were *L. tridentata*, *H. monogyra* and *L. palmeri*, with mostly inhibitions from 41 (++) to ≥ 81% (+++) with water and ethanol solvents (1000 mg L^{-1}). The next most effective plants with values from 21 to 40% were *Aristolachea montícola*, *Cyrtocarpa edulis*, *Haplopappus sonorensis* and *Ambrosia ambrosoides.* The ethanol extracts of these selected plants significantly showed higher activity compared to aqueous extracts. Thus, ethanol and aqueous extracts with the highest activity were selected for further analysis of 50%-effective doses (ED_{50}) and spore inhibition tests.

Table 2. *In vitro* screening of mycelial radial growth and spore germination inhibition of the most effective extracts by the SDD method against *Fusarium oxysporum* f. sp. *basilici* after six days.

Tabla 2. Detección *in vitro* del crecimiento radial micelial y la inhibición en la germinación de esporas mediante el método SDD contra *Fusarium oxysporum* f. sp. *basilici* después de seis días.

Plant species	Range of inhibition (%)[1]		
	Radial growth mycelium [2]		Spores [3]
	Ethanol	Water	Ethanol
Larrea tridentata	++	++	+++
Hymenoclea monogyra	++	+	+++
Lippia palmeri	++	++	++
Trixis californica var. *peninsularis*	+	+/-	+++
Aristolachia montícola	+/-	+/-	+
Cyrtocarpa edulis	+/-	+/-	+/-
Haploppapus sonorensis	+	+/-	+
Ambrosia ambrosoides	+/-	+/-	+/-
Baccharis glutinosa	+/-	-	+
Crescencia alata	+/-	-	+/-
Artemisia absinthium	+	-	+/-
Heliotropium curassavicun	+/-	+/-	++
Ricinus communis	+/-	+/-	-
Euphorbia nutans	+/-	+/-	++
Jatropha cinérea	+/-	-	+
Mirabilis jalapa	+/-	-	+
Proboscidea althaefolia	-	+	++
Acalypha comonduana	-	+	+
Anaphalis margaritacea	+/-	+	-
Borago officinalis L.	+/-	+	-
Ambrosia ludoviciana (Cav.) Payne	+/-	+	-
Perityle californica Benth	+	+	-
Benomyl (1000 mg L^{-1})	+++	+++	+++
Control 1 (ethanol 70%)	-	-	-
Control 2 (water)	-	-	-

[1] The inhibition symbology (-, +) in the table was according the observed ranges: (-) ≤ 10% growth inhibition, (±) 11 to 20%, (+) 21 to 40%, (++) 41 to 80%, and (+++) ≥ 81% (n = 6). [2] All extracts for mycelial inhibition were extracted from the leaves at standard concentrations of 1000 mg L^{-1}. [3] Spore germination inhibition test was carried out at concentrations of 5 mg mL^{-1}.

[1] La simbología de inhibición en la tabla (-, +) es de acuerdo a los rangos observados: (-) ≤ 10% de inhibición del crecimiento, (±) 11 a 20%, (+) 21 a 40%, (++) 41 a 80% y (+++) ≥ 81% (n = 6). [2] Todos los extractos para la inhibición del micelio fueron extraídos de las hojas a concentraciones estándar de 1000 mg L^{-1}. [3] La prueba de inhibición de la geminación de esporas se realizó a una concentración de 5 mg mL^{-1}.

Spore germination inhibition analysis

At least 17 plant extracts showed some inhibitory effect from 11 to 20% at low doses of 5 mg mL^{-1}. (table 2). The best treatments for the inhibition of the fungus were *L. tridentata*, *H. monogyra* and *Trixis peninsularis.* They were the most effective with inhibition above 81% (+++). Similar response at 5 mg mL^{-1} has been observed in other ethanol extracts against the *in vitro F. oxysporum* test (6, 29). The extracts of *L. palmeri*, *Heliotropium curassavicun*, *Euphorbia nutans* and *Proboscidea althaefolia* showed an inhibition of sporulation in a range from 41 to 80%, although the effect on mycelial inhibition was moderate (11 to 20%). The tendency of progressive increase in sporulation inhibition with the increase of concentrations was also observed (table 2, page 239).

Effectiveness of dose response (ED_{50})

Model parameters of ED_{50} indicated 50% of effective biocontrol (dose-response). ED_{50} response of the most effective plant extracts by the ADL method were *L. tridentata, H. monogyra* and *L. palmeri* with values ranging from 220 to 3000 mg L^{-1} (table 3). The three tested extracts showed significant differences among the effective doses (one-way ANOVA, F = 136.6, $P < 0.001$). The PostHoc Tukey HSD test indicated that ED_{50} of the *L. tridentata* (ED_{50} = 220 mg L^{-1} and *H. monogyra* (ED_{50} = 303 mg L^{-1}) were more efficient to inhibit the fungus (figure 1A, page 241). The predictive trend of the three extract curve analysis, focusing on dose-response at the logarithmical concentration studied (10-10000 mg L^{-1}), were more pronounced in *L. tridentata* than *H. monogyra* and *L. palmeri* (figure 1B, page 241). However, no significant differences were observed between ED_{50} of *L. tridentata* and *H. monogyra* ($P > 0.05$). This result indicated that *L. tridentata* was 13.6 times more effective than *L. palmeri* and 1.3 times more than *H. monogyra.*

Table 3. Percentage of inhibition of the radial mycelial growth of *Fusarium oxysporum* f. sp. *basilici* of the extracts with the highest antimicrobial activity in the SDD tests, showing the effective dose (ED_{50}), logarithmical concentration and the fiducial limits

Tabla 3. Porcentaje de inhibición del crecimiento radial micelial de *Fusarium oxysporum* f. sp. *basilici* de los extractos con mayor actividad en las pruebas SDD, mostrando la dosis efectiva (ED_{50}), las concentraciones logarítmicas y los límites fiduciales.

Logarithmical concentration (mg L^{-1})	**Mycelial radial inhibition (%)[2]**		
	Lippia palmeri	*Larrea tridentata*	*Hymenoclea monogyra*
40	5.25± 0.4	33.91± 1.74	
60	10.36± .38		30.78±0.68
80	9.69± 0.86	42.81± 1.13	36.02±0.93
200	14± 0.23	52.66± 1	46.77±0.81
400	26.65± 0.81	65.16± 2.11	54.3±0.94
600	32.97± 0.59	73.28± 0.66	59.01±1.06
800	40.38± 0.36	78.25±0.9	63.98±0.51
2000	53.97± 3.01	87.81±0.37	73.12±1.09
4000	68.64± 3.52	100	79.03±1.69
6000	79.27± 0.45	100	84.54±0.41
8000	85.87± 0.61	100	84.11±1.07
ED_{50}[1]	3000.28±979.75	220.87±31.01	303.46±33.73
P	0.008	0.0022	0.0026

[1] ED50= effective dose which inhibits 50% of the population. [2] Fiducial limits of the standard error (±) adjusted to the ANOVA model (P > 0.05).

[1] ED50= dosis efectiva para inhibir el 50% de la población. [2] Limites fiduciales del error estándar (±) ajustado al modelo ANOVA (P > 0,05).

The ED_{50} of the BCS plant extracts were lower than those of other of similar reported species (*Origanum* spp.), extracted as essential oils, where ED_{50} was from 450 to 8000 mg L^{-1} (3). The literature has reported that the effectiveness *in vitro* of the aqueous-ethanol extracts of medicinal plants has a wide range from 100 to 5000 mg L^{-1} to inhibit fungi of different genus; *Aspergillus, Colletotrichum, Phytophthora, Penicillium, Botrytis, Alternaria* and *Fusarium* (15, 35). On the other hand, the formulation of botanical fungicides with good potential to inhibit a wide range of filamentous fungi ranges from 1000 to 2000 mg L^{-1} (44). In spore germination assays, the extracts of *L. tridentata, H. monogyra* and *T. peninsularis* were the most effective to inhibit spore germination at 5000 mg L^{-1} by more than 81%, followed by the extracts of *L. palmeri, H. curassavicun* and *P. althaefolia,* which presented values of 41 to 80% (table 2, page 239). The results of this study were in agreement with Obongoya *et al.* (2010) who showed the phytotoxic effect selecting crude extracts of some Mexican plants against soil-borne *F. oxysporum* f. sp. *phaseoli* ranging from 2.5 to 10 mg mL^{-1}.

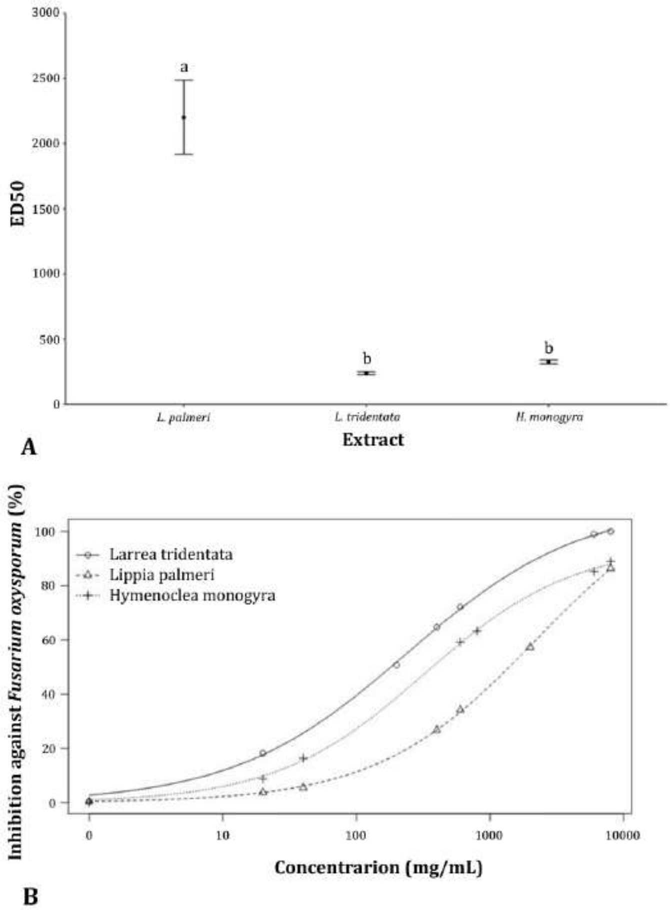

Figure 1. Dose-response of inhibition of ethanol-aqueous extracts of medicinal plants from BCS, Mexico with greater antimicrobial activity against *Fusarium oxysporum* f. sp. *basilici*. A.

Figura 1. Dosis-respuesta de inhibición de los extractos etanólicos-acuosos de plantas medicinales de BCS, México con mayor actividad antimicrobiana contra de *Fusarium oxysporum* f. sp. *basilici.* A.

Tukey's PostHoc test indicated that *H. monogyra* and *L. tridentata* are more effective (ED_{50} <500 mg L^{-1}) than *L. palmeri* (ED_{50} ~3000 mg L^{-1}). B. Trend line due to the effect of logarithmic doses (20, 40, 60, 80, 200, 400, 600, 800, 2000, 4000, 6000, 8000 mg L^{-1}), observing the most marked effect among the 100 and 1000 mg L^{-1}).

La prueba PostHoc de Tukey indicó que la *H. monogyra* y *L. tridentata* son más efectivos (ED_{50}<500 mg L^{-1}) que *L. palmeri* (ED_{50} ~3000 mg L^{-1}). B. Línea de tendencia por efecto de las dosis logarítmicas (20, 40, 60, 80, 200, 400, 600, 800, 2000, 4000, 6000, 8000 mg L^{-1}), observándose el efecto más marcado entre los 100 y 1000 mg L^{-1}).

This research showed that three species used in traditional medicine in BCS could potentially control fusarium disease. However, for this particular species (*F. oxysporum* f. sp. *basilici*), very few studies have documented the antifungal activity, and the most documented are especially related to antimicrobial activity with some bacteria of clinical interest (12, 26). By far, *L. tridentata* locally known as "gobernadora" (governor), creosote bush or chaparral, is one of the most documented species with inhibitory effects. For approximately half a century, *L. tridentata* has been the most studied plant in Mexico and the world and reported for the treatment of more than 50 diseases (2). The effect of this species as anti-parasitic, antiseptic and control of foot fungi and bacteria and treatment of kidney and gynecological infections are well documented (16). One of the most active ingredients, nordhydroguaiaretic acid (NDGA) and numerous lignans have been characterized. This species was found to be the most promising in this study based on values of 220 mg L^{-1} and values ≥ 81% to inhibit sporulation at 1000 mg L^{-1}. Oregano is one of the most interesting cases from its nomenclature, which can be referred to as Mexican or wild oregano (*L. palmeri, Verbenaceae* family) or in the world known as common oregano (*Origanum vulgare; Lamiaceae* family). The microbial properties of both species are well documented, and it appears to be an interesting promise because of its availability as an agricultural domesticated species (27, 32). In addition to their wide use in the food industry, active compounds, such as eugenol, carvacrol, carvone, p-cymene and thymol inhibit mycelial growth and sporulation of different types of *Fusarium* species in a variety of crops (13). The effectiveness of

L. palmeri as an aqueous-ethanol extract has been observed in other medicinal and culinary plants in the state of BCS, such as sage (*Salvia officinalis*) and where this type of extraction maximizes antifungal components (10). On the other hand, its multiple use as an export crop (41) could be interesting for the industry as essential oil or dry for the food industry or for the formulation of natural antifungal solutions for agricultural use (8). *Hymenoclea monogyra* seems to be the least documented; however, different species of the genus have shown antimicrobial properties due to the presence of flavonoids and sesquiterpene lactones. *H. monogyra* has been reported against enteropathogenic bacteria in BCS and in other regions of northwest Mexico (25). A diversity of active compounds can be extracted from medicinal plants using ethanol-water methods, which makes it an interesting model for the semi-pilot scale extractions, providing confidence for the optimization processes in the formulation of botanical fungicides.

Mexico is a country with an agricultural vocation where certified agriculture activities in the production of vegetables and aromatic herbs occur mainly in the states of Sinaloa, Sonora and BCS (23). The most important regions of the peninsula are mainly in Ensenada, which is the largest municipality with an organic agricultural vocation. Other producing areas in the southern part of the peninsula, such as Santo Domingo, Valle de Vizcaino, La Paz and San Jose del Cabo are known for the production of annual vegetables and for the certified organic production of aromatic herbs (14). Basil is the most commercialized gourmet herb species that generates a good economic performance (39). Although losses due to fusarium in this crop have not been estimated in BCS, the pressure of the disease and the lack of botanical fungicides for its control are a constant risk for production systems. This study has reported *F. oxysporum* f. sp. *basilici* associated with crown and root rot and wilt diseases in basil grown in BCS for the first time. Crude aqueous-ethanol extracts of L. tridentata, L. palmeri and H. monogyra, can efficiently inhibit mycelial growth and spore germination of F. oxysporum f. sp. basilici with potential use in phytosanitary management programs for organic basil production.

Conclusions

The *in vitro* effectiveness of *Fusarium*-growth inhibition (ED_{50} ranges from 220 to 3000 mg L^{-1}) and inhibition of sporulation (80% at doses of 5000 mg L^{-1}) of native medicinal plants (*L. tridentata*, *H. monogyra* and *L. palmeri*) from the Baja California peninsula, opens the possibility of using them as an ethanol-aqueous extract for long-term control of the fusarium disease caused by *F. oxysporum* f. sp. *basilici* in organic basil in BCS, Mexico and in other producing areas of the world.

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Arthropods of the Limarí River basin (Coquimbo Region, Chile): taxonomic composition in agricultural ecosystems

Artrópodos de la cuenca del Río Limarí (Región de Coquimbo, Chile): composición taxonómica en agroecosistemas

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Abstract

The Limarí valley, located in the Coquimbo Region of Chile, is an important agricultural area that is immersed in the transverse valleys of the Norte Chico. In recent decades, the continuous expansion of agriculture towards dry land zones has favored the migration and establishment of potential pests, such as arthropods, that may affect crops or be zoonotic agents. Based on the limited knowledge we have about the arthropod group present in the Limarí basin, our objective is to describe the taxonomic composition of the assemblage of economically important arthropods inhabiting this basin of the semiarid region of Chile. After reviewing historical data, specimen collections, and the specialized literature, a total of 414 arthropod species were recorded. Of the total number of species recorded, 92.5% were insects, the most diverse taxon, with 11 orders. Arachnids, in turn, were represented only by Acari with 31 species. The most widely represented orders of insects were Coleoptera, Hemiptera, and Lepidoptera. Within Coleoptera the most species-rich families were, in decreasing order of importance, Curculionidae, Coccinellidae, Cerambycidae, Scarabaeidae, Chrysomelidae (Bruchinae), Ptinidae, and Bostrichidae; within Hemiptera these were Aphididae, Diaspididae, Coccidae, Pseudococcidae, Pentatomidae and Rhopalidae; and within Lepidoptera they were Noctuidae and Tortricidae. We hope this study serves as a starting point for identifying the most diverse arthropod groups and developing pest monitoring and control programs.

Keywords
taxonomic structure • insects • Norte Chico • agricultural pests • transverse valleys • arid zones

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Resumen

El valle del río Limarí, situado en la Región de Coquimbo de Chile, es un importante foco de producción agrícola del Norte Chico. En las últimas décadas, la constante expansión de los cultivos agrícolas hacia zonas de secano ha permitido la migración y el establecimiento de potenciales plagas, como los artrópodos, que pueden afectar las producciones agrícolas o constituir agentes en la transmisión de zoonosis. Debido al escaso conocimiento que se tiene sobre el grupo de artrópodos presentes en la cuenca del Limarí, el objetivo del presente trabajo fue describir la composición taxonómica de los artrópodos de importancia económica presentes en esta cuenca del semiárido chileno. Mediante la revisión de bases de datos históricas, colecciones y literatura disponible, se registró un total de 414 especies de artrópodos. El 92,5% de las especies registradas fueron insectos, los que corresponden al grupo más diverso, con 11 órdenes, mientras que los arácnidos estuvieron representados solo por Acari con 31 especies. Los órdenes de insectos con mayor representación fueron Coleoptera, Hemiptera y Lepidoptera. Las familias con mayor riqueza fueron, en orden decreciente, las siguientes: dentro de Coleoptera, Curculionidae, Coccinellidae, Cerambycidae, Scarabaeidae, Chrysomelidae (Bruchinae), Ptinidae y Bostrichidae; dentro de Hemiptera, Aphididae, Diaspididae, Coccidae, Pseudococcidae, Pentatomidae y Rhopalidae; y dentro de Lepidoptera, Noctuidae y Tortricidae. Se espera que el presente estudio sirva como un catastro inicial para desarrollar programas de monitoreo y control de potenciales plagas.

Palabras clave
estructura taxonómica • insectos • Norte Chico • plagas agrícolas • valles transversales • zonas áridas

Introduction

In Chile, the Norte Chico region extends from 27° to 32° S and encompasses the administrative regions of Atacama and Coquimbo. The area is characterized by the presence of an intermediate depression interspersed with mountain ranges that give origin to transverse valleys that extend from the Andes to the Pacific Ocean (14). These valleys, namely the valleys of Copiapó (27° to 28° S) and Huasco (28° to 29° S) in the Atacama region and the valleys of Elqui (30° S), Limarí (31° S) and Choapa (32° S) in the Coquimbo region, form a semiarid matrix characterized by scarce and disperse rainfall and the presence of permanent, mixed-regime rivers (14).

Significant among the transverse valleys of semiarid Chile is the Limarí basin, considered to be an economically important food and agricultural area (22) with secondary production activities such as small-scale agriculture, cattle raising and small-scale mining (13). Nowadays the valley surface is covered by forage (25,456 ha), fruit (20,151 ha), grapevine (8,353 ha) and vegetable (4,753 ha) cultivation lands (22). Based on export volumes, the most economically important fruit crops include grapevines (7,321.7 ha), avocado trees (4,128.0 ha), olives (2,511.2 ha) and mandarin trees (1,573.4 ha), which together with other fruit crops account for 46.9% of the fruit tree species of the Norte Chico (12, 22).

The agri-food activity in the Limary valley is directly dependent on the irrigation water storage capacity provided by its three dams (*i.e.*, La Paloma Dam, 748 million m^3; Cogotí Dam, 150 million m^3; Recoleta Dam, 100 million m^3) (15). However, as a result of the introduction of novel irrigation techniques, cultivation lands have expanded from the river bank to slopes located at higher altitudes (4), that is, hill sides, that naturally host vegetation whose productivity varies greatly with the seasons and is highly dependent on climate phenomena (37). Once resources in the natural areas become scarce, the newly cultivated lands, which maintain stable vegetable productivity due to artificial irrigation, become favorable areas for the migration of potential pests (1, 2).

The establishment of large-scale cultivation systems in a semiarid basin translates into increased vegetable diversity and, consequently, greater availability of habitats and resources for both pests and natural enemies (3, 7). For this reason, taxonomical characterization of pest species, including arthropods, is key to get a better understanding of their nature and of the potential biological risks of cultivation systems, particularly agricultural ones (38), data which is essential for identifying biological and entomological vulnerabilities in this large agricultural area. Based on the limited knowledge we have about the arthropod group present in the Limarí basin, our objective is to describe the taxonomic composition of the assemblage of economically important arthropods inhabiting this basin of the semiarid region of Chile.

Materials and methods

Study area

The study area encompasses the basin of the Limarí River, including the coastal fringe extending north from the mouth of the Elqui River to the mouth of the Choapa River, following the limits of the water divide for the Limarí River basin, and the coastal fringe south of 31°W (figure 1); the corresponding limits were defined using 1:250.000 scale maps from the Instituto Geográfico Militar (29, 30). The predominant soil types in the area are entisols, aridisols, and inceptisols, all of which show some influence from the vegetation (26). The climate is steppe type, ranging from steppe with abundant clouds in the coast to cold steppe in mountainous areas (27).

The mean annual precipitation exceeds 300 mm in mountainous areas and reaches 60 mm to 240 mm in the lowlands near the coast (16).

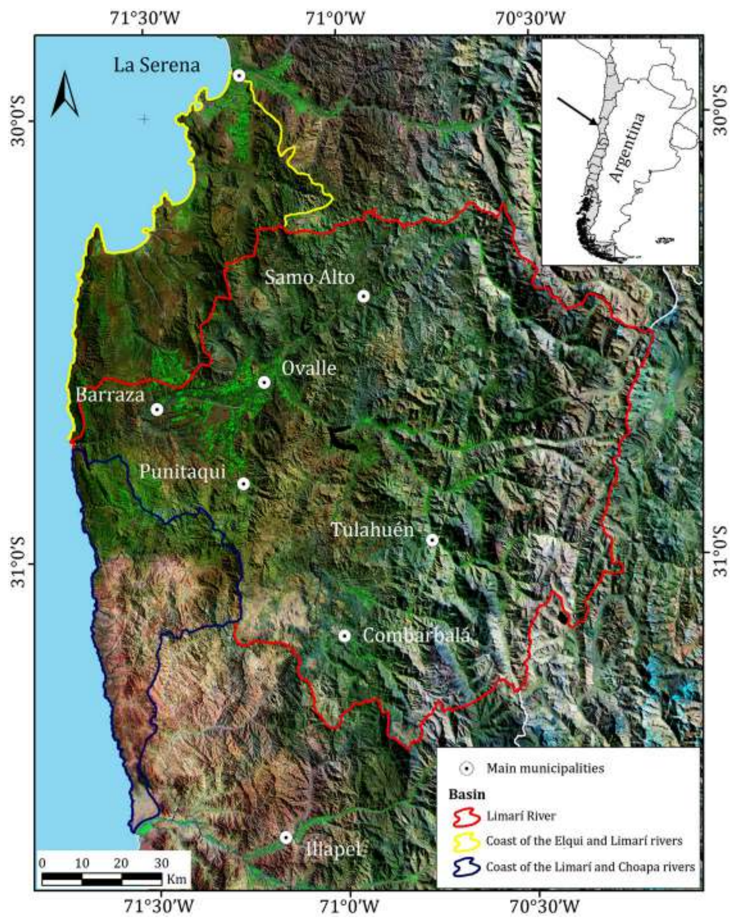

Figure 1. Geographical location of the Limarí basin and coastal basins surrounding. (Coquimbo Region, Chile).

Figura 1. Localización geográfica de la cuenca del Limarí y cuencas costeras circundantes. (Región de Coquimbo, Chile).

The annual temperature is homogeneous in the coast, but varies in the interior valleys and mountainous areas (16). Far from the influence of the sea, the vegetation in the interior areas corresponds to an interior steppe scrubland (19, 37).

Capture methods and data collection

The taxonomic characterization of economically important arthropods in the Limarí basin was based on distributional data obtained from reference material deposited in the following entomological collections: Juan Enrique Barriga's personal collection (JEBC); Laboratorio de Entomología Ecológica, Universidad de La Serena, La Serena, Chile (LEULS); and Museo Entomológico Luis Peña, Departamento de Sanidad Vegetal, Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago, Chile (MEUC). Additionally, the Servicio Agrícola y Ganadero de Chile (SAG) provided data from entomological prospections conducted between 2009 and 2015 in the Limarí Province. These records were supplemented with distributional data from the literature and data collected using insect-capturing devices, including nets, umbrellas, and fans, between June and October 2015. Additionally, farmers, stakeholders, community leaders, agricultural and orchard workers were interviewd. The information was checked and complemented with literature reviews. The captured material was cleaned, dried, and preserved in alcohol (70%) until processing and mounting as per Pizarro-Araya *et al.* (2019 a y b). All the collected material is deposited at the Laboratorio de Entomología Ecológica of Universidad de La Serena (LEULS).

Within Arachnida, Acari was taxonomically identified based on Krantz (1978) and Rojas (2000), whereas Araneae was characterized following Ramírez (1999) and the World Spider Catalog (2020). For Insecta, the taxonomic identification of Coleoptera followed Barriga (1990, 1993 y 2020), Elgueta & Marvaldi (2006), Vidal & Guerrero (2007), Solervicens (2014), Moore & Vidal (2015), González (2019); for Lepidoptera, Artigas (1994), Estay & Bruna (2002), Angulo *et al.* (2006); for Hemiptera, Ripa & Rodríguez (2000), Estay & Bruna (2002), Castro da Costa (2010); for Diptera, Estay & Bruna (2002); and for Hymenoptera, Rojas (2005).

Results

A total of 414 economically important arthropods species were recorded, 92.5% (383 species) of them insects (Insecta). Arachnids (Arachnida) were only represented by the order Acari (31 species). Insects were the most diverse group, with 11 orders, among which Coleoptera, Hemiptera, Lepidoptera and Hymenoptera had the highest number of taxa (table 1 and figure 2, page 249). The taxonomic composition (genus/species) for these latter groups is as follows: for Coleoptera, the main taxa were Curculionidae (15/21), Coccinelidae (14/21), Chrysomelidae: Bruchinae (7/13), and Scarabaeidae (9/11) (figure 2a, page 249); for Hemiptera, these were Aphididae (17/29), Diaspididae (11/17), Pseudococcidae (4/10), and Pentatomidae (7/8) (figure 2b, page 249); for Hymenoptera, these were Vespidae (3/5) and Pompilidae (1/3) (figure 2c, page 249); and for Lepidoptera, these were Noctuidae (11/14), Tortricidae (4/6), Pieridae (3/4), Gelechidae (3/3), and Hesperiidae (3/3) (figure 2d, page 249).

According to the economic importance given for each of the species analyzed in this study, a large percentage of phytophagous species (59.9%, table 2, page 250), mainly belonging to Acari, Lepidoptera, Hemiptera and Coleoptera, were registered in the basin. Some families of agricultural importance, such as Aleyrodidae, Aphididae, Coccidae, Diaspididae, Margarodidae, and Pseudococcidae (Hemiptera) (figure 2b, page 249), were observed in large agricultural crops in the basin (*e.g.*, vines, oranges, mandarins, lemon trees, avocado trees, walnuts, olive trees, vegetable crops). These organisms can cause direct damage to the crops, since the different stages (adults, nymphs and/or larvae) can feed on both vegetative and fruitful organs of the plants. A smaller but no less important fraction corresponded to the group of predators (14.7%) and parasitoids (2.2%), mainly represented by Coleoptera (*i.e.*, Coccinellidae), Neuroptera (*i.e.*, Chrysopidae) and Hymenoptera (*i.e.*, Braconidae, Encyrtidae, Ichneumonidae, Platygastridae, Signiphoridae) (table 2, page 250).

Table 1. Taxonomic composition (families, genera, and species) of economically-important arthropod orders in the Limarí basin (Coquimbo Region, Chile).

Tabla 1. Composición taxonómica (familias, géneros y especies) de órdenes de artrópodos de importancia económica de la cuenca del Limarí (Región de Coquimbo, Chile).

Order	Families	Genera	Species
Acari	**9**	**19**	**31**
Coleoptera	**26**	**95**	**139**
Dermaptera	**2**	**3**	**3**
Diptera	**8**	**14**	**15**
Hemiptera	**23**	**82**	**125**
Hymenoptera	**14**	**21**	**25**
Lepidoptera	**17**	**38**	**45**
Neuroptera	**2**	**5**	**5**
Orthoptera	**4**	**6**	**8**
Phasmatodea	**1**	**1**	**1**
Psocoptera	**2**	**2**	**2**
Thysanoptera	**3**	**11**	**15**
Total	**111**	**297**	**414**

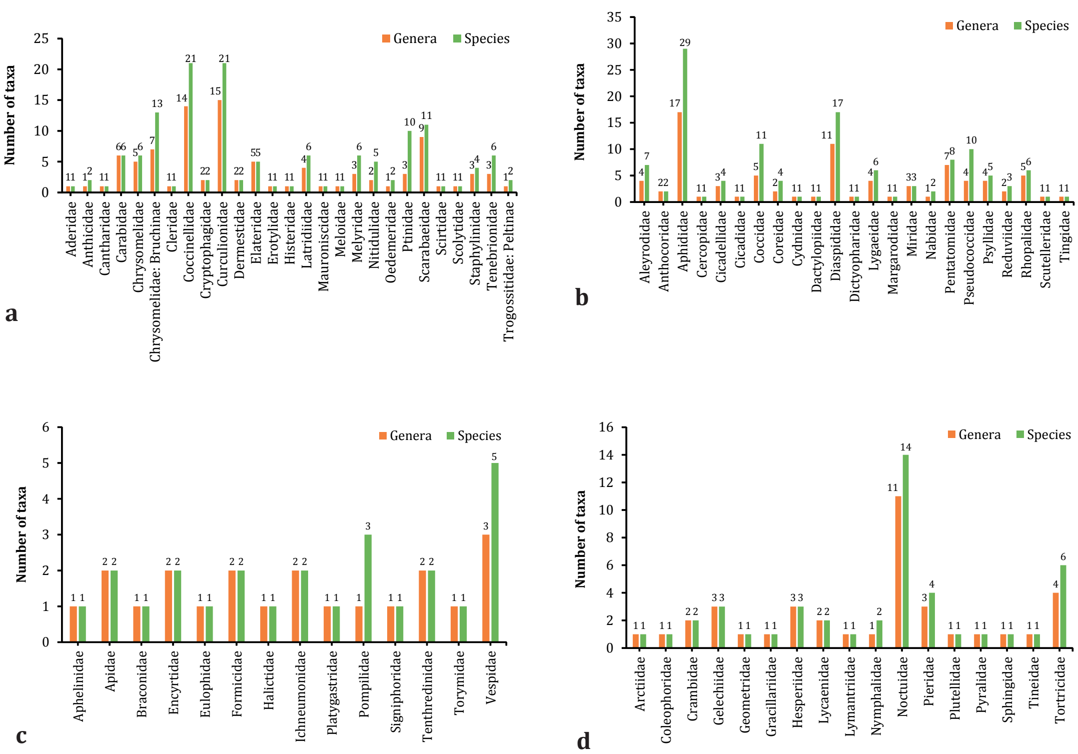

Figure 2. Taxonomic composition (genera and species) of economically-important arthropod orders in the Limarí basin (Coquimbo Region, Chile). a) Coleoptera; b) Hemiptera; c) Hymenoptera; d) Lepidoptera.

Figura 2. Composición taxonómica (géneros y especies) para los órdenes de artrópodos de importancia económica de la cuenca del Limarí (Región de Coquimbo, Chile). a) Coleoptera; b) Hemiptera; c) Hymenoptera; d) Lepidoptera.

Table 2. Economic importance of the different arthropod species of the Limarí river basin (Coquimbo Region, Chile).

Table 2. Importancia económica de las diferentes especies de artrópodos de la cuenca del Limarí (Región de Coquimbo, Chile).

Economic importance	Species	Percentage
Anthophagous	1	0.2
Attack stored products	24	5.8
Carpophagus	1	0.2
Comercial	2	0.5
Detritivorous	1	0.2
Hematophagous	2	0.5
Mycophago	5	1.2
Omnivorous	2	0.5
Parasitoid	9	2.2
Phytophagous	248	59.9
Pollinator	17	4.1
Predator	61	14.7
Quarantine pest	19	4.6
Saprophagus	8	1.9
Spermophagus	14	3.4
Total	414	100

Some species may constitute quarantine pests for the main markets that import Chilean products. In this study we recorded approximately 19 elements (table 2) that may correspond to species, genera and/or families of quarantine importance for both the US and the European Union. Among these species, insects such as *Lobesia botrana* (Denis & Schiffermuller) (Lepidoptera: Tortricidae), *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae), *Aleurothrixus floccosus* Maskell, *Aleurothrixus porteri* Quaintance & Baker and *Aleurothrixus* sp. (Hemiptera: Aleyrodidae), these last three correspond to whitefly in citrus, cucurbitaceae and solanaceae.

Other groups of quarantine importance widely registered in the basin were Pseudococcidae (Hemiptera), with the species *Pseudococcus calceolariae* (Maskell), *P. longispinus* (Targioni & Tozzetti), *P. meridionalis* (Prado), *P. viburni* (Signoret) and the native mite phytophagus *Brevipalpus chilensis* Baker (Acari: Tenuipalpidae).

Discussion

One of the first studies to document potential pests in a valley of Chile's Norte Chico was Pizarro-Araya *et al.* (2009), who recorded 181 arthropod species (spiders and insects) in three localities of the Elqui valley. Based on a review of historical data, specimen collections, literature data, and entomological captures, Pizarro-Araya *et al.* (2019a) examined the spatial distribution of economically important arthropods in the Limarí basin (Coquimbo Region, Chile), recording 5,418 arthropod specimens, most of them insects, particularly homopterans and coleopterans. The richness and spatial records were mostly concentrated between the city of Ovalle and the estuary of Punitaqui - the areas with most intense agricultural activity. Also, Pizarro-Araya *et al.* (2019b) examined the richness of arthropods of forestry and medical-veterinary importance in the Limarí valley, recording 10 families, 39 genera and 51 arthropod species of forestry importance; the xylophagous species of quarantine importance for the US included *Cryptotermes brevis* (Kalotermitidae), *Neoterius mystax* (Bostrichidae), *Tyndaris planata* (Buprestidae), *Rhyephenes humeralis* (Curculionidae) and *Phoracantha recurva* (Cerambycidae). Taxa of medical-veterinary importance were represented by 18 species, 14 genera, and 11 families, 9 of them arachnids and 9 insects. The most important arachnid genera were *Loxosceles* (Sicariidae), *Latrodectus, Steatoda* (Theridiidae) and *Rhipicephalus* (Ixodidae), whereas within insects the most important ones were *Triatoma* and *Mepraia* (Reduviidae). Some species identified in this study corresponded to predators and parasitoids, both groups considered natural enemies of certain pests. These species can help control and reduce pest populations in agricultural crops (20, 32).

At present, the Servicio Agrícola y Ganadero (SAG) has under official control six agricultural pests according to the impacts they generate both in international markets and in the environment and biodiversity; these are *Lobesia botrana, Bagrada hilaris, Halymorpha halys* Stal, *Phyllocnistis citrella* Stainton, *Homalodisca vitripennis* (Germar) and *Dactylopis coccus* (Costa) (35), of which the first two have been registered in the Limarí basin.

As of now, a total of 385 insect species and 24 mite species considered potential agricultural pests have been documented for Chile (6, 23, 33). In spite of these works, continuous update to these taxonomic inventories is required to identify the inlet pressure and pest establishment in this transverse valley of agricultural and productive importance. We hope that this taxonomic report may be useful for monitoring and controlling pests in newly established crops in a highly modified basin.

Conclusions

The economically important arthropods in the Limarí basin included mostly insects (orders Coleoptera, Hemiptera, Lepidoptera and Hymenoptera) and, to a lesser extent, arachnids, the latter represented only by the order Acari. The majority of the species registered in the basin were phytophagous groups (table 2, page 250) that make up a large assemblage of arthropods specialized in and adapted to agricultural crops (*e.g.*, fruit and vegetables crops) of great importance in the Limarí. The total richness recorded in the study area was higher compared to the richness documented for the Elqui basin, probably due to differences in the collection methods used. These data may be complemented with future studies incorporating detailed information on the major economically important groups per crop or sub-basin for a more realistic analysis of the richness level observed in the Limarí basin.

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Development and validation of diagrammatic scales to evaluate damage by the two-spotted spider mite (*Tetranychus urticae*) in peanut

Desarrollo y validación de escalas diagramáticas para evaluar el daño por arañuela roja (*Tetranychus urticae*) en maní

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Abstract

Argentina is the second largest peanut (*Arachis hypogaea* L.) exporter in the world. The main peanut pest in our country is the two-spotted spider mite (*Tetranychus urticae* Koch 1836). To date, there is no validated method to quantify this pest. The aim of this work was to develop and validate a logarithmic diagrammatic scale to assess damage by the two-spotted spider mite in peanut. In 2015-16, 200 leaflets were collected from a plot infested with the pest. Damaged leaf area (DLA) was calculated using SisCob software. Six-, seven-, eight- and nine-class scales were proposed. Mean values for each class and scale were obtained with 2-LOG. Leaflets were analyzed by 13 raters who used the four proposed scales. Precision and accuracy were determined by simple linear regression between the DLA and estimated damaged leaf area. Reproducibility was determined by linear regression between estimates of raters combined in pairs. The seven-class scale was the best validated one for all the parameters. Most raters showed constant deviations and overestimated DLA, whereas only one rater presented systematic deviations. This seven-class scale is the first developed and validated one to evaluate two-spotted spider mite damage to peanut in Argentina.

Keywords
diagrammatic scale • *Tetranychus urticae* • peanut

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Resumen

Argentina es el segundo exportador mundial de maní (*Arachis hypogaea* L.). La principal plaga del cultivo en nuestro país es la arañuela roja (*Tetranychus urticae* Koch 1836) no existiendo un método validado para su cuantificación, planteándose como objetivo desarrollar y validar una escala logarítmica diagramática de daño para *T. urticae* en maní. En 2015/16, en un lote con presencia de la plaga, se colectaron 200 folíolos, a los que se les calculó el área foliar dañada (AFD) a través del software SisCob. Se plantearon escalas de seis, siete, ocho y nueve clases obteniendo el valor medio de cada clase con el programa 2-LOG. Los folíolos fueron analizados por 13 evaluadores con el uso de las cuatro escalas. La validación por precisión y exactitud se realizó por regresión lineal entre el AFD y la estimada, y la reproducibilidad por regresión lineal entre las estimaciones de los evaluadores combinados de a pares. La escala de siete clases fue la mejor validada por todos los parámetros. La mayoría de los evaluadores presentaron desvíos constantes; mientras que solo un evaluador presentó desvíos sistemáticos. Esta escala de siete clases es la primera desarrollada y validada para evaluar el daño por arañuela roja en maní en Argentina.

Palabras clave

escala diagramática • *Tetranychus urticae* • maní

Introduction

World production of peanut (*Arachis hypogaea* L.) is expanding, with more than 40 million t of in-shell peanut and 6 million tons of oil having been produced in the last decade. Argentina is one of the main peanut exporter countries worldwide; more than 90% of the area of Córdoba province is cultivated with peanut, and almost all the processing industry is concentrated in that province (1).

In Argentina, peanut crop is affected by a low number of arthropod pests; however, under certain climatic conditions, especially in years of low precipitations, the two-spotted spider mite *Tetranychus urticae* Koch 1836 becomes an important concern in several peanut plots (6). Under prolonged dry and hot weather, this pest exhibits fast development and overlapping generations, reaching important infestation levels over a short period (23).

The two-spotted spider mite causes damage directly and indirectly to peanut: by ingesting leaf cell contents with needle-like mouthparts, producing white or yellow specks called stipples, and through the weaving of webs, which contribute to the reduction of the crop photosynthetic process, respectively (6, 35).

In decision-making in pest control, it is necessary to generate an economic injury level (EIL). Although some EIL has been calculated based on the number of individuals per leaf or leaflet (32), it is important to have an objective tool for the quantification of the damage caused by it. One of the best ways to evaluate injury by a pest or disease affecting the leaf area is using a scale that represents the damage that has being caused by that pest or disease (29, 33).

Diagrammatic scales are useful for monitoring pests and diseases, supporting decision making about chemical control, assessing cultivar behavior, developing disease progress curves, and evaluating management practices (3, 4, 8, 38).

In Argentina, scales have been developed to quantify foliar diseases in peanut (19, 31), but only one scale quantifies damage caused by insect pests in this crop (10). Scales that evaluate the spider mite in other crops have been reported (13, 36), but none evaluates damage caused by two-spotted spider mite to peanut, the main pest damaging this crop in Argentina. For this reason, the aim of this work was to develop and validate a logarithmic diagrammatic scale of damage by the two-spotted spider mite in peanut.

Materials and methods

The study was conducted in a plot heavily infested with the two-spotted spider mite, located in Río Cuarto department, south of Córdoba province, Argentina, (33º 02´ 32.33" S, 63º 30´ 14,02"W), during the 2015-2016 crop season. Two hundred leaflets were collected with the aim of having a representative sample of the different degrees of damage. Each leaflet was photographed; photographs were processed using the software SisCob v1.0 (18), which measures the healthy leaf area (HLA) and the damaged leaf area (DLA). Percentage of damage of each leaflet was calculated based on those measurements. In addition, images with a schematic representation of each leaflet were generated.

Scales of different numbers of damage classes (six, seven, eight and nine) were proposed, considering that these numbers of classes were suitable for estimating disease damage and consequent losses (2, 15, 37, 38, 39). Considering the maximum real value of DLA obtained by the software and the number of classes selected to develop the scales, the intervals of each class were defined using the program 2-LOG v1.0 for Windows® (30). This program calculates the values for the upper limit (UL), lower limit (LL) and the midpoint (MP) of each class of the proposed scales.

Diagrammatic scales were performed using the images generated with the software SisCob v1.0, selecting the leaflet that represented the average DLA value for each of the classes. Image digitalization allows a rapid sample processing and enhances the capacity to discriminate between healthy and damaged area (4, 37).

Scales were validated using the field-collected leaflets, which were analyzed by 13 raters. Each leaflet was evaluated using the four scales and was assigned to a class, then the midpoint value corresponding to that class was assigned to each leaflet. Besides being of easy and rapid application, a scale needs to be validated in terms of precision, accuracy and reproducibility (8, 20, 25, 38). Accuracy and precision of visual estimation of each rater, for the different scales proposed, were determined via a linear regression analysis considering the actual damage data as independent variable and the estimated damage data as dependent variable, using InfoStat statistical software (12).

The accuracy of each rater's estimates was determined via the "t" test applied to the intercept of the regression line *a*, to confirm the hypothesis H0: a=0, and to the slope of the regression line *b*, to confirm the hypothesis H0: b=1, at 5% probability. Values of *a* different from 0 indicate constant deviations, and *b* values different from 1 indicate systematic errors in the evaluations. The precision of evaluations was estimated using the R^2 coefficient of determination and absolute error distribution (estimated damage minus actual damage) (24, 37). Reproducibility, which indicates if the scale can be efficiently applied by other raters, was determined using the R^2 coefficient of determination obtained from the linear regressions between damage values estimated by the different raters combined in pairs (9, 13, 28).

Results and discussion

The sampled leaflets exhibited between 0 and 96.1% of leaf area damaged by the two-spotted spider mite; the proposed scales (six-, seven-, eight- and nine-class scales) (table 1, page 257) were developed based on those values. The maximum DLA value recorded is usually found in the field (7, 21).

Precision was high for all the scales with R^2 values ranging between 0.78 and 0.96 (table 2, page 257). A value above 0.75 is considered appropriate for validation in terms of precision (15, 25, 26, 28). In the seven-class scale, the R^2 value ranged between 0.82 and 0.90; this value was higher than 0.80 for all the raters, which confers high precision to leaf damage estimation. Values higher than 80% are indicated in scales considered of high precision (9, 16, 22). Accuracy, determined by the proximity between a mean estimate and the actual value, was higher for the seven-class scale, considering both *a* and *b* parameters (table 2, page 257).

The analysis of the value *a* for the six-, seven-, eight- and nine-class scales showed that 12, 10, 11, and 9 raters, respectively, obtained values different from 0. Regarding the *b* value, only one of the 13 raters obtained a value significantly different from 1 for the six-, seven-, and eight-class scales. By contrast, for the nine-class scale, seven raters found a value of *b* significantly different from 1 (table 2).

Values of *a* obtained by the linear regression between the actual damage and the damage estimated using the seven-class scale varied from -1.01 to 10.39; in turn, 12 of the 13 raters had constant deviations and tendency to overestimate, which agrees with previous works that validated severity scales for leaf diseases (2, 9, 14, 15, 22).

Table 1. Upper limit (UL), lower limit (LL) and midpoint (MP) of each class of the six-, seven-, eight- and nine-class scales.

Tabla 1. Límite superior (LS), inferior (LI) y punto medio (PM) de cada clase, para las escalas de seis, siete, ocho y nueve clases.

Scale	Class	Lower limit (%)	Midpoint (%)	Upper limit (%)	Scale	Class	Lower limit (%)	Midpoint (%)	Upper limit (%)
Six-Class	1	0	0	0	Seven-Class	1	0	0	0
	2	0	3.64	6.95		2	0	2.94	5.03
	3	6.95	13.27	25.32		3	5.03	8.62	14.78
	4	25.32	48.32	72.89		4	14.78	25.32	43.39
	5	72.89	85.80	92.56		5	43.39	66.37	80.38
	6	92.56	96.10			6	80.38	88.55	93.32
						7	93.32	96.10	
Eight-Class	1	0	0	0	Nine-Class	1	0	0	0
	2	0	2.52	3.99		2	0	2.24	3.36
	3	3.99	6.34	10.06		3	3.36	5.03	7.54
	4	10.06	15.96	25.32		4	7.54	11.29	16.90
	5	25.32	40.17	60.78		5	16.90	25.32	37.92
	6	60.78	75.28	84.42		6	37.92	55.98	70.61
	7	84.42	90.18	93.81		7	70.61	80.38	86.90
	8	93.81	96.10			8	86.90	91.25	94.16
						9	94.16	96.10	

Table 2. Intercept *a*, slope *b* and determination coefficient R^2 of linear regression between actual and estimated values of damage caused by the two-spotted spider mite to peanut crop, for 13 raters with the use of six-, seven-, eight- and nine-class scales.

Tabla 2. Ordenada al origen *a*, pendiente *b* y coeficientes de determinación R^2 de la regresión lineal entre valores de daño real y valores de daño estimado de arañuela roja en el cultivo de maní, para 13 evaluadores con la utilización de las escalas de seis, siete, ocho y nueve clases.

Class	6			7			8			9		
Rater	*a*	*b*	R^2	*a*	*b*	R^2	*a*	*b*	R^2	*a*	*b*	R^2
1	3.17*	1.04	0.87	3.48*	1.01	0.88	2.74*	1.01	0.90	1.90*	1.03*	0.94
2	7.45*	0.96	0.83	8.97*	0.91*	0.83	6.55*	0.96	0.86	8.01*	0.98*	0.90
3	3.24*	1.06*	0.87	2.74*	1.04	0.88	2.21*	1.02	0.89	1.37	1.07*	0.93
4	9.43*	1.00	0.78	10.39*	0.98	0.82	7.77*	1.01	0.84	9.45*	1.05	0.88
5	3.62*	1.00	0.88	0.40	0.98	0.90	1.59*	0.96	0.90	0.56	0.98	0.85
6	8.02*	0.99	0.85	4.11*	0.95	0.89	4.04*	0.94*	0.89	2.14*	0.96	0.88
7	6.67*	1.01	0.85	3.89*	0.97	0.89	5.33*	0.95	0.88	4.07*	1.02	0.94
8	4.50*	1.05	0.87	1.15	1.03	0.89	2.26*	1.02	0.90	1.62*	1.05*	0.94
9	7.55*	0.99	0.82	5.47*	0.95	0.82	3.00*	0.98	0.86	0.80	1.01*	0.95
10	-0.39	1.05	0.88	-1.01	1.03	0.90	-1.28	1.04	0.90	-2.06*	1.05*	0.96
11	2.61*	1.04	0.88	4.30*	1.02	0.88	3.50*	1.03	0.88	3.18*	1.06*	0.95
12	5.42*	0.99	0.84	7.26*	0.96	0.84	4.72*	0.99	0.87	4.57*	1.01	0.94
13	3.82*	1.03	0.86	1.75*	1.02	0.90	1.51	1.02	0.89	0.06	1.05*	0.96

* Indicates that the null hypotheses (H0: a = 0 - H0: b = 1) were rejected by the T test: p≤0.05.

* Representa situaciones donde la hipótesis nula (H_0: *a*=0 – H_0: b=1) fueron rechazadas por el test T: p≤0,05.

In the seven-class scale, the value of *b* ranged between 0.91 and 1.04, with a mean of 0.99, and only one rater showed a tendency to make systematic errors. The narrow range of *b* and its mean value similar to 1 indicate that the seven-class scale is highly accurate, with similar values to those reported in the validation of other scales (5, 10, 13, 17, 38).

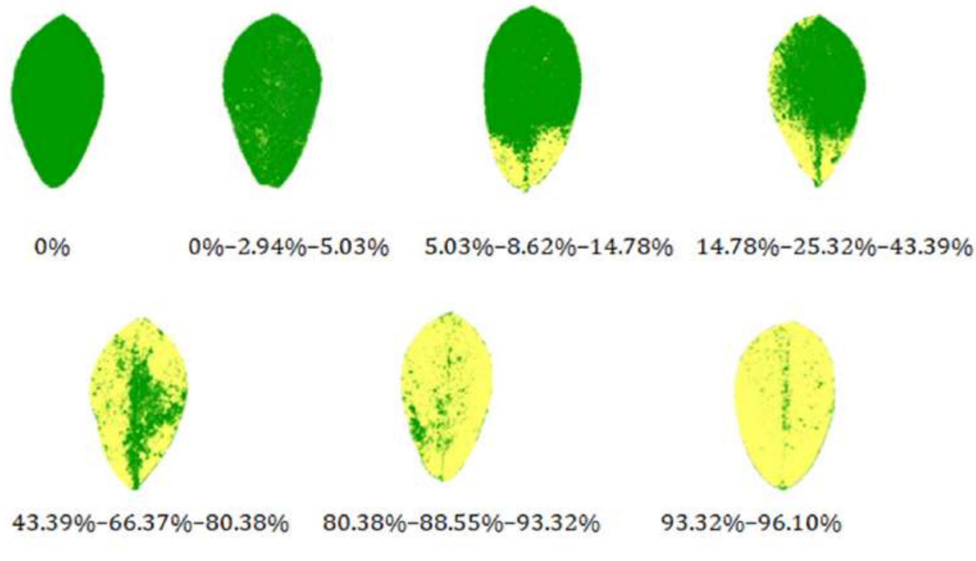
According to Sherwood *et al*. (1983) and Godoy *et al*. (2006), leaves with similar severity but different number of lesions generate a tendency to overestimate the disease, mainly when the number of lesions is very high and leaf size is small. This tendency was not observed in the validation of the present scale, despite the damage caused by the two-spotted spider mites (35).

The number of classes of the best validated scale (seven classes) is appropriate, considering that the number should not be too small, since it would have low resolution power, or too high, since it would be difficult to decide on the most appropriate class, as indicated by Campbell and Madden (1990). Overall, most diagrammatic scales range between five and eight classes (15, 37, 38, 39).

Figure 1 shows the seven-class diagrammatic scale generated from the leaflet images, indicating the upper and lower limits and the midpoint values for each class.

Figure 1. Seven-class diagrammatic scale of damage by two-spotted spider mite to peanut elaborated with software images indicating the lower limit, middle point and upper limit for each class.

Figura 1. Escala diagramática de siete clases de daño por arañuela roja en maní confeccionada con las imágenes generadas por el programa, detallando para cada clase el límite inferior, punto medio y límite superior.



Absolute errors or residuals, determined as the difference between visually estimated damage and actual damage, for the 13 raters are presented in figure 2 (page 259). Residuals are randomly dispersed around the prediction line, indicating an appropriate fit of the model (8). Most of the absolute errors of the 13 raters were about 10%, a value considered adequate for validation of diagrammatic scales for pests and diseases by several authors (2, 5, 9, 14, 27).

The comparison of the estimations of damage among rater pairs, using the seven-class scale, showed R^2 values ranging between 0.78 and 0.96 (table 3, page 259). Of all the crossed correlations among raters, 100% yielded R^2 values higher than 75%, which confirms reproducibility of evaluations using this scale. Similar reproducibility percentages were repeatedly reported with the use of diagrammatic scales (2, 5, 9, 11, 22, 26).

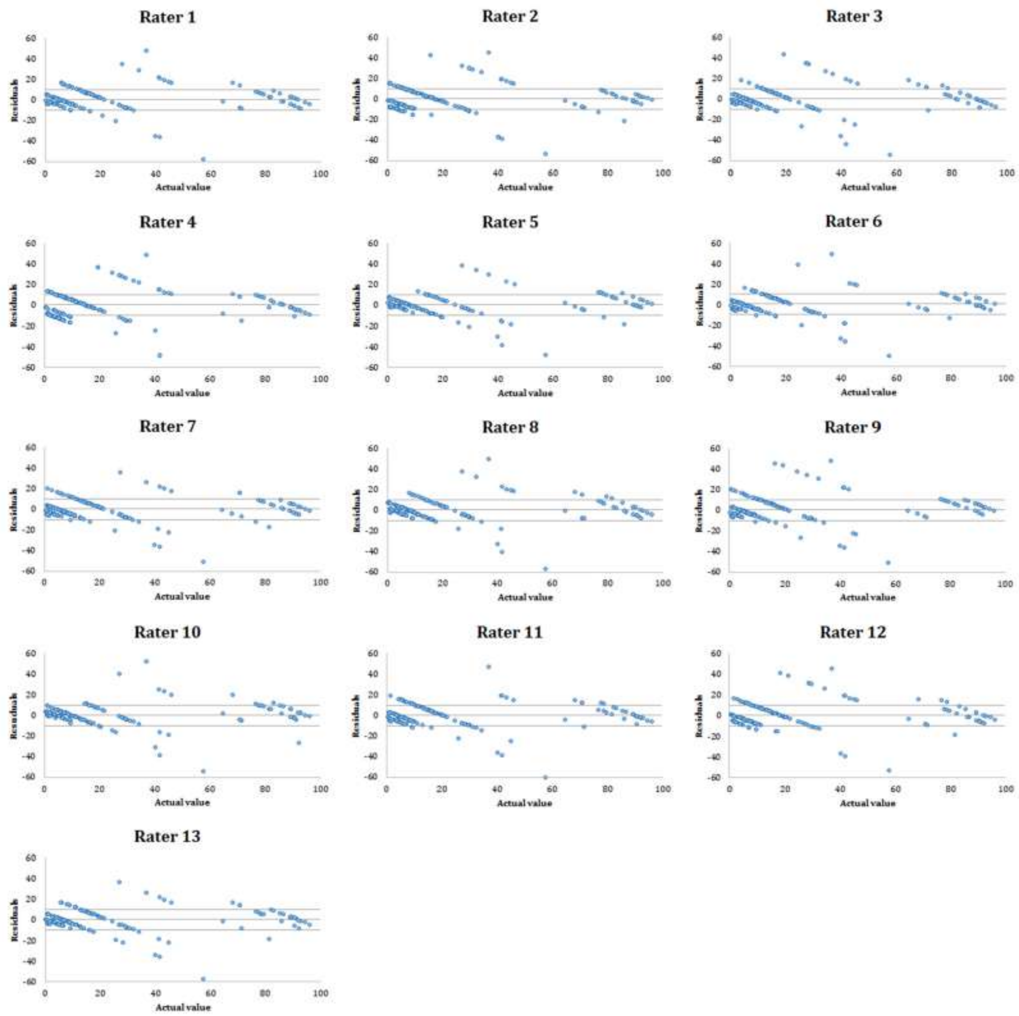


Figure 2. Residues distribution (estimated damage-actual damage) based on actual damage, for the 13 raters, obtained with the use of the seven-class scale of damage by the two-spotted spider mite to peanut.

Figura 2. Distribución de los residuos (daño estimado-daño real) en función del daño real, de los 13 evaluadores, obtenidos con el uso de la escala de siete clases para arañuela roja en maní.

Table 3. R^2 determination coefficients obtained from linear regression analyses between estimated damage values by 13 raters, combined in pairs.

Tabla 3. Coeficientes de determinación R^2 obtenidos de los análisis de regresión lineal entre los valores de daño estimados por los 13 evaluadores, combinados de a pares.

Rater	2	3	4	5	6	7	8	9	10	11	12	13
1	0.86	0.91	0.87	0.88	0.90	0.90	0.91	0.84	0.91	0.92	0.88	0.90
2		0.83	0.86	0.82	0.85	0.83	0.84	0.78	0.85	0.87	0.87	0.84
3			0.88	0.90	0.88	0.91	0.90	0.83	0.90	0.89	0.84	0.92
4				0.85	0.85	0.84	0.87	0.82	0.86	0.85	0.84	0.85
5					0.90	0.92	0.94	0.84	0.95	0.89	0.84	0.92
6						0.90	0.91	0.84	0.90	0.91	0.87	0.90
7							0.92	0.82	0.92	0.93	0.85	0.96
8								0.82	0.93	0.90	0.86	0.93
9									0.84	0.85	0.79	0.82
10										0.92	0.87	0.93
11											0.87	0.93
12												0.86

Conclusions

According to the validation results, the seven-class diagrammatic scale was the best one in terms of accuracy, precision and reproducibility with values above 0.80, 0.75 and 0.75 respectively.

This is the first scale developed and validated for evaluating damage by the two-spotted spider mite in peanut crop in Argentina. With this scale a loss function will be calculate which will allow the estimation of the EIL of this pest, fundamental parameters for integrated pest management.

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Salmea scandens (Asteraceae) extracts inhibit *Fusarium oxysporum* and *Alternaria solani* in tomato (*Solanum lycopersicum* L.)

Extractos de *Salmea scandens* (Asteraceae) inhiben el crecimiento de *Fusarium oxysporum* y *Alternaria solani,* patógenos del tomate (*Solanum lycopersicum* L.)

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Abstract

Phytopathogenic fungi from *Fusarium* and *Alternaria* genders affect tomato crops, reducing fruit production and quality. Some plant extracts may constitute an alternative for fungal control. In this regard, this research studied the antifungal effect of *Salmea scandens* extracts against *F. oxysporum* and *A. solani.* The functional groups of the chemical compounds involved in fungal control, were identified. Plant extracts were obtained by three techniques (soxhlet, ultrasound assisted and maceration) using three solvents (water, acetone and ethyl ether). Their biological effectiveness was evaluated against *F. oxysporum* in treated medium, and *A. solani* in tomato fruit. while the functional antifungal groups were identified by the Fourier Transform Infrared Spectroscopy (FTIR) technique. The soxhlet technique resulted the best extraction method for *S. scandens*, using the three solvents. Maceration-acetone extracts at concentrations of 4000 and 5000 ppm showed greater antifungal activity against both phytopathogens. The FTIR analysis confirmed the presence of carboxylic acids, aldehydes, ketones and aromatic compounds in *S. scandens* extracts, constituting probable responsible compounds for antifungal activity. *Salmea scandens* extracts resulted an efficient preventive treatment for *F. oxysporum* and *A. solani*.

Keywords
antifungal activity • FTIR spectroscopy • post-harvest fungi • soxhlet • solvents

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Resumen

Hongos de los géneros *Fusarium* y *Alternaria* afectan significativamente el cultivo de tomate, reduciendo la producción y calidad de los frutos. Los extractos vegetales son una alternativa para el control de hongos fitopatógenos, por lo que, en este trabajo, se evaluó el efecto antifúngico de los extractos de *Salmea scandens* contra *F. oxysporum* y *A. solani* y se identificaron los grupos funcionales de los compuestos químicos. Los extractos se obtuvieron por tres técnicas (soxhlet, ultrasonido asistido y maceración) utilizando tres solventes (agua, acetona y éter etílico). La efectividad biológica de estos extractos, se evaluó contra *F. oxysporum* en medio PDA suplementado con el extracto y *A. solani* en frutos de tomate. Asimismo, los distintos grupos funcionales se identificaron por la técnica de Espectroscopía Infrarroja con Transformación de Fourier (FTIR por sus siglas en inglés). La técnica soxhlet resultó el mejor método de extracción para *S. scandens,* utilizando los tres disolventes. Los extractos de maceración-acetona a concentraciones de 4000 y 5000 ppm, presentaron mayor actividad antifúngica contra ambos fitopatógenos. El análisis de FTIR confirmó la presencia de ácidos carboxílicos, aldehídos, cetonas y compuestos aromáticos en los extractos como los probables responsables de la actividad antifúngica. Se concluye que los extractos de *S. scandens* podrían ser utilizados como tratamiento preventivo en el control de enfermedades provocadas por *F. oxysporum* y *A. solani.*

Palabras clave

actividad antifúngica • espectroscopía FTIR • hongos de poscosecha • soxhlet • disolventes

Introduction

Tomato (*Solanum lycopersicum* L.) is one important vegetable crop worldwide, with high nutritional and industrial value, being Mexico one of the main producing countries (23). This crop is affected by various diseases caused, mainly, by fungal pathogens of the genera *Fusarium* (Link)*, Rhizoctonia* (Kuhn), *Phytophthora (*de Bary), *Pythium* (Pringsheim) and *Alternaria* (Nees) (16, 38). *Fusarium oxysporum* (Schltdl) causes vascular wilt disease (9, 30, 36), decreasing yields up to 60 % (3), while, *Alternaria solani* (Cooke) infects ripe fruit, decreasing its commercial value (18).

Plant extracts constitute an important alternative for fungal control. Their high content of secondary metabolites (flavonoids, phenols, terpenes, alkaloids, saponins, polypeptides, among others) inhibit fungal growth and activate plant defense system (40). The Asteraceae family is widely used against fungal pathogens due to its demonstrated anti-phytopathogenic effects. *Flourensia cernua* DC. inhibited *Alternaria alternata* (Keissl), *Penicillum digitatum* (Pers.) and *Colletrotichum gloeosporioides* (Penz) by 91% (21). *Xanthium strumarium* L. inhibited *A. alternata* (5), and *Artemisia annua* L. leaves showed antifungal effects against *F. oxysporum* and *F. solani* (Mart.), presumably due to leaf organic compounds such as camphor, camphene, β-caryophyllene and germacrene D (31). On the other hand, the fungicide effect of garlic plants (*Allium sativum* L.), neem seeds (*Azadirachta indica* A. Juss.), lemon grass (*Cymbopogon proxims* Stapf.), cumin (*Carum carvi* L.) and clove (*Eugenia caryophyllus* Spreng.), against *F. oxysporum* has also been documented (2). The inhibition of *Colletotrichum musae* (Berk and Curtis) Arx growth by *Acacia albida* Delile A. Chev. and *Prosopis juliflora* (Sw.) DC, has been also demonstrated (6), *Aloe vera* L., *A. sativum* L., *Capsicum annuum* L. and *Ginkgo biloba* L. present antifungal activity against *Chalara paradoxa* and *Fusarium guttiforme* (41), while *Annona squamosa* L. extract successfully inhibits growth of *A. alternata*, *Candida albicans* (C.P.Robin), *F. solani*, *Microsporum canis* (Bodin) and *Aspergillus niger* (P.E.L. Van Tieghem) (26).

"Chilemecate", *Salmea scandens* L. is a neotropical plant, with edible leaves widely consumed in Oaxaca, Mexico (48). However, its antimicrobial properties have been slightly studied and the antimicrobial activity of its essential oils has been only documented against *Pseudomonas syringae*, *Clavibacter michiganensis*, *Erwinia carotovora*, *F. oxysporum* and *Phytophthora infestans*, and attributed to its high levels of D-germacrene and elemol (48).

This project aimed to evaluate the antifungal effect of *Salmea scandens* extracts against *Fusarium oxysporum* and *Alternaria solani,* identifying the functional groups of the chemical compounds. The hypothesis stated that the obtained extracts inhibited *in vitro* growth of *F. oxysporum* and *A. solani*.

Materials and methods

Collection and identification of botanical material

Plant tissues were collected in the municipality of Villa Corzo, Chiapas, Mexico, from January to March 2018, coordinates 16° 5' 21" N; 93° 19' 9" W. Plant identification followed known taxonomic keys (17, 30, 32). The identified specimen was registered under number 53106 and deposited in the CHIP Herbarium of the Ministry of the Environment, Housing and Natural History, located in Tuxtla Gutiérrez, Chiapas.

Extraction of plant compounds

A two kg sample of *S. scandens* stems was firstly washed with five liters of tap water, followed by five liters of 2 % NaClO solution for one minute, and three final washes with five liters of sterile distilled water (SDW) for three minutes, respectively. The tissue was dried at 40 °C for 24 h in a hybridization oven (FinePCR Combi-SV12DX, USA), pulverized in an M20 universal mill, IKA, (Staufen, Germany), sieved into a 20 mesh (0.84 mm) and stored in hermetically sealed ziploc plastic bags (Monterrey, N.L, Mexico) at 25 °C and darkness, until use (29).

The extracts were obtained using three solvents, (water, acetone and ethyl ether), and three extraction techniques (soxhlet, ultrasound-assisted and maceration). For the soxhlet extraction, 40 g of pulverized plant material were mixed with 200 ml of solvent, and refluxed (water 100 °C, acetone 56 °C and ethyl ether 34.6 °C). All solvent contained in the extracts was evaporated in a drying oven at 40 °C (20). For ultrasound-assisted extraction (UAE), 40 g of sample were mixed with 200 ml of solvent and incubated in an ultrasound bath at 20 kHz, for 45 min. The extract was filtered (Super Premium #2 filter paper, Melitta brand, USA) and concentrated in a drying oven (FinePCR Combi-SV12DX, USA) at 40 °C (12, 24). For maceration, pulverized plant material was mixed in a 1:4 ratio, with each solvent and incubated at 25 °C, for 24 h and constant shaking (200 rpm). The solvent was filtered and removed in a drying oven at 40 °C (12, 24). The obtained extracts were stored in Pyrex (USA) amber vials at 4 °C until use (34). All the extractions were made in triplicate.

In vitro fungistatic activity of *S. scandens* extracts against *F. oxysporum*

Fungal conidia were obtained from the ceparium of the Laboratory of Plant Pathology, Biological Control and Post-harvest of the Research Center for Food Development (CIAD., A.C., Cd. Cuauhtémoc, Chihuahua, México) and preserved in potato dextrose agar (PDA; BD Difco, USA) medium.

S. scandens extracts were resuspended in dimethyl sulfoxide (DMSO) obtaining a 10 % mass/volume solution (10). Dilutions were prepared at concentrations of 1000, 2000, 3000, 4000 and 5000 ppm using DMS0 as solvent (25), and added to the PDA medium where the final concentrations of DMSO were 0.1, 0.2, 0.3, 0.4 and 0.5 %. Imazalil 44.65 %. CS (Fungaflor 500 EC, Valent, Zapopan, Jalisco, México) at 4 ml/L of water and PDA-DMSO 0.1 - 0.5 %, respectively, were used as controls.

PDA dishes containing the different extract concentrations were inoculated with 10 µl of 1×10^6 conidia ml^{-1} of *F. oxysporum,* resuspended with 0.05 % Tween 60 and incubated at 28 °C. Fungal radial growth was measured every 24 h, until mycelium growth of the control treatment completely covered the PDA dish (34). Inhibition of Radial Growth (IRG), was determined using the formula $IRG=[(R1-R2)/R1]\times100$, where R1 represents control pathogen diameter and R2 is pathogen diameter with *S. scandens* extracts, expressed in millimeters (17). The experiment was conducted in triplicate.

Post-harvest bioassay of *S. scandens* extract against *A. solani*

The *A. solani* strain was obtained from the same laboratory previously mentioned and grown in PDA at 25 °C for 14 d. Conidia were collected with SDW, added with 0.05 % Tween 20, and adjusted to 1×10^6 conidia ml^{-1} (50).

Thirty ripe Saladette tomatoes were used to monitor treatment effects. Fruits were washed with SDW (~5 L) for 2 min and disinfected with 70 % ethanol for 30 s (37). Three longitudinal lesions (~2 mm in diameter and depth) were made on each fruit, later inoculated with 20 µl of *S. scandens* maceration-acetone extract, using the concentration of 5000 ppm, which resulted to be the most effective treatment in the *in vitro* inhibiting of *F. oxysporum*. Thirty percent hymexazol LS (Thachigaren 30 SL, Summit agro, México) and 0.5 % DMSO were used as controls. Subsequently, each lesion was inoculated with 10 µL of *A. solani* spore suspension (1×10^6 spores ml^{-1}). Inoculated fruits were placed in 25×13×10 cm plastic trays (Baquelita brand, México City, México), hermetically sealed, supplemented with moist cotton and incubated under controlled conditions (26 ± 2 °C and 14:10 LD photoperiod) for five days (37). A randomized block design with three replicates per treatment and 10 fruits per replicate, was used. Symptoms incidence caused by *A. solani* was determined as follows: Incidence (%) = (number of damaged fruits/total number of fruits) × 100 (27). Severity was estimated by Alternaria rot injury % = 100 × (Ø of injury on treated fruits/ Ø of untreated fruits), where Ø is diameter (37). Lesion size was determined from five random fruits from each replicate in each treatment according to: Lesion size (mm^2) = π x a/2 × b/2, a: length of the disease spot (mm), b: width of the disease spot (mm) (26). Percentage of fungal inhibition in treatments was determined by: % inhibition = (A-B) /A*100, where A is control pathogen diameter and B is pathogen diameter under treatment (44). The experiment was conducted in triplicate.

Analysis of *S. scandens* extracts by Fourier Transform Infrared Spectroscopy (FTIR)

A spectrophotometer (Spectrum Two, Perkin Elmer, Massachusetts, USA) was used. FTIR spectra were recorded from 400 to-4000 with a resolution of 4 cm^{-1} by 24 scans (14). Finally, a pure extract sample (solid extract obtained with water and oily extract obtained with acetone and ethyl ether solvents) was placed in the cell and covered with the metal cap applying a pressure of 60 Pa. The respective spectra were obtained using attenuated total reflection (ATR). Analyses were conducted in triplicate.

Statistical analysis*.*

All data obtained were subjected to ANOVA and a Tukey test ($p < 0.05$) using the minitab 18 program (Minitab, State College, PA, USA).

Results

Extraction of plant compounds

The highest mass/volume extraction efficiency yield was 3.94 % with the soxhlet technique, followed by UAE (2.97%) and maceration (2.12%) using water as a solvent (figure 1, page 266).

In vitro* fungistatic activity of *S. scandens* extracts, against *F. oxysporum

Extracts obtained by maceration-acetone technique at 5000, 4000 and 3000 ppm, caused the strongest pathogen inhibition with IRG values of 76.72, 73.32 and 57.77 %, respectively. Soxhlet-acetone extraction obtained, IRG values of 56.86 and 38.6 % at 5000 and 4000 ppm, respectively. The ultrasound-water technique showed low IRG values (11.83 %) obtained at 5000 ppm (figures 2, page 266 and 3, page 267). The extracts inhibited mycelial growth, without affecting conidia production.

Different letters show statistical differences in techniques and solvents (Tukey; *p*≤ 0.05).

Letras diferentes muestran las diferencias estadísticas entre técnicas y solventes (Tukey; *p*≤ 0,05).

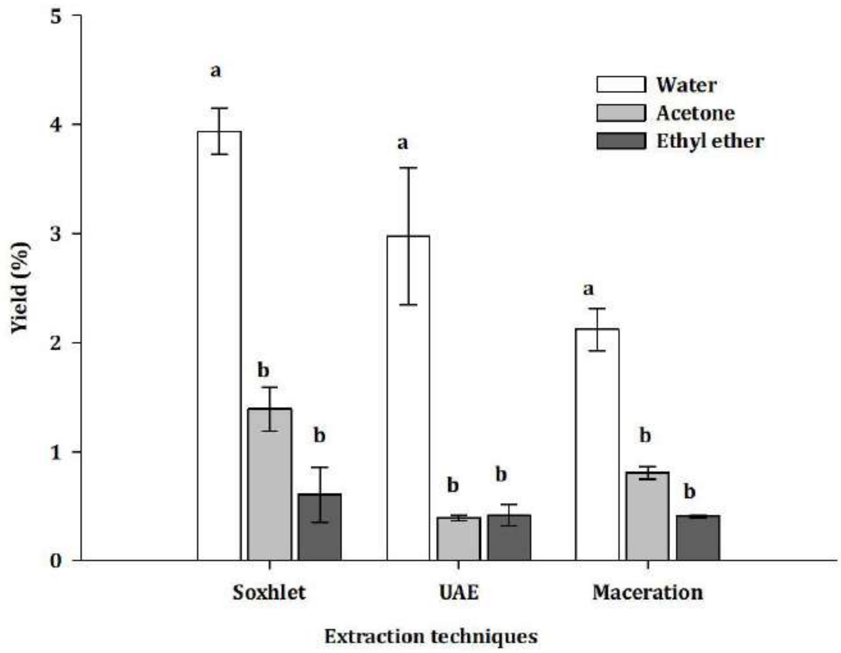


Figure 1. Percentage yield (mass/volume) from *S. scandens* plant extracts obtained by three extraction techniques, soxhlet, UAE and maceration using three solvents (water, acetone and ethyl ether).

Figura 1. Porcentaje de rendimiento (masa/volumen) de los extractos vegetales de *S. scandens* obtenido mediante tres técnicas de extracción soxhlet, ultrasonido asistido (UA) y maceración, usando tres solventes (agua, acetona y éter etílico).

Different letters indicate statistical differences for techniques and solvents (Tukey; *p*≤ 0.05).

Letras diferentes indican las diferencias estadísticas entre técnicas y solventes (Tukey; *p*≤ 0,05).

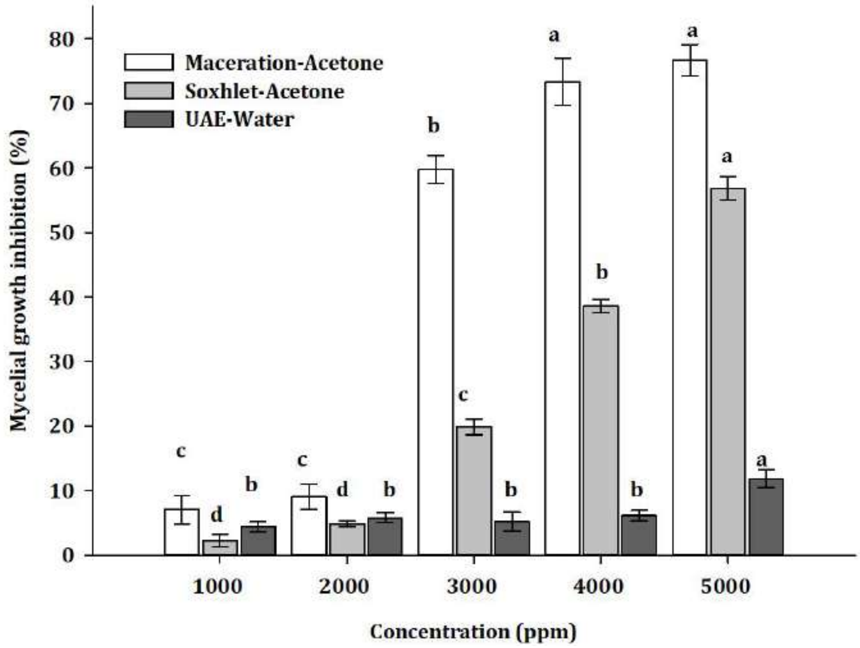


Figure 2. Inhibition of radial growth (IRG) of *F. oxysporum* by different concentrations of *S. scandens* extracts, obtained by different extraction techniques and solvents (maceration-acetone) (soxhlet-acetone) (UAE-water), after 12 days of interaction.

Figura 2. Inhibición del crecimiento radial (ICR) de *F. oxysporum* por diferentes concentraciones de extractos de S. scandens, obtenido mediante diferentes técnicas extracción y solventes (Maceración-acetona) (Soxhlet-acetona) (UAE-Agua) después de 12 días de interacción.

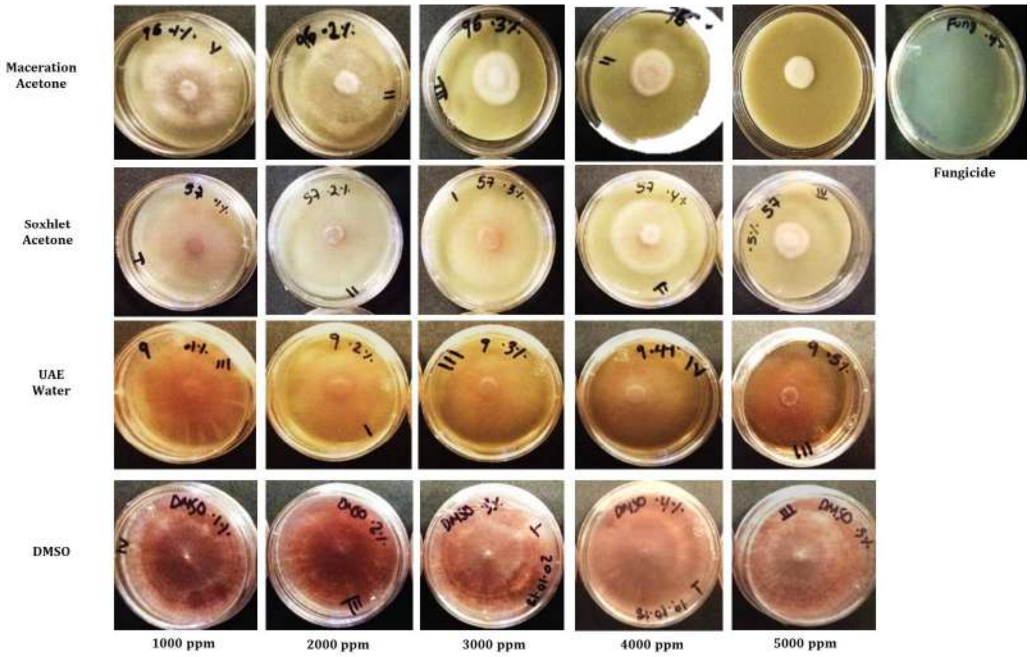


Figure 3. Schematic representation of *in vitro* inhibition of radial growth (IRG) of *F. oxysporum* in PDA medium with increasing concentrations (1000, 2000, 3000, 4000 and 5000 ppm) of *S. scandens* extracts, obtained by maceration-acetone, soxhlet-acetone and UAE-water. DMSO was used as absolute control after 12 days of interaction.

Figura 3. Representación esquemática de la inhibición del crecimiento radial (ICR) *in vitro* de *F. oxysporum* en medio de PDA adicionado con diferentes concentraciones (1000, 2000, 3000, 4000 y 5000 ppm) de extractos de *S. scandens* obtenido mediante Maceración-acetona, Soxhlet-acetona y UAE-agua. El DMSO se utilizó como control absoluto después de 12 días de interacción.

Biological effectiveness of *S. scandens* extract against *Alternaria solani* in post-harvest

In this experiment, the maceration-acetone extract at 5000 ppm was evaluated against *A. solani,* showing comparatively higher inhibition of mycelial growth (>92%) than the commercial fungicide Hymexazol, after five days of interaction (table 1; figure 4A-F, page 268). The extract inhibited mycelial growth, without affecting conidia production.

Identification of functional groups in *S. scandens* extracts by Fourier Transform Infrared Spectroscopy (FTIR*)*

The FTIR analysis showed strong peaks in extracts obtained by the soxhlet and maceration techniques using the acetone solvent. At 2927 cm^{-1} and 2849 cm^{-1}, peaks belonged to the C–H group, whereas peaks at 1734 cm^{-1} and 1656 cm^{-1} belonged to(C=O)and limited amine group (–NH), respectively. Spectrums at 1459cm^{-1}, 1273 cm^{-1} and 1161 cm^{-1} (CO), are characteristic of the vibrational modes of carboxylic groups, while at 1387 cm^{-1} and 1124 cm^{-1}, C=O, hydrolysable tannins have been documented. The extract obtained by water-assisted ultrasound technique showed weak peaks at 2892 cm^{-1}, 1545 $cm-^{1}$, 1249 $cm-^{1}$ and 1084 $cm-^{1}$ indicating the presence of amides, alkynes, alkanes, carboxylic acids, alkenes and aromatic compounds (figure 5, page 269).

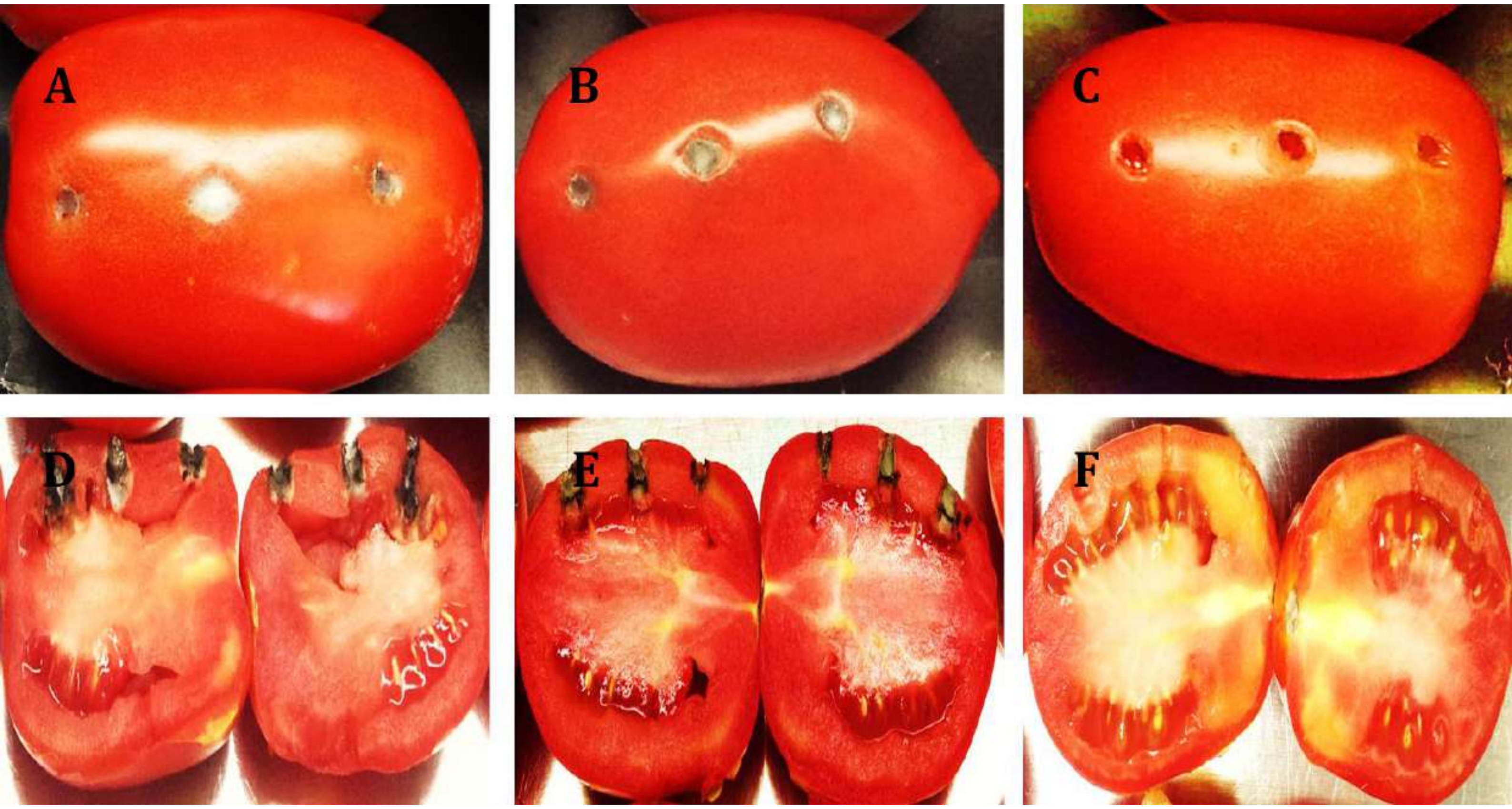
Table 1. Biological effectiveness of *S. scandens* maceration-acetone extract at 5000 ppm against *A. solani* in tomato fruit after five days of interaction.

Tabla 1. Efectividad biológica del extracto de maceración-acetona de *S. scandens* 5000 ppm contra *A. solani* en frutos de tomate después de cinco días de interacción.

Treatments	Injury size (mm^2)	Injury diameter (mm^2)	Severity of injury (%)	Incidence (%)	Inhibition (%)
DMSO + *A. solani* Control (+)	19.74±3.75 a	1.69±0.10 B	100 a	100	0.0 a
Hymexazol + *A. solani* Control (-)	15.39±0.43 a	4.38±0.07 A	94.94±3.79 a	100	5.06±3.71 a
S. scandens extract Maceration-Acetone 5000 ppm	0.91±0.29 b	0.33±0.15 C	7.36±3.71 b	13.33	92.64±3.71 b

Different letters between rows show significant difference (ANOVA, Tukey test, $p<0.05$).

Letras diferentes entre filas muestran diferencia significativa (ANOVA, prueba de Tukey, $p<0,05$).



(A, D). fruits inoculated with DMSO-*A. solani*, (B, E), fruits inoculated with *A. solani*-Himexazol; (C, F), fruits inoculated with *A. solani*-extract of *S. scandens* at 5000 ppm.

(A, D). frutos inoculados con DMSO-*A. solani*, (B, E). frutos inoculados con *A. solani* y el fungicida comercial (Himexazol); (C, F). frutos inoculados con *A. solani* y extracto de *S. scandens* a 5000 ppm.

Figure 4. Schematic representation of the effects of *S. scandens* extract on growth inhibition of *A. solani* in tomato.

Figura 4. Representación esquemática de los efectos del extracto de *S. scandens* sobre la inhibición del crecimiento de *A. solani* en jitomate.

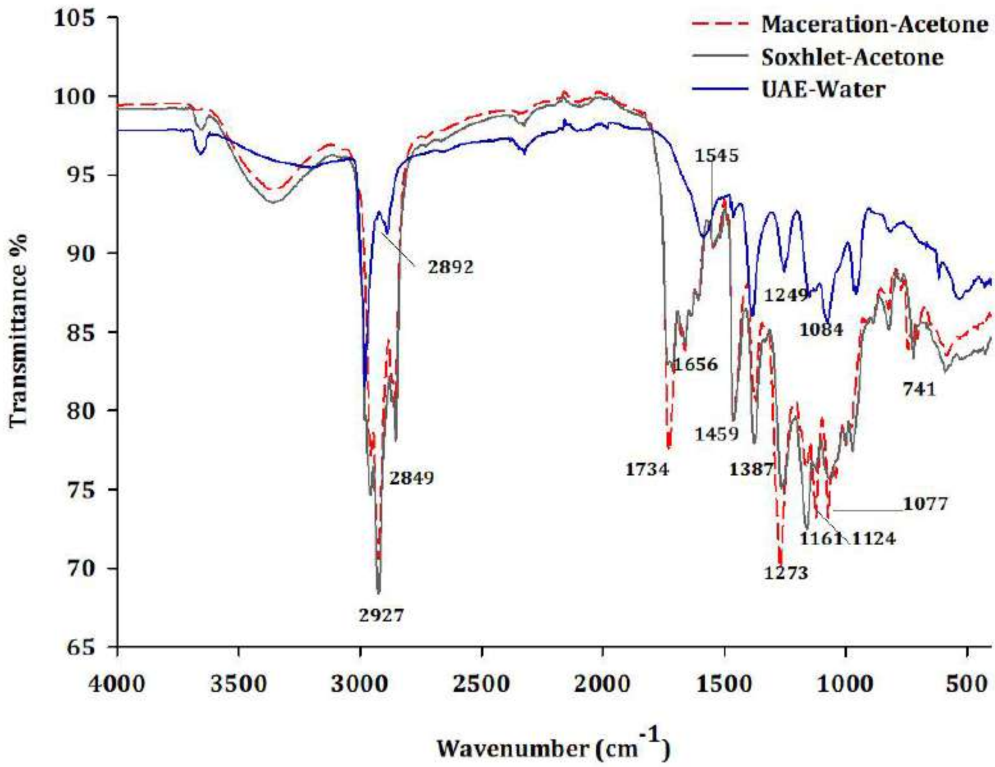


Figure 5. FTIR spectrum of *S. scandens* extracts obtained by the maceration-acetone, soxhlet-acetone and UAE-water techniques.

Figura 5. Espectro FTIR de los extractos de *S. scandens* obtenido mediante las técnicas de Maceración-acetona, Soxhlet-acetona y UAE-Agua.

Discussion

The higher extract yield obtained with the soxhlet technique, might have been given by water low volatility, compared to acetone and ethyl ether (4). In this regard, different yield percentages of *Mentha spicata* L. extracts had been also found by the soxhlet technique using different solvents; methanol (267.33 mg/g), ethanol-water (70:30) (257.66 mg/g) and petroleum ether (30.47 mg/g) (7). Extraction efficiency can also be increased by rising water content, facilitating protein and carbohydrate solubility, (13). Extract yield is affected by several factors like sample preparation, extraction methods and time, boiling temperature, and the specific nature of the bioactive compound (22). In this sense, water and acetone solvents are frequently used for hydrophilic compounds extraction from plants (46). Therefore, polar compounds result easier to extract with polar solvents, leading to the assumption that the most abundant compounds of *S. scandens* are hydrophilic ones (7).

Although maceration and ultrasonic-assisted extraction techniques showed lower yield than soxhlet, they demonstrated interesting attributes such as low operating temperature by using less solvent, no compounds degradation and faster and more selective extraction of target compounds (7). According to our best knowledge, this is the first comparative study of different extraction techniques using different organic and aqueous solvents for the obtention of bioactive compounds from *S. scandens*.

The 76.72 % inhibition of *F. oxysporum* growth could have been due to solvent natural polarity and extraction capacity (29). Acetone extracts of *Cinnamomum burmanni* (Nees & T. Nees) Blume, *Nerium oleander* L. and *Parrotia persica* (DC.) C. A. Mey. inhibited *F. oxysporum* f.sp. *lycopersici* (Sacc.) radial growth by 48.43 %, given their content of antimicrobial metabolites like flavonoids, alkaloids, terpenes, tannins and saponins (42, 43). Likewise, *S. scandens* essential oil is known to inhibit *F. oxysporum* and *P. infestans* growth (48), attributed to high levels of germacrene D (47.1 %) and elemol (15.3 %), and to N-isobutyl-(2E, 4E, 8Z, 10E/Z)-dodecatetraenamide isomers (39.7 %) contained in the stem bark (48).

The use of plant extracts for post-harvest disease control in fruits and vegetables constitutes a sustainable alternative in relation to synthetic fungicides (49). In the present study, the inhibitory effect of acetone-soluble extract of *S. scandens* against *A. solani*, in tomato plants and fruits, is documented for the first time, providing an alternative for protection and control during transport and storage (1). Antifungal plant derived compounds had already shown to be advantageous for *Alternaria* control in cherry tomatoes (47).

Regarding the identification of functional groups, similar results were also obtained with the extract from *Chamaemelum nobile* (L.) identifying several functional groups (C–H, –NH, phenolic groups of tannins, C-O, amines, carboxylic acids, aldehydes and ketones) (15). On the other hand, absorption intensity variation between1743 cm^{-1} (C=O) and 1160 cm^{-1} (CO) are characteristic of the vibrational modes of carboxylic groups (45). In this experiment, close bands of 1734 and 1161 cm^{-1} were obtained, while in the 1722–1702, 1368 and 1157 cm^{-1} peaks, the C=O stretching of esters from hydrolysable tannins were found, especially those derived from gallic acid, since condensed tannins show intense signals of C=C-C stretching between the 1555–1503, 1361-1340 and 1284-1283 cm^{-1} regions (39). In this sense, *S. scandens* extracts showed absorption bands at 1545 cm^{-1}, predicting the presence of hydrolyzed tannins. Stretching of C-C aldehyde (1459 cm^{-1}), CN nitrile (1273 cm^{-1}) and C-C aromatic hydrocarbons (741 cm^{-1}) had previously been found, determining that the stretching vibration of C=O at 1554 cm^{-1} in aldehydes and ketones, indicates the presence of phenolic acid and/or terpenoid (51). Other functional compounds of *Albizia lebbeck* (L) Benth had also been identified, confirming the presence of amides, alkines, alkanes, carboxylic acids, alkenes, aromatic compounds and aliphatic amines, showing main peaks at 2918.44 and 2849.92, 1643.73, 1454.46, 1054.13 and 510.34 cm^{-1}, respectively (8). Finally, other easily identified stretches are the aromatic bonds C=C-C in the region of 1611–1444 cm^{-1} and C-O between 1368–1157 cm^{-1} and 1031–1023 cm^{-1} (11).

Therefore, the FTIR analysis on *S. scandens* indicated the presence of carboxylic groups, hydrolysable tannins, amides, alkenes, alkylic acids, alkenes, aromatic compounds, aliphatic amines and alkyl halides as possible antifungal molecules. Aromatic compounds, monoterpenes, sesquiterpenes, alkylamides and aliphatic and oxygenated hydrocarbons in *S. scandens,* were identified (48). However, few studies about the characterization of the biological activity of the compounds of this plant species, are available. Additional work characterizing the antimicrobial activity of the compounds already reported should be conducted.

Conclusions

Salmea scandens extracts demonstrated strong antifungal effects against *Fusarium oxysporum* and *Alternaria solani.*

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Mixtures between glyphosate formulations and ACCase-inhibiting herbicides in the control of *Chloris elata*

Mezclas de formulaciones de glifosato y herbicidas inhibidores de ACCase em el control de *Chloris elata*

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Abstract

Chloris elata is an important weed for grain and sugarcane crops. In addition to its aggressiveness, it may show resistance to glyphosate herbicide. In this context, the aim of this study was to assess the effectiveness of glyphosate formulations, isolated or in association with ACCase inhibitors, in controlling *C. elata* (putatively resistant to glyphosate) at different developmental stages. Four experiments were conducted in a completely randomized design. Treatments consisted of glyphosate application under different formulations with ACCase inhibitors, isolated or in mixtures. Applications were carried out at the stages of four fully expanded leaves in Experiment I, four tillers in Experiment II, and at regrowth of the four-tiller plants in Experiments III and IV. Applications of glyphosate isopropylamine salt associated with sethoxydim or clethodim showed to be among the best treatments in Experiments I, II, and III, presenting control scores equal to or greater than 90%. However, not even these treatments could provide successful control in Experiment IV. Moreover, and regardless of the formulation, isolated glyphosate, showed 85% (Experiment III) and 50% (Experiment IV) maximum controls. Associations between glyphosate and ACCase-inhibiting herbicides showed to be effective in controlling *C. elata*, especially at early developmental stages. In general, isolated herbicides provided lower percentages of control, as well as higher values of dry matter. Sole herbicide applications were not effective in controlling *C. elata* (putatively resistant to glyphosate), regardless of the developmental stage.

Keywords
chemical control • clethodim • haloxyfop • quizalofop • sethoxydim • tank mix

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Resumen

Chloris elata es una maleza importante para cultivos de cereales y caña de azúcar. Además de su agresividad, presenta casos de resistencia al herbicida glifosato. El objetivo de este estudio fue evaluar la efectividad de formulaciones de glifosato, solas o en mezclas con inhibidores de la ACCase, en el control de *C. elata* (supuestamente resistente al glifosato) en diferentes estadios. Se realizaron cuatro experimentos en un diseño completamente al azar. Los tratamientos consistieron en la aplicación de glifosato en diferentes formulaciones con inhibidores de ACCase, solo o en mezclas. Las aplicaciones de los tratamientos se llevaron a cabo en las etapas de cuatro hojas completamente expandidas en el Experimento I y cuatro macollos en el Experimento II, y en el rebrote de las plantas de cuatro macollos en los Experimentos III y IV. La aplicación de glifosato sal de isopropilamina, en mezclas con setoxidim o cletodim, siempre estuvo entre los mejores tratamientos en los Experimentos I, II y III, presentó puntuaciones de control iguales o superiores a 90%. Sin embargo, incluso estos tratamientos no proporcionaron un control satisfactorio en el Experimento IV. En general, los herbicidas solos proporcionaron porcentajes de control más bajos, así como valores más altos de materia seca. Además, el herbicida de glifosato solo, independientemente de la formulación, mostró 85% (Experimento III) y 50% (Experimento IV) de controles máximos. Las mezclas entre glifosato y los herbicidas inhibidores de la ACCase demostraron ser efectivas en el control de *C. elata*, especialmente en estadios iniciales. En general, las aplicaciones solas de herbicidas no fueron efectivas para controlar *C. elata* (supuestamente resistente al glifosato), independientemente del estadio.

Palabras clave

control químico • cletodim • haloxifop • quizalofop • setoxidim • mezcla de tanque

Introduction

Species of the genus *Chloris,* family Poaceae, presents annual or perennial plants of C4 physiology with well-developed Kranz anatomy and a high adaptive capacity to different habitats (17, 21). The species *Chloris elata* (Syn.: *Chloris polydactyla,* native to America, presents seed and/or rhizome reproduction (11, 17). One single plant can produce more than 96,000 seeds (8).

Populations of *C. elata* present cases of glyphosate resistance (4, 6, 9). Other species of this genus, such as *C. barbata* (7), *C. virgata* (20), *C. truncata* (19), and *C. distichophylla* (30) are also reported as resistant. In the last years, these species have gained importance in different crops, mainly grains and sugarcane, thus increasing the number of studies on their biology and management (2, 3, 9, 12, 13, 25). In addition, no specific recommendations of glyphosate as chemical control of *C. elata* are available (4). Rotational mechanisms of action and herbicide associations have significant importance in the management of hard-to-control weeds, as well as in the prevention of herbicide-resistant biotypes selection (16). Thus, the hypothesis is that glyphosate and ACCase-inhibiting mixture effectively controls *C. elata* in different stages of development. Therefore, this study aimed to assess the effectiveness of glyphosate formulations with ACCase-inhibiting herbicides, against *C. elata* (putatively resistant to glyphosate) at different development stages.

Materials and methods

Greenhouse experiments

The experiments were conducted in a greenhouse, from November to December 2014, located in the Department of Crop Science of the University of São Paulo, “Luiz de Queiroz” College of Agriculture (USP/ESALQ), Piracicaba, State of São Paulo (SP), Brazil. During the conduction period of the study, an average temperature of 25.3°C and 13 h daylight^{-1} was observed.

Seeds of *C. elata* population identified with a possible resistance were collected according to Burgos *et al*. (2013) from a citrus area located in Matão, SP, Brazil (21.61°S, 48.44°W), with a history of repetitive glyphosate applications. Seeds from this collection were sown in pots filled in a 1:1 ratio of substrate and medium-textured soil with the characteristics described in table 1.

Table 1. Chemical and physical soil analysis.

Tabla 1. Análisis químico y físico del suelo.

pH	P	K	Ca	Mg	H + Al	SB	CEC	V	Clay	Silt	Sand
6.2	5.0	4.2	41.0	15.0	< 26.0	60.1	72.5	83.0	276	86	638

Unities: H + Al, K, Ca, Mg, SB (sum of bases), and CEC (cation exchange capacity): $mmol_c\ dm^{-3}$; P: $mg\ dm^{-3}$; V (base saturation): %; clay, silt, and sand: $g\ Kg^{-1}$.

Unidades: H + Al, K, Ca, Mg, SB (suma de bases), and CEC (capacidad de intercambiar cationes): $mmol_c\ dm^{-3}$; P: $mg\ dm^{-3}$; V (saturación de bases): %; arcilla, limo y arena: $g\ Kg^{-1}$.

Four experiments were conducted in a completely randomized design with 19 herbicide treatments and a control without application (table 2). Treatment applications were carried out at the stages of four fully expanded leaves in Experiment I and four tillers in Experiment II. In both experiments, four replications were used per treatment in 5 L pots. For Experiments III and IV, the applications were conducted in the regrowth of the four-tiller plants, with three replications per treatment in 5 L (Experiment III) and 7 L pots (Experiment IV).

[1] Commercial products: Roundup Ready® - glyphosate IS (isopropylamine salt); Roundup® WG - glyphosate AS (ammonium salt); Zapp® QI 620 - glyphosate PS (potassium salt); Panther® 120 EC - quizalofop; Verdict® R - haloxyfop; Poast® - sethoxydim; Select® 240 EC - clethodim. For quizalofop and sethoxydim applications it was used adjuvant Joint® Oil (2 L ha^{-1}); for haloxyfop, Joint® Oil (1 L ha^{-1}); for clethodim, Lanzar® (1 L ha^{-1}). [2] Rates in grams of acid equivalent (g a.e. ha^{-1}) for glyphosate and haloxyfop, and grams of active ingredient (g a.i. ha^{-1}) for the other herbicides.

[1] Productos comerciales: Roundup Ready® - glifosato IS (sal de isopropilamina); Roundup® WG - glifosato AS (sal de amonio); Zapp® QI 620 - glifosato PS (sal de potasio); Panther® 120 EC - quizalofop; Verdict® R - haloxifop; Poast® - setoxidim; Select® 240 EC - cletodim. Para las aplicaciones de quizalofop y setoxidim se utilizó adyuvante Joint® Oil (2 L ha^{-1}); para haloxifop, Joint® Oil (1 L ha^{-1}); for cletodim, Lanzar® (1 L ha^{-1}). [2] Dosis en gramos de equivalente de ácido (g e.a. ha^{-1}) para glifosato y haloxifop, y gramos de ingrediente activo (g i.a. ha^{-1}) para los otros herbicidas.

Table 2. Treatments for control of *C. elata* with different formulations of glyphosate and ACCase inhibitors, alone and in mixtures.

Tabla 2. Tratamientos para el control de *C. elata* con diferentes formulaciones de glifosato e inhibidores de ACCase, solas o en mezcla.

Treatments [1]	Rate [2]	
	Exp. I	Exp. II. III and IV
1. glyphosate IS	720	720
2. glyphosate IS + quizalofop	720 + 60	720 + 120
3. glyphosate IS + haloxyfop	720 + 48	720 + 60
4. glyphosate IS + sethoxydim	720 + 184	720 + 368
5. glyphosate IS + clethodim	720 + 84	720 + 108
6. glyphosate AS	720	720
7. glyphosate AS + quizalofop	720 + 60	720 + 120
8. glyphosate AS + haloxyfop	720 + 48	720 + 60
9. glyphosate AS + sethoxydim	720 + 184	720 + 368
10. glyphosate AS + clethodim	720 + 84	720 + 108
11. glyphosate PS	720	720
12. glyphosate PS + quizalofop	720 + 60	720 + 120
13. glyphosate PS + haloxyfop	720 + 48	720 + 60
14. glyphosate PS + sethoxydim	720 + 184	720 + 368
15. glyphosate PS + clethodim	720 + 84	720 + 108
16. quizalofop	60	120
17. haloxyfop	48	60
18. sethoxydim	184	368
19. clethodim	84	108
20. control (without application)	-	-

Treatment applications were carried out in an experimental herbicide spraying chamber, powered by an electric motor, with a constant pressure of 40 psi, and equipped with spray tips flat-fan Teejet® 8002 positioned at 50 cm from the target and with a spray solution volume of 200 L ha^{-1}.

Data gathering

The control of *C. elata* was assessed for each treatment at 7, 14, 21, and 28 days after application (DAA) through visual assessments in which percentage scores ranging from 0 to 100% were assigned to each experimental unit, where 0 represents no injury and 100% represents plant death (31).

At 28 DAA, the aerial part of each plant was collected to determine the dry matter by using a forced air ventilation oven at 65 °C for 72 h and an analytical balance with accuracy to three decimal places.

Statistical analyses

The data were submitted to analysis of variance by F-test ($P < 0.01$), while the means of treatments were compared by the Scott and Knott (1974) test ($P < 0.01$) (24).

Results

Experiment I

At 7 DAA, some differences were observed in *C. elata* control. However, with absolute values not exceeding 16.25%. At 14 DAA, the application of glyphosate isopropylamine salt (IS) + clethodim provided a control of 81.25%, which was higher than those observed in all other treatments. At 21 DAA, greater control results were observed for the application of glyphosate IS + clethodim, glyphosate IS + haloxyfop, glyphosate IS + sethoxydim, and glyphosate potassium salt (PS) + clethodim, with control scores higher than 85%, which was higher than those observed in all other treatments. Similarly, it was observed at 28 DAA, with control scores equal to or greater than 90%. All treatments presented a reduction in *C. elata* dry matter in relation to the control (without application). While the application of glyphosate IS reduced the *C. elata* dry matter only in relation to the control, presenting values superior to all other treatments (table 3, page 278).

Experiment II

At 7 DAA, some differences were observed in *C. elata* control. However, control percentages were low, with absolute values not exceeding 18.75%. At 14 DAA, the application of glyphosate IS + sethoxydim provided a control of 83.75%, which was higher than those observed in all other treatments. At 21 DAA, the best control results were observed for the application of glyphosate IS + sethoxydim, glyphosate ammonium salt (AS) + quizalofop, glyphosate AS + sethoxydim, and glyphosate AS + clethodim, with control scores equal to or greater than 86.25%, which was higher than those observed in all other treatments.

At 28 DAA, greater control results were observed for the application of glyphosate IS + sethoxydim, glyphosate IS + clethodim, glyphosate AS in association with any of the graminicides, glyphosate PS + haloxyfop, glyphosate PS + sethoxydim, and glyphosate PS + clethodim, with control scores equal to or greater than 87.50%, which was higher than those observed in all other treatments. In addition, all treatments showed a reduced dry matter value in relation to the control, and no differences were observed between herbicide treatments (table 4, page 278).

Table 3. Control (%) at 7, 14, 21 and 28 DAA, and dry matter (mg) of *C. elata* (Exp. I).
Tabla 3. Control (%) a 7, 14, 21 y 28 DAA, y materia seca (mg) de *C. elata* (Exp. I).

Treatments[1]	Control (DAA)				Dry mass
	7	14	21	28	
1. glyphosate IS	11.25 a	56.25 d	76.25 c	81.25 c	165.00 c
2. glyphosate IS + quizalofop	12.50 a	58.75 d	82.50 b	87.50 b	133.25 a
3. glyphosate IS + haloxyfop	12.50 a	70.00 c	88.75 a	92.50 a	105.00 a
4. glyphosate IS + sethoxydim	11.25 a	67.50 c	86.25 a	91.25 a	115.00 a
5. glyphosate IS + clethodim	12.50 a	81.25 a	88.75 a	93.75 a	85.00 a
6. glyphosate AS	12.50 a	65.00 c	72.50 c	81.25 c	136.50 b
7. glyphosate AS + quizalofop	16.25 a	72.50 b	81.25 b	87.50 b	102.50 a
8. glyphosate AS + haloxyfop	12.50 a	71.25 c	81.25 b	86.25 b	120.00 b
9. glyphosate AS + sethoxydim	13.75 a	67.50 c	78.75 b	83.75 b	95.00 a
10. glyphosate AS + clethodim	12.50 a	67.50 c	80.00 b	86.25 b	96.50 a
11. glyphosate PS	8.75 a	60.00 d	72.50 c	77.50 c	103.25 a
12. glyphosate PS + quizalofop	12.50 a	56.25 d	73.75 c	80.00 c	117.50 b
13. glyphosate PS + haloxyfop	10.00 b	66.25 c	75.00 c	82.50 c	120.00 b
14. glyphosate PS + sethoxydim	12.50 a	67.50 c	81.25 b	85.00 b	103.25 a
15. glyphosate PS + clethodim	10.00 b	73.75 b	85.00 a	90.00 a	102.50 a
16. quizalofop	10.00 b	55.00 d	70.00 c	77.50 c	106.50 a
17. haloxyfop	5.00 b	53.75 d	70.00 c	78.75 c	115.00 a
18. sethoxydim	7.50 b	52.50 d	65.00 d	71.25 c	122.50 b
19. clethodim	6.25 b	48.75 d	62.50 d	75.00 c	130.00 b
20. control (without application)	0.00 c	0.00 e	0.00 e	0.00 d	270.00 d
CV (%)	25.23	7.55	7.03	6.02	13.64
F	7.44**	52.25**	52.89**	67.43**	22.15**
P>F	0.00	0.00	0.00	0.00	0.00

[1] IS (isopropylamine salt); AS (ammonium salt); PS (potassium salt). ** Means followed by the same letter in the column did not differ between the Scott and Knott (1974) test (*P* < 0.01).

[1] IS (sal de isopropilamina); AS (sal de amonio); PS (sal de potasio). ** Los medios seguidos por la misma letra en la columna no difirieron por el teste de Scott y Knott (1974) (*P* < 0,01).

Table 4. Control (%) at 7, 14, 21 and 28 DAA, and dry matter (mg) of *C. elata* (Exp. II).
Tabla 4. Control (%) a 7, 14, 21 y 28 DAA, y materia seca (mg) de *C. elata* (Exp. II).

Treatments[1]	Control (DAA)				Dry mass
	7	14	21	28	
1. glyphosate IS	8.75 b	55.00 d	66.25 c	81.25 b	180.00 a
2. glyphosate IS + quizalofop	11.25 b	58.75 d	76.25 c	85.00 b	162.50 a
3. glyphosate IS + haloxyfop	12.50 b	65.00 c	80.00 b	86.25 b	162.50 a
4. glyphosate IS + sethoxydim	18.75 a	83.75 a	91.25 a	95.00 a	150.00 a
5. glyphosate IS + clethodim	16.25 a	70.00 b	83.75 b	91.25 a	160.00 a
6. glyphosate AS	10.00 b	62.50 c	71.25 c	80.00 b	210.00 a
7. glyphosate AS + quizalofop	15.00 a	67.50 c	88.75 a	92.50 a	180.00 a
8. glyphosate AS + haloxyfop	12.50 b	66.25 c	80.00 b	88.75 a	195.00 a
9. glyphosate AS + sethoxydim	13.75 a	76.25 b	87.50 a	92.50 a	185.75 a
10. glyphosate AS + clethodim	13.75 a	70.00 b	86.25 a	92.50 a	205.00 a
11. glyphosate PS	8.75 b	56.25 d	72.50 c	78.75 b	210.00 a
12. glyphosate PS + quizalofop	13.75 a	62.50 c	76.25 c	86.25 b	195.00 a
13. glyphosate PS + haloxyfop	12.50 b	60.00 d	78.75 c	87.50 a	195.00 a
14. glyphosate PS + sethoxydim	17.50 a	63.75 c	81.25 b	90.00 a	190.00 a
15. glyphosate PS + clethodim	15.00 a	62.50 c	75.00 c	87.50 a	206.50 a
16. quizalofop	13.75 a	62.50 c	73.75 c	81.25 b	206.50 a
17. haloxyfop	11.25 b	57.50 d	75.00 c	80.00 b	205.00 a
18. sethoxydim	15.00 a	65.00 c	73.75 c	85.00 b	210.00 a
19. clethodim	11.25 b	60.00 d	73.75 c	82.50 b	203.25 a
20. control (without application)	0.00 c	0.00 e	0.00 d	0.00 c	548.75 b
CV (%)	20.43	7.78	7.16	6.40	13.45
F	9.54**	44.74**	49.04**	57.44**	34.67**
P>F	0.00	0.00	0.00	0.00	0.00

[1] IS (isopropylamine salt); AS (ammonium salt); PS (potassium salt).** Means followed by the same letter in the column did not differ between the Scott and Knott (1974) test (*P* < 0.01).

[1] IS (sal de isopropilamina); AS (sal de amonio); PS (sal de potasio). ** Los medios seguidos por la misma letra en la columna no difirieron por el teste de Scott y Knott (1974) (*P* < 0,01).

Experiment III

Control scores equal to or greater than 81.67% were observed at 7 DAA for the treatments with glyphosate IS + clethodim, glyphosate AS + haloxyfop, glyphosate AS + sethoxydim, glyphosate PS + haloxyfop, glyphosate PS + clethodim, with values higher than those observed in all other treatments. In the subsequent assessments, all herbicide treatments showed control scores higher than 75%, except for the application of haloxyfop, which provided a control of 63.33% at the 14 DAA. At 28 DAA, all herbicide treatments showed control scores higher than 83%. For dry matter, no differences were observed between herbicide treatments, with lower values in relation to the control (table 5).

Table 5. Control (%) at 7, 14, 21 and 28 DAA, and dry matter (mg) of *C. elata* (Exp. III).
Tabla 5. Control (%) a 7, 14, 21 y 28 DAA, y materia seca (mg) de *C. elata* (Exp. III).

Treatments[1]	Control (DAA)				Dry mass
	7	14	21	28	
1. glyphosate IS	68.33 c	76.33 b	81.67 c	85.00 b	205.00 a
2. glyphosate IS + quizalofop	63.33 c	78.33 b	86.67 b	88.33 b	195.00 a
3. glyphosate IS + haloxyfop	75.00 b	88.33 a	91.67 a	94.33 a	168.33 a
4. glyphosate IS + sethoxydim	78.33 b	88.33 a	91.67 a	91.67 a	185.00 a
5. glyphosate IS + clethodim	81.67 a	86.67 a	91.67 a	94.33 a	180.00 a
6. glyphosate AS	68.33 c	76.33 b	80.00 c	83.33 b	205.00 a
7. glyphosate AS + quizalofop	65.00 c	76.67 b	86.67 b	91.67 a	202.33 a
8. glyphosate AS + haloxyfop	85.00 a	88.33 a	88.33 b	94.33 a	180.00 a
9. glyphosate AS + sethoxydim	83.33 a	91.67 a	95.00 a	96.00 a	175.00 a
10. glyphosate AS + clethodim	78.33 b	81.67 a	88.33 b	94.33 a	185.00 a
11. glyphosate PS	68.33 c	75.00 b	78.33 c	83.33 b	205.00 a
12. glyphosate PS + quizalofop	78.33 b	81.67 a	83.33 c	86.67 b	195.00 a
13. glyphosate PS + haloxyfop	83.33 a	86.67 a	91.67 a	94.33 a	187.33 a
14. glyphosate PS + sethoxydim	78.33 b	88.33 a	93.33 a	94.33 a	175.00 a
15. glyphosate PS + clethodim	83.33 a	91.67 a	95.00 a	96.00 a	160.00 a
16. quizalofop	73.33 b	80.00 b	83.33 c	85.00 b	205.00 a
17. haloxyfop	55.00 d	63.33 c	76.67 c	83.33 b	202.86 a
18. sethoxydim	73.33 b	85.00 a	86.67 b	88.33 b	198.14 a
19. clethodim	70.00 c	76.67 b	80.00 c	86.67 b	195.43 a
20. control (without application)	0.00 e	0.00 d	0.00 d	0.00 c	480.71 b
CV (%)	6.11	4.92	4.39	4.62	16.30
F	54.55**	79.60**	93.57**	81.81**	22.81**
P>F	0.00	0.00	0.00	0.00	0.00

[1] IS (isopropylamine salt); AS (ammonium salt); PS (potassium salt). ** Means followed by the same letter in the column did not differ between the Scott and Knott (1974) test ($P < 0.01$).
[1] IS (sal de isopropilamina); AS (sal de amonio); PS (sal de potasio). ** Los medios seguidos por la misma letra en la columna no difirieron por el teste de Scott y Knott (1974) ($P < 0,01$).

Experiment IV

Greater control results of *C. elata* at 28 DAA were observed in glyphosate applications (regardless of the formulation) associated with sethoxydim or clethodim and in glyphosate AS or PS with haloxyfop. However, control values did not exceed 68.33% (table 6, page 280). Isolated herbicide applications presented the worst control percentages, reaching a maximum of 51.67% for clethodim application at 28 DAA. Among all herbicide treatments, the lowest control values were observed for the isolated application of haloxyfop (38.33%) and quizalofop (33.33%). All herbicide treatments reduced plant dry matter when compared to the control. However, satisfactory controls of *C. elata* were not observed over the assessments.

Table 6. Control (%) at 7, 14, 21 and 28 DAA, and dry matter (mg) of *C. elata* (Exp. IV).
Tabla 6. Control (%) a 7, 14, 21 y 28 DAA, y materia seca (mg) de *C. elata* (Exp. IV).

Treatments[1]	Control				Dry mass
	7	14	21	28	
1. glyphosate IS	23.33 c	30.00 c	38.33 d	45.00 b	3466.67 b
2. glyphosate IS + quizalofop	25.00 c	35.00 c	43.33 c	50.00 b	3453.33 b
3. glyphosate IS + haloxyfop	38.33 b	46.67 b	53.33 b	58.33 b	3416.67 b
4. glyphosate IS + sethoxydim	48.33 a	61.67 a	65.00 a	68.33 a	2736.67 a
5. glyphosate IS + clethodim	43.33 b	53.33 b	61.67 a	66.67 a	2033.33 a
6. glyphosate AS	25.00 c	31.67 c	38.33 d	48.33 b	2740.00 a
7. glyphosate AS + quizalofop	28.33 c	35.00 c	45.00 c	51.67 b	2666.67 a
8. glyphosate AS + haloxyfop	53.33 a	56.67 a	65.00 a	68.33 a	1960.00 a
9. glyphosate AS + sethoxydim	48.33 a	56.67 a	61.67 a	68.33 a	2263.33 a
10. glyphosate AS + clethodim	46.67 a	51.67 b	58.33 a	66.67 a	2230.00 a
11. glyphosate PS	30.00 c	36.67 c	43.33 c	50.00 b	2596.67 a
12. glyphosate PS + quizalofop	31.67 c	40.00 c	46.67 c	55.00 b	2390.00 a
13. glyphosate PS + haloxyfop	40.00 b	46.67 b	58.33 a	68.33 a	2033.33 a
14. glyphosate PS + sethoxydim	40.00 b	50.00 b	56.67 a	68.33 a	2283.33 a
15. glyphosate PS + clethodim	51.67 a	58.33 a	60.00 a	65.00 a	1990.00 a
16. quizalofop	26.67 c	28.33 c	30.00 d	33.33 c	2770.00 a
17. haloxyfop	31.67 c	33.33 c	36.67 d	38.33 c	2546.67 a
18. sethoxydim	31.67 c	35.00 c	40.00 c	46.67 b	1936.67 a
19. clethodim	31.67 c	40.00 c	48.33 b	51.67 b	1856.67 a
20. control (without application)	0.00 d	0.00 d	0.00 e	0.00 d	4826.67 c
CV (%)	14.03	12.27	9.48	8.68	10.96
F	19.79**	23.74**	34.17**	38.76**	3.38**
P>F	0.00	0.00	0.00	0.00	0.00

[1] IS (isopropylamine salt); AS (ammonium salt); PS (potassium salt). ** Means followed by the same letter in the column did not differ between the Scott and Knott (1974) test ($P < 0.01$).

[1] IS (sal de isopropilamina); AS (sal de amonio); PS (sal de potasio). ** Los medios seguidos por la misma letra en la columna no difirieron por el teste de Scott y Knott (1974) ($P < 0,01$).

Discussion

In Experiments I, II, and III, the application of glyphosate IS associated with sethoxydim or clethodim stood out at 28 DAA, being always among the best treatments, with control scores equal to or greater than 90%. In this sense, haloxyfop was only inferior in Experiment II. However, even these treatments did not provide a satisfactory control in Experiment IV. These lower control percentages for Experiment IV can be explained using larger pots (7 L), which, in hypothesis, may have favored a greater root development of plants, leading to lower control percentages. In another study, this weed showed high growth as well as interference with soybean crop, when grown in 7 L pots (3). This possible greater root development, coupled with the fact that they are plants from regrowth, also helps to explain the higher values of dry matter, in relation to the control at 28 DAA.

In general, the isolated herbicides and associations presenting quizalofop provided lower control percentages. Moreover, the isolated glyphosate, regardless of the formulation, showed maximum controls of 85.00% for Experiment III and 50.00% for Experiment IV. Placido *et al*. (2016) observed a 100% control of *C. elata* biotypes when applying glyphosate IS (720 g a.e. ha^{-1}) + clethodim (108 g a.i. ha^{-1}). Nonemacher *et al*. (2017) verified a 97% control of *D. ciliaris* at 21 DAA in the application of glyphosate IS (1080 g a.e. ha^{-1}) + clethodim (96 g a.i. ha^{-1}). Mixtures of glyphosate PS (900 g a.e. ha^{-1}) with clethodim (60 g a.i. ha^{-1}) or sethoxydim (300 g a.i. ha^{-1}) were also effective in controlling volunteer maize, with percentages above 95%, but an association of this same glyphosate formulation with quizalofop (72 g a.i. ha^{-1}) was not effective (29). The results aforementioned reinforce the use of glyphosate and inhibitors of ACCase mixtures to effectively control *C. elata* and other grasses.

Nunes *et al*. (2007) observed a control of *C. distichophylla* equal to or greater than 85% for the application of glyphosate and fluazifop, but the clethodim application did not present a satisfactory control (10%), again with the most effective mixtures. The herbicides clodinafop (48 g a.i. ha^{-1}), haloxyfop (60 g a.e. ha^{-1}), clethodim (120 g a.i. ha^{-1}), fluazifop (187.5 g a.i. ha^{-1}), tepraloxydim (100 g a.i. ha^{-1}), sethoxydim (221 g a.i. ha^{-1}), and quizalofop (60 g a.i. ha^{-1}) were effective in controlling *C. elata* of 20 cm in height and with six leaves. For flowering plants (85 cm in height), only glyphosate (1440 g a.e. ha^{-1}) provided a satisfactory control (8). These results suggest that the effectiveness of ACCase inhibitors may vary with the stage and development of *Chloris* spp.

As previously mentioned, seeds of the *C. elata* population identified with possible resistance were collected in an area with a history of more than 10 years of successive glyphosate applications. The species *C. elata* was reported as resistant to glyphosate (6, 9) and as having a differential susceptibility (3). Herbicide mixture has a great importance in the management of weeds difficult-to-control, as well as in the prevention of selection of herbicide-resistant biotypes (16, 18). The results of our study indicate the association of glyphosate with ACCase-inhibiting herbicides as an effective alternative in controlling *C. elata*, as also observed by other authors for *Chloris* spp., (15, 27), also highlighting the herbicide glufosinate (GS inhibitor) in these mixtures, which indicates that other mechanisms of action should be considered in the management of *Chloris* spp. (28).

Weed management to prevent the selection of herbicide-resistant biotypes should be preventive (1, 5, 14, 18), using the herbicide combination highlighted in this study, herbicide rotation of different mechanisms of action, groups of ACCase herbicides, and other management strategies beyond chemical control, such as those cultural, physical, and mechanical.

Conclusion

Mixtures between glyphosate and ACCase-inhibiting herbicides have been shown to be effective in controlling *C. elata*, especially in the early development stages. In general, isolated herbicide applications were not effective in controlling *C. elata* (putatively resistant to glyphosate), regardless of the development stage.

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First report of *Berkeleyomyces basicola* (synonymous: *Thielaviopsis basicola*) on roots of sweet potato (*Ipomoea batatas* (L.) Lam) in Argentina

Primer reporte de Berkeleyomyces basicola (sinónimo: Thielaviopsis basicola) en raíces de batata (*Ipomoea batatas* (L.) Lam) en Argentina

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Abstract

Symptomatic sweet potato cv Arapey INIA samples were collected from a commercial production field in Colonia Molina, Guaymallén department, Mendoza province, Argentina. They showed dark rounded lesions, sometimes coalescing with white granular mycelium. Fungus was obtained from symptomatic sweet potatoes, which represented the generalized infection that affected the crop. They were seeded in PDA with streptomycin sulfate and incubated for seven days at 21°C, alternating white/black (UV400nm) light. Observations with an optical microscope revealed the presence of hyaline, not septated, cylindrical endoconidia with rounded ends. They were 8-16 μm length and 4–6 μm width. Phialides were 43-46 μm length, rounded bases (7-9 μm width) and tapering to the neck´s tip (4-6 μm width). Brown chlamydospores (aleuriospores), 9-13 μm length and 8-12 μm width, in chains of 2-8 spores were observed. For molecular identification, total genomic DNA was extracted. ITS fragment of 565 pb was amplified using ITS5/ITS4 primers and sequenced. The sequence indicated 99% identity with *Berkeleyomyces basicola* (synonymous: *Thielaviopsis basicola*). This was deposited in GenBank as (KX580957) (CBS: C430.74, Gen Bank accession number AF275482.1). This is the first report of *B. basicola* in sweet potato in Argentina, a potential threat to storage root yields.

Keywords

Ipomoea batatas • root rot • *Chalara elegans* • potato • sweet potato fungal disease

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Resumen

Muestras sintomáticas de batata cv Arapey INIA, fueron recolectadas en lotes de producción comercial en Colonia Molina, Guaymallén, Mendoza, Argentina. Estas presentaban lesiones redondeadas, oscuras, frecuentemente coalescentes, con micelio granuloso blanco. El hongo fue aislado a partir de batatas sintomáticas que representaban la infección generalizada del cultivo. Los aislamientos fueron sembrados en APG con sulfato de estreptomicina e incubados durante siete días a 21°C con alternancia de luz blanca/luz negra (UV-400 nm). Las observaciones al microscopio óptico revelaron la presencia de endoconidios hialinos, no-septados, cilíndricos con extremos redondeados (8-16 x 4-6 μm), fiálides (43-46 μm de largo) de base redondeada (7-9 μm) y cuello que se estrecha hacia la punta (4-6 μm). También se observaron clamidosporas marrones (aleuriesporas) de 9-13 x 8-12 μm, en cadenas con 1-7 septos. El ADN genómico fue extraído para la identificación molecular. Un fragmento ITS de 565 pb fue amplificado usando los iniciadores ITS5/ITS4 y secuenciado. La secuencia reveló 99 % de identidad con *Berkeleyomyces basicola* (sinónimo: *Thielaviopsis basicola*) y está depositada en el GenBank como KX580957 (CBS: C430.74, GenBank accession number AF275482.1). Esta constituye el primer reporte de *B. basicola* en el cultivo de batata en Argentina y una amenaza potencial para la producción de raíces reservantes.

Palabras clave
Ipomoea batatas • podredumbre de raíz • *Chalara elegans* • enfermedad fúngica en batata

Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam) is among the seven most important food crops worldwide. It has great potential not only for human consumption, but also for animal feeding and industrial use (4). It has been considered as a calorie source in many circumstances, such as in Japan when typhoons destroyed rice crops, or during the 1930s depression in the USA. Besides, storage roots can also be used for fermentation, as a source of starch for food processing, or production of chemical stocks, including organic acids (16). It is considered a subsistence crop as it can be produced with few inputs. Moreover, sweet potato tolerates stresses such as drought and, in the absence of frost, it can be left in the field to be harvested when needed (14).

The fungus *B. basicola* (synonymous *T. basicola* and *Chalara elegans*) (20, 21) is the causal agent of black root rot. This fungus infects a wide range of hosts, including plants from at least 15 families worldwide, including Argentina (9). On sweet potato (*I. batatas*), it was isolated from black rotted roots in China, Japan, Indonesia, Papua New Guinea, New Zealand and the USA (15).

B. basicola is a soilborne hemibiotrophic fungus. The infection cycle begins with a biotrophic phase followed by a necrotrophic phase (18). It can also be associated with hosts in a nonpathogenic manner (26) and is capable of saprophytic utilization of soil organic matter (10, 22). The life cycle of *B. basicola* has been described for tobacco (12), pansy (18) and cotton (17). Based on these descriptions, this cycle is divided into six major steps: (i) germination of spores; (ii) growth of the germ tube towards roots; (iii) attachment to root surface-the first contact and initial host-pathogen recognition; (iv) pathogen differentiation into infection structures and penetration into host cells; (v) establishment of a biotrophic phase; and (vi) conversion to necrotrophic phase (root rotting) and production of new spores.

B. basicola produces two types of spores, endoconidia (also known as phialospores) and chlamydospores (or aleuriospores), that could be produced singly or clustered as chains (1, 22). Two complex cell-wall systems have been observed in chlamydospores. An outer cell wall encloses the whole chlamydospore chain and each individual compartment possesses its own separate cell wall. Both cell walls are composed of an outer highly electron-dense layer and an inner layer, which appeares to be electron-transparent (8). In culture, endoconidia have been produced within 24 h and chlamydospores, within 3 days (24); each of these with some variations depending on the isolate and culture conditions.

The presence of the two types of spores and the shape of the chlamydospores are diagnostic morphological tools for the identification of the species. Since the endoconidia have the typical features of *Chalara* complex (19, 21), *T. basicola* was placed into this genus just like *C. elegans*. Nel *et al.* (2017), *T. basicola* was described as *Berkleyomyces* gen nov. and the current name is *B. basicola.*

Healthy nursery materials, fungicides, and crop rotations are important elements for disease control, but they require early diagnosis and pathogen detection in either plant, soil or water. Rapid and accurate identification and detection of *B. basicola* would improve diagnosis and prophylaxis (13).

Previously published works have shown black root rot caused by *B. basicola* on other crops in Argentina (3, 5). However, these crops do not share the same production environment with sweet potato in Mendoza. The symptoms observed in field sweet potatoes, clearly match those described for black root rot. Therefore, the hypothesis was that these symptoms are produced by *B. basicola*. The objective of this study was to identify the causal agent of sweet potato root rot in Argentina.

Materials and Methods

Storage roots of sweet potato cv. Arapey INIA with black root rot symptoms were collected from a commercial field in Colonia Molina, Mendoza province, Argentina (-32.8316311,-68.7238142), in May 2017.

The fungus was isolated from three symptomatic roots, which represented the generalized infection that affected the crop. The isolates were obtained by removing mycelium of the rounded black, sometimes coalescing superficial lesions using a sterile needle (figure 1a, page 286). The lesions were 3-10 mm diameter, not deeper than 3 mm and with granular white mycelium dispersed on the surface (figure 1.b, page 286). The fungus isolates, morphologically identified as *B. basicola* (2, 6, 11) were transferred to plates containing potato dextrose-agar plus streptomycin sulfate (150 mg/l). They were incubated for 7 days, at 21°C with 12 h alternation of white/black (UV-400 nm) light. An optical microscope (NIKON *eclipse* T*i*) was used to observe specific structures developed on colonies. The monosporic isolates were deposited in Instituto de Patología Vegetal-INTA mycobank (collection in process of registration). Molecular identification was performed through DNA extraction from a pure culture (N°118 strain) according to Dellaporta *et al.* (1983). A Polymerase Chain Reaction (PCR) of rDNA was carried out using ITS5/ITS4 primers (25) according to previously published conditions (23). The obtained PCR product was purified for sequencing and checked by BLAST analysis.

Results and Conclusions

Colonies initially white, turned brown after 7 days of incubation. Morphological features were consistent with those previously described (2, 6, 11): non septated, cylindrical, hyaline, endoconidia with rounded ends and 8-16 μm length x 4-6 μm width. They emerged from 43-46 μm length phialides with rounded bases 7-9 μm width, tapering to the neck´s tip (4-6 μm in width). Detected chlamydospores (aleuriospores) were 8-12 x 9-13 μm, arranged in chains of 2-8 spores, with thick brown walls and hyaline ends (figure 2, page 286).

An ITS fragment of 565 bp from isolate, was amplified. The alignment analysis indicated 99 % identity with the *B. basicola* collection, and the sequence was sent to GenBank (KX580957) (CBS: C430.74, GenBank accession number AF275482.1).

According to morphological and molecular features, *B. basicola* is the causal agent of sweet potato black root rot. This is the first report about sweet potato black root rot in Argentina, a potential threat to this crop in the Mendoza production area.

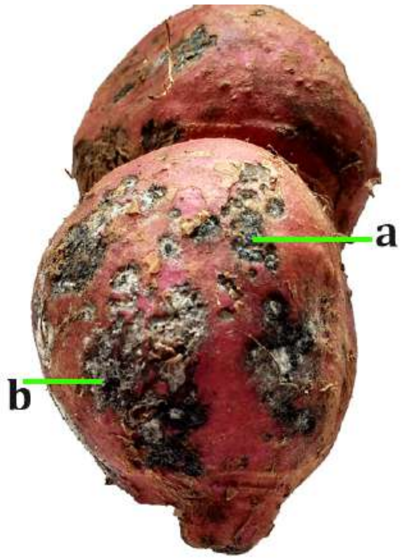

Figure 1. Sweet potato with typical black root rot symptoms. a- Black lesions. b- White mycelium on lesions.

Figura 1. Batata con síntomas típicos de podredumbre negra de raíz. a- Lesiones negras. b- Micelio blanco sobre lesiones.

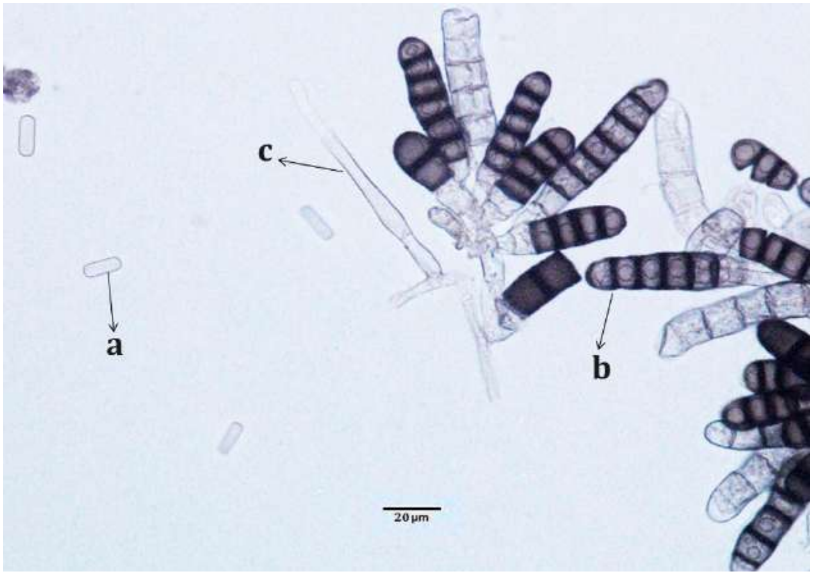

Figure 2. a- Endoconidium. **b-** Chain of chlamydospores. **c-** Phialide.

Figura 2. a- Endoconidio. **b-** Cadena de clamidosporas. **c-** Fílide.

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Ruminal ammonia concentration and fermentation kinetics of commercial herbal feed additives with amino acids

Cinética de liberación de nitrógeno amoniacal y fermentación ruminal de aditivos para piensos a base de hierbas con aminoácidos

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Abstract

The objective of this study was to characterize the chemical composition of rumen fermentation while estimating it's *in vitro* protein degradation (from ruminal ammonia concentration) and kinetics regarding two herbal feed plant additives. The tested herbal mixtures were elaborated with *Phaseolus mango* and *Linum usitatissimum,* providing lysine (Lys) and *Trigonella foenum-graecum* and *Allium sativa,* providing Methionine (Met). They were compared to alfalfa (*Medicago sativa*) and solvent extracted soybean meal (*Glicine max*), as standard sources of protein using the *in vitro* gas production technique modified to estimate $N\text{-}NH_3$, recording fermentation kinetics and dry matter digestibility (72 h), in a completely randomized design followed by Tukey test. Ruminal ammonia concentration in the herbal mixtures was lower ($P<0.05$) than in the standard protein sources, indicating that protein from herbal mixtures could resist ruminal degradation. Herbal additives with Lys or Met showed minimum $N\text{-}NH_3$ concentration in the first 4 h of incubation. At 8 h, the concentration was 0.27 and 0.54 mg dL^{-1} for the herbal products with Lys and Met, significantly lower than solvent extracted soybean meal and alfalfa (1.15 and 2.24 mg dL^{-1} respectively, $P<0.05$).

Keywords
Allium sativa • Feed plant additive • *Linum usitatissimum* • lysine • methionine • ammonia nitrogen • *Phaseolus mango* • protein • rumen • *Trigonella foenum-graecum*

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Resumen

El objetivo del estudio fue caracterizar la composición química, estimar la degradación de la proteína *in vitro* (a partir de la concentración de N amoniacal) y los parámetros de cinética de fermentación ruminal de dos aditivos herbales. Las mezclas herbales probadas están elaboradas con *Phaseolus mango* y *Linum usitatissimum*, para aportar lisina (Lis), y con *Trigonella foenum-graecum* y *Allium sativa*, para aportar metionina (Met), mismas que fueron comparadas con alfalfa (*Medicago sativa*) y harina de soya extraída con solvente (*Glycine max*), como fuentes estándar de proteína, usando la técnica de producción de gas *in vitro* modificada para liberación de $N\text{-}NH_3$, estimando la cinética de fermentación y la digestibilidad de materia seca (72 h) en un diseño completamente al azar, con prueba de medias de Tukey. La concentración de N amoniacal de las mezclas herbales fue menor (P<0,05) que las fuentes de proteínas estándar, lo que indica que su proteína podría resistir la degradación ruminal. Los aditivos herbales con Lis o Met mostraron una concentración mínima de $N\text{-}NH_3$ en las primeras 4 h de incubación; a las 8 h la liberación, para dichos productos fue de 0,27 y 0,54 mg dL^{-1} respectivamente, menor (P<0,05) que la harina de soya extraída con solvente y alfalfa (1,15 y 2,24 mg dL^{-1} respectivamente).

Palabras clave

Allium sativa • Aditivo herbal • *Linum usitatissimum* • lisina • metionina • nitrógeno amoniacal • *Phaseolus mango* • proteína • rumen • *Trigonella foenum-graecum*

Introduction

Estimation of protein degradation for evaluation of feeds and calculation of the escape protein value of a particular protein, is necessary for formulating and meeting ruminant requirements (15, 39). Amino acids absorbed in the small intestine of ruminants derive from microbial protein and from dietary protein that escape ruminal degradation. In weaned young ruminants, a smaller rumen size (compared to the adult) limits dry matter intake and consequently microbial protein synthesis, resulting in the duodenal flow of microbial amino acids theoretically not being adequate to reach maximum growth potential (38, 39). Protein composition reaching the small intestine is also of great relevance given that microbial protein is considered limited in certain amino acids such as lysine, methionine and threonine, for growing ruminants (9, 47). Therefore, dietary undegradable rumen protein should contain amino acids that could complement the microbial contribution (38, 44).

Protein in plant products degrades extensively in the rumen. However, several herbaceous legumes such as *Phaseolus* spp and *Trigonella* spp have high concentrations of polyphenolic compounds (mainly tannins) and other secondary metabolites, that allow some protection from ruminal microorganisms. These tannins affect bacterial populations, improving ruminal fermentation efficiency, performing as natural feed additives (22, 25). These plants could increase amino acid absorption in the intestine. However, evidence about ruminal degradation and characterization of this type of species, is scarce. Therefore, the objective of the present study was to characterize two sources of herbal mixtures containing limiting amino acids (Lys and Met), analyze their chemical compositions, estimate ruminal protein degradation using ruminal ammonia concentration compared with known feeds as standard sources of protein (alfalfa and solvent extracted soybean meal) and characterize the kinetics of rumen dry matter degradation with the *in vitro* gas production technique.

Materials and methods

Two herbal mixtures were tested: one from *Phaseolus mango* and *Linum usitatissimum* (as contributor of herbal lysine; HL) and another from *Trigonella foenum-graecum* and *Allium sativa* (as herbal methionine; HM), in addition to alfalfa (*Medicago sativa*) and solvent extracted soybean meal (*Glycine max*), as standard sources of protein. The four protein substrates (HL, HM, alfalfa and solvent extracted soybean meal) were independently tested.

OptiMethionine and OptiLysine, corresponding to HM y HL respectively, were provided by Nuproxa Mexico (Indian Herbs and Nuproxa Switzerland). These feed plant additives are standardized products and have certified mixtures, following ISO 9001 and GMP (Good Manufacturing Practices).

Herbal products composition was determined using the methodology established by the A.O.A.C. (3): humidity percentage (method 934.01), crude protein by Kjeldahl method (N x 6.25) (method 954.01), ether extract (method 920.39) and ashes by calcination at 550 °C (method 923.03). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the Van Soest *et al.* procedures (1991).

In vitro gas production was determined according to Theodorou *et al.* (1994) modified in order to measure ammoniacal nitrogen. Rumen liquor was collected from a cannulated Holstein bull, about five years old and weighing 600 kg. Ruminal fluid was filtered through eight layers of cheesecloth and held under CO_2 in a water-bath at 39 °C. A 0.5 g sample was placed in 125 mL amber flasks. Gas pressure was measured at 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 42, 48, 60 and 72 h of incubation, using a manual pressure gauge (scale 0 to 1 kg cm^{-2}). Gas pressure was then transformed into volume through the linear equation proposed by Ørskov and McDonald (1979). Gas production kinetics, namely maximum volume of gas produced (Vmax), Lag phase (L), gas production rate (S) and time required to reach half Vmax ($K_{0.5}$), were obtained using the logistic model of Pitt *et al*. (1999). *In vitro* dry matter digestibility (IVDMD) was estimated by filtering the residue from the flasks after 72 hours of incubation with a filter paper Waltham No. 41 and dried at 55 ° C for 48 h.

$N\text{-}NH_3$ concentration was determined by the *in vitro* gas production test (described above), extracting 1.2 mL of ruminal fluid from each incubated flask at 2, 4, 8, 12 and 24 h of incubation and mixing with 0.3 mL of metaphosphoric acid, kept at -18 °C. Later, the samples were centrifuged at 12,100 x g for 5 minutes in a Mini-spin eppendorf centrifuge. The supernatant was used to measure $N\text{-}NH_3$ concentration with the phenol-hypochlorite reaction (26). Readings were made at 630 nm with a spectrophotometer (Cary 100 UV-VIS). The results were multiplied by the sample dilution factor (4:1).

Incubation was performed twice. Each assay contained three replicates per tested substrate and their respective blanks. The experimental design was completely randomized with 4 treatments (HL, HM, solvent extracted soybean meal and alfalfa) and 6 repetitions per variable (Vmax, L, S, $K_{0.5}$, IVDMD, as well as the $N\text{-}NH_3$ concentration at 4, 8, 12 and 24 hours). Each flask used in gas production tests was considered an experimental unit. Data were analyzed with the JMP statistical software (42) and the means were compared with the Tukey test (P< 0.05).

Results and discussion

Chemical composition was similar between herbal mixtures (table 1, page 291). Considering that their formulation contains at least one legume (*P. mango* or *T. foenum-graecum*), crude protein percentage could have been higher than the value determined (20, 43, 44). However, the exact proportion in which these legumes and their plant parts are present in the herbal mixture, are unknown. Pinto *et al.* (2010) mentioned that forage chemical composition can be affected by specie, forage age, plant organ (fruits or leaves), growth site and soil type, among others. The tested herbal mixtures are certified by an amino acid formulation (lysine or methionine) analysis.

† NDF: neutral detergent fiber, ADF: acid detergent fiber, ME: metabolizable energy.
¶ Estimated with *in vitro* digestibility equations (33) .

† NDF: fibra detergente neutro, ADF: fibra detergente ácido, ME: energía metabolizable.
¶ Estimada con ecuaciones de la digestibilidad *in vitro* (33) .

Table 1. Partial chemical composition of herbal mixtures.

Tabla 1. Composición química parcial de las mezclas herbales.

Item	Herbal lysine (HL)	Herbal methionine (HM)
Dry matter (%)	96.0	97.8
Organic matter (%)	92.6	85.0
Crude protein (%)	8.2	9.2
NDF (%)†	52.6	44.1
ADF (%)	33.1	25.9
Ether extract (%)	6.7	4.8
Calcium (%)	1.44	0.97
Phosphorus (%)	0.44	0.54
Methionine (g/100g protein)	1.74	5.23
Lysine (g/100g protein)	11.19	0.81
ME (Mcal kg^{-1}) ¶	2.57	3.02

Kassi *et al.* (2000) explained that high concentrations of neutral detergent fiber (NDF) in forage are associated with lower intake, and high concentrations of acid detergent fiber (ADF) are associated with low ruminal digestibility, both undesirable characteristics reflected in the results of IVDMD in table 2. Legumes like *P. mango* have an advantage over grasses, because they mainly lignify their stems and not their leaves, as most grasses used for grazing. Therefore, greater stability in forage nutritional quality of woody legume species, is observed over time (7). These herbal mixtures are composed by finely ground herbs, therefore the effective NDF contribution is low, with minimal intake or digestion effects.

Legumes are considered a good source of protein, essential amino acids (such as lysine, leucine, isoleucine, phenylalanine and valine), unsaturated fatty acids (like linolenic and linoleic acids), dietary fiber, minerals (Ca, Fe and Zn) and vitamin C (4, 13, 14, 20), as well as various secondary metabolites. The species included in the mixtures evaluated, provide good metabolite diversity. The genus *Phaseolus* contains protease inhibitors, phytic acid (11) and polyphenols, as well as condensed tannins and flavonoids (anthocyanin glycosides: cyanidine, definidine and pelargonidine) (11, 20). *L. usitatissimum* contains polyunsaturated oils, mainly linolenic acid, as well as polyphenolic compounds, called lignans, with antioxidant activity (6, 12). The legume *T. foenum-graecum*, has antioxidant effects given by glutathione, β-carotenes and α-tocopherol, as well as tannins, alkaloids and saponins, particularly diosgenin (30, 50). Finally, *A. sativum* is a rich source of essential oils and organosulphurous compounds such as allicin, with specific antimicrobial activities. Total phenols, condensed tannins and essential oils present in this plant appear to be biologically active secondary metabolites, which modify rumen fermentation (22, 23, 34) and explain the resistance to protein degradation in the *in vitro* incubation.

Table 2. Kinetic parameters of ruminal fermentation and *in vitro* digestibility from herbal mixtures and protein sources.

Tabla 2. Parámetros de cinética de fermentación ruminal *in vitr*o y digestibilidad de mezclas herbales y fuentes proteicas.

Parameter	Source †				
	HL	HM	SM	A	SEM
$Vmax^{72}$(mL g^{-1})	292.07^{b}	327.77^{ab}	340.67^{a}	290.43^{b}	7.541
S (h^{-1})	0.038^{c}	0.044^{ab}	0.039^{bc}	0.046^{a}	0.0012
L (h)	0.83^{c}	1.06^{c}	2.22^{b}	2.97^{a}	0.265
$K_{0.5}$ (h)	18.46^{a}	15.59^{bc}	17.47^{ab}	15.04^{c}	0.472
IVDMD ^{72} (%)	75.60^{d}	83.89^{b}	97.69^{a}	78.51^{c}	2.565

† HL: herbal lysine, HM: herbal methionine, SM: solvent extracted soybean meal, A: alfalfa, Vmax: maximum volume, S: fractional rate, L: Lag phase, $K_{0.5}$: time required to reach half of Vmax, IVDMD: *in vitro* dry matter digestibility, SEM: Standard error of mean. abc Means with different literals in the same row are different (P<0.05).

HL: Lisina herbal, HM: metionina herbal, SM: harina de soya extraída con solvente, A: alfalfa, Vmax: volumen máximo, S: tasa fraccional, L: fase lag, $K_{0.5}$: tiempo requerido para alcanzar la mitad de Vmax, IVDMD: digestibilidad *in vitro* de la materia seca, SEM: error estándar de la media. abc Medias con diferente literal en la misma hilera son diferentes (P<0,05).

Ruminal fermentation parameters are shown in table 2 (page 291). Solvent extracted soybean meal generated the highest gas production, probably due to the type of carbohydrates of the substrate since cellulose predominates in alfalfa and herbal mixtures. Soto *et al.* (1994) found that peptides and amino acids addition had no effect on ruminal fermentation parameters *in vitro* when bacteria is grown on cellulose-rich substrates. However, bacterial growth was stimulated with cellobiose or glucose. Although not statistically significant, gas production of herbal lysine was lower than that of herbal methionine, coinciding with Hernández *et al*. (2010), who explainedthat leaves with lower energy value generate less gas *in vitro*.

It has generally been observed that forages have a longer *lag* phase, especially when being of poor quality (2). However, although the herbal compounds analyzed in the present study have similar composition to that of any other forage, they have a very short *lag* phase, shorter than that determined for solvent extracted soybean meal and alfalfa, which can be explained by the proportion of its cellular contents.

Table 3 shows that N-NH_3 concentration achieved by herbal mixtures (HL and HM) was very low during the first 12 h of incubation compared to protein standards, remaining constant from the 4th hour of incubation. Raab *et al.* (1983) and Lorenz *et al*. (2011) reported that N-NH_3 concentration can be an estimator of protein degradation in the rumen. Therefore, these results would imply that part of the protein contained in these forages is resistant to rumen degradation.

Table 3. Ruminal N-NH_3 concentration (mg dL^{-1}) from herbal mixtures and protein sources incubated *in vitro.*

Tabla 3. Concentración ruminal de N-NH_3 (mg dL^{-1}) de mezclas herbales y fuentes proteicas incubadas *in vitro.*

Time h	Source †				
	HL	HM	SM	A	SEM
4	0.00[c]	0.02[c]	1.15[b]	2.24[a]	0.154
8	0.27[b]	0.54[b]	2.76[a]	3.33[a]	0.339
12	0.34[b]	0.57[b]	5.19[a]	4.75[a]	0.245
24	4.23[b]	3.43[b]	16.94[a]	6.74[b]	1.233

† HL: herbal lysine, HM: herbal methionine, SM: solvent extracted soybean meal, A: alfalfa, SEM: Standard error of mean. [abc] Means with different literals in the same row are different ($P<0.05$).

† HL: Lisina herbal, HM: metionina herbal, SM: harina de soya extraída con solvente, A: alfalfa, SEM: error estándar de la media. [abc] Medias con diferente literal en la misma hilera son diferentes ($P<0,05$).

Rates of rumen protein degradation depend on multiple factors. Bach *et al*. (2005) mention protein structure, interaction with other nutrients and proteolytic activity of the ruminal microbiota as key factors, among others. However, enzymatic inhibitors or anti-nutritional factors may influence protein degradation (27, 40). The most studied antinutritional factors are tannins, followed by secondary plant metabolites such as saponins, cyanogenic compounds, lecithins, alkaloids, oxalic acid and flavonoids (18, 19, 29). These factors can influence rumen protein degradation and synthesis, either by directly affecting the ruminal microorganisms, or by their interaction with nutrients (8, 22, 27).

Plants like *P. mungo*, *T. foenum-graecum* and *A. sativum*, contain significant amounts of condensed tannins, that react with proteins forming tannin-protein complexes through hydrogen bonds, hydrophobic interactions, ionic bonds and covalent bonds (41). These complexes can affect bacterial enzymes inhibiting carbohydrate and protein fermentation (16). In addition, tannins affect rumen proteolysis given that they can associate with soluble dietary proteins, protecting them from microbial action (8, 16, 41).

It has been reported that 100 g of *T. foenum-graecum* provides 4.63 g of saponins (1, 50). Hu *et al.* (2005) found that when the amount of saponins in an *in vitro* fermentation test is increased, the concentration of ammonia decreases significantly. Wang *et al.* (2000) also observed a decrease in N-NH_3 by including *Yucca schidigera* (a product rich in saponins), both *in vivo* (sheep feeding) and *in vitro*.

Various secondary metabolites have been identified when reducing the protozoan population. Total polyphenols, condensed tannins and saponins, were all reported as secondary metabolites of forages used in the herbal mixtures of the present study. Galindo and Marrarero (2005) reported the effect of *Leucaena leucocephala*, *Enterolobium cyclocarpum*, *Gliricidia sepium*, *Sapindus saponaria* and other plants on rumen ciliated protozoa. Given that protozoa have high protein requirements, and ruminal N-NH_3 concentrations decrease with defaunation, this has been considered advantageous in low protein diets (31).

Decreasing rumen protein degradation of herbal mixtures (table 3, page 292) represents an advantage. Coomer *et al.* (1993) and De Almeida *et al.* (2015) statedthat supplementing young ruminants' diet with undegradable rumen protein, can increase protein and amino acid flow to the lower gastrointestinal tract and, therefore, increase the metabolizable protein supply. These herbal mixtures could be used as additives, not as protein supplement. To feed diets that maintain the concentration of N-NH_3 for microbial protein synthesis between 4 to 10.0 mg of N-NH_3/100 mL of rumen fluid, as well as to provide available energy for the ruminal ecosystem (41), is important. Ammonia values from the herbal mixtures in the first 12 h were below these values compared to the standard protein (table 3, page 292). Galindo and Marrarero (2005) mention that some legumes have soluble proteins, highly degradable in rumen, making it necessary to guarantee enough energy for adequate synthesis of microbial protein. Ruminal N-NH_3 concentration has been used to estimate rumen degradation (37) and lambs supplemented with herbal lysine have improved growth (28) confirming resistance to rumen degradation of the feed plant additive.

Conclusions

Ammonia concentrations found in the present study would imply that part of the protein contained in these forages is resistant to rumen degradation. Herbal mixtures evaluated could be used in ruminants feed as a source of undegradable rumen protein. However, the potential use as amino acid bypass for ruminants, needs further evaluation, in order to assess its degradation throughout the entire digestive tract.

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The effect of residual nitrogen fertilization on the yield components, forage quality, and performance of beef cattle fed on Mombaça grass

Efecto residual de la fertilización nitrogenada sobre los componentes de la producción, la calidad de forraje y la performance del ganado de carne en la gramínea mombaça

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Abstract

The objective of this study was to evaluate the effect of residual nitrogen (N) on pastures of Mombaça grass and its impact on pasture structure and the nutritive value and performance of beef cattle. The experiment analyzed randomized blocks subdivided into plots, with three replications. The research focused on a number of pastures having received three annual doses of N (100, 200 and 300 kg ha^{-1}) from 2015 to 2017, with no N fertilization in 2018. The results indicated that pastures under residual effect of 300 kg ha^{-1} of N were characterized by higher ($P<0.05$) rates of forage accumulation than those under the residual effect of 100 kg ha^{-1} N. Furthermore, this study indicated no effect ($P>0.05$) on the pasture nutritive value and average daily gain (0.490 kg day^{-1}). The stocking rate was higher ($P<0.05$) in pastures under the residual effect of 300 kg ha^{-1} of N, and lower in those at 100 kg ha^{-1} of N, while weight gain per area followed the trend observed in the stocking rate. The results thus suggest that the suspension of N fertilization for one year after three years of sequential fertilization was responsible for an immediate loss of 50 and 55% of the productivity of plants and animals, respectively.

Keywords
pasture fertilization • forage accumulation • *Panicum maximum* • stocking rate

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Resumen

El objetivo fue evaluar el efecto del nitrógeno residual y su impacto en la estructura y valor nutritivo del forraje y variables productivas del ganado vacuno de carne en pasturas de Mombaça. El diseño experimental fue en bloques al azar, en parcelas subdivididas, con tres repeticiones. Las pasturas recibieron tres dosis anuales de nitrógeno (N) (100, 200 y 300 kg ha^{-1}) de 2015 a 2017. La fertilización se suspendió en 2018, para estimar el efecto residual del nutriente. Las pasturas que recibieron 300 kg ha^{-1} de N mostraron un efecto residual en tasa de acumulación de forraje más alto ($P<0,05$) que las que recibieron 100 kg ha^{-1} N. No hubo efecto ($P>0,05$) para valor nutricional de forraje ni ganancia diaria promedio (0,490 kg $día^{-1}$). La carga animal fue mayor ($P<0,05$) en pasturas bajo el efecto residual de 300 kg ha^{-1} de N, y menor en el efecto residual de 100 kg ha^{-1} de N, y el aumento de peso por área siguió la tendencia observada en la carga animal. La suspensión de la fertilización nitrogenada durante un año después de tres años de fertilización secuencial en Mombaça pastoreados por ganado promueve pérdidas inmediatas de 50 y 55% de la productividad de plantas y animales, respectivamente.

Palabras clave

fertilización de pasturas • acumulación de forraje • *Panicum maximum* • carga animal

Introduction

Beef cattle production in Brazil represents an essential segment of the economy. The high potential for the use of tropical grasses established since the 1990s, is the responsible factor for the increase of productivity indexes in livestock. Despite the large pasture area, many production units do not yet fulfill their full production capacity due to poor management of the soil-plant-animal system. Failures in grazing management (stocking rate adjustments) and pasture maintenance fertilization lead to an unsuccessful activity, which reduces the ability to generate income making it financially unsustainable over the years.

Nitrogen (hereinafter N) fertilization along with maintenance fertilization are fundamental to ensure pasture productivity (22, 35), and the failure to adopt these technologies is considered one of the primary triggers of degradation. The productivity of a pasture is evaluated based on the individual performance of the animals and the stocking rate used (13, 18). The individual performance depends on the genetic potential of the animal (19) and the quantity, quality, and form of forage that it is fed (15). Also, the N fertilization promotes increases in forage accumulation (5), altering pasture dynamics (20, 28) with direct reflexes in animal and per area production (16).

Although forage production can improve with N fertilization (6, 20), increasing secondary productivity (9), the use of N has been limited by cost, due to the extension of the areas involved and the need for frequent applications. In addition, N can be easily lost when not associated with the organic fraction of the soil (37). Research evaluating the residual effect of nitrogen has shown promising results for agricultural crops (29, 36). Thus, a better understanding of the responses of forage plants and animals to residual fertilization with N may suggest an alternative way to reduce costs by managing inputs, mechanization and labor, in addition to revealing the impact of not using N fertilization on productivity (35).

The current study tests the hypothesis that suspending N fertilization of Mombaça grass for one year after three years of sequential fertilization with the same doses, promotes a residual effect of N on pasture structure, as well as on the forage nutritive value and the performance of beef cattle. To determine this, the research evaluates the residual effect of N on Mombaça grass and the impact of this effect on structure and nutritive value of the sward and performance of beef cattle.

Material and methods

Experiment location and edaphoclimatic monitoring

The experiment was carried out at Embrapa Beef Cattle in Campo Grande, Mato Grosso Sul (figure 1). The experimental area is situated at latitude 20°27' S and longitude 54°37' W and boasts an average altitude of 530 m above sea level. The experiment took place over 196 days from 11/07/2017 to 05/22/2018.

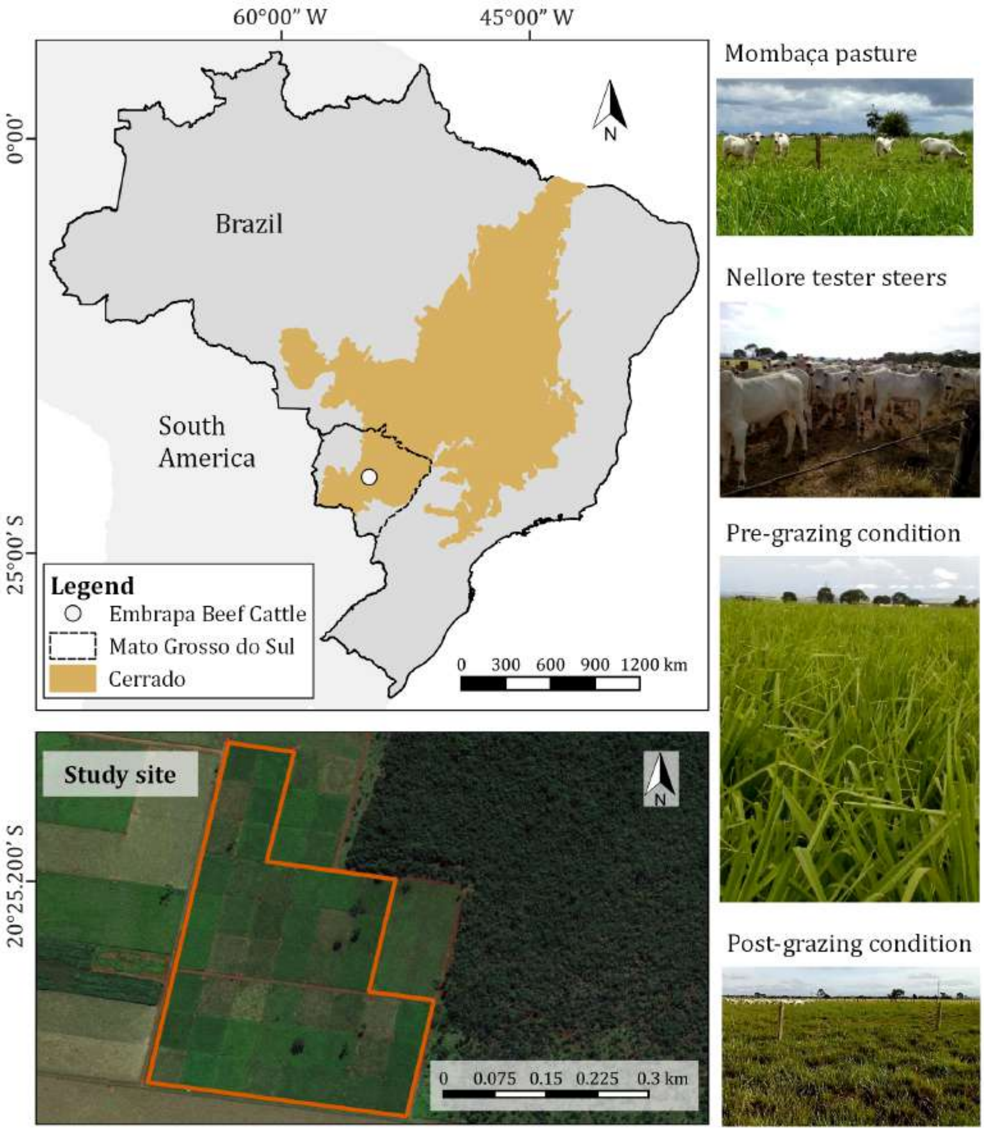

Figure 1. Geographic location of the experimental area and experimental conditions.
Figura 1. Ubicación geográfica del área experimental y condiciones experimentales.

According to the Köppen climate classification, this region is of type AW: a tropical, rainy savannah, with a dry period lasting from May to September, the historical average temperature is 23.4°C. All precipitation that occurred in the area during the experimental period was documented (figure 2, page 299). Climatic data was extracted from the National Institute of Meteorology (2018) database and gathered by the meteorological station located at Embrapa Beef Cattle.

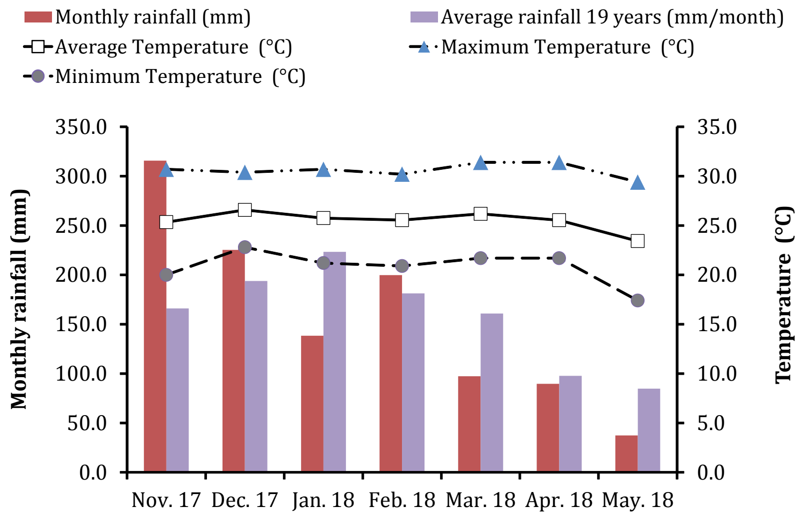

Figure 2. Precipitation and average, minimum, and maximum temperatures during the experimental period and average rainfall for the period year 1 - year 19.

Figura 2. Precipitación y temperaturas promedio, mínima y máxima durante el período experimental y precipitación promedio el período año 1 - año 19.

The average temperature and monthly precipitation were used to calculate the water balance (figure 3). The soil water storage capacity was determined to be 75 mm. The soil of the experimental area is Red Latosol, with clay contents between 30 and 35%. Before the start of the experiment, the soil was sampled in the 0-10, 0-20 and 20-40 cm layers for chemical analysis (table 1, page 300).

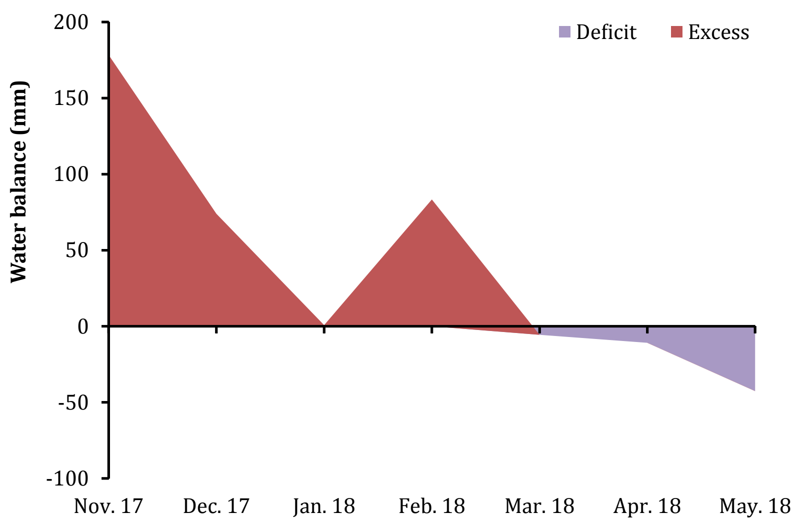

Figure 3. Water deficit and excess in the soil during the experimental period from 11/07/2017 to 05/11/2018.

Figura 3. Déficit y exceso de agua en el suelo durante el período experimental del 07/11/2017 al 11/05/2018.

Table 1. Chemical characteristics of the soil in the experimental area, in the depths of the layers of 0 - 10, 0 - 20 and 20 - 40 cm.

Tabla 1. Características químicas del suelo en el área experimental, en las profundidades de las capas de 0-10, 0-20 y 20-40 cm.

Layer	pH	Ca^{++}	Mg^{++}	K^{+}	Al^{+3}	H+Al	BS	T	t	V	m	OM	P*
	$CaCl_2$	----------- $cmolc/dm^{-3}$-----------								%			mg/dm^{-3}
0-10	5.7	2.5	1.3	0.4	0.2	3.5	4.1	7. 6	4.1	54.2	0.4	4.2	8.4
0-20	5.6	2.4	1.2	0.3	0.1	3.1	3.9	7.0	3.9	5 6.0	0.3	3.9	4.9
20-40	5.5	1.3	0.9	0.2	0.2	3.0	2.4	5.4	2.4	44.9	0.8	2.9	2.9

Ca^{++}: Calcium; Mg^{++}: Magnesium; K^{+}: Potassium; Al^{+3}: Aluminum; BS: Base sum; T: Cation exchange capacity (CEC) at pH 7.0; t: Effective CEC; V: saturation by base; m: saturation by aluminum; OM: Organic matter; P*: Phosphorus by method - Mehlich -1.

Ca^{++}: calcio; Mg^{++}: magnesio; K^{+}: potasio; Al^{+3}: aluminio; BS: suma base; T: capacidad de intercambio catiónico (CEC) a pH 7,0; t: CEC efectiva; V: saturación por base; m: saturación por aluminio; OM: materia orgánica; P*: Fósforo por método - Mehlich -1.

Experiment area and management

The Mombaça grass (*Panicum maximum cv.* Mombaça) studied was initially planted in January 2008 (Blocks I and II) and November 2010 (Block III) and has since been used for the intermittent grazing of beef cattle. The experimental area of 13.5 ha was divided into three blocks, with each block further subdivided into three modules of 1.5 ha, each module into six paddocks of 0.25 ha each.

Over three consecutive years 2014/2015, 2015/2016, and 2016/2017, the pastures received three annual doses of N in the form of urea (100, 200, and 300 kg ha^{-1} of N) and cover fertilization (80 kg ha^{-1} of P2O5 and 80 kg of K2O) (2, 3). Starting in the rainy season of 2017, there was no maintenance or N fertilization.

Intermittent grazing with a variable stocking rate was used, involving five days of occupation and 25 days of rest in the first five grazing cycles (summer) and seven days of occupation and 35 days of rest in the sixth grazing cycle (fall). Fifty-four Nellore male, uncastrated bovine animals initially weighing 205 ± 26.0 kg (the data is displayed average ± standard error) were used and remained in the paddocks during the entire experimental period.

A group of six steers serving as test subjects were also present in each paddock during the experimental period. Twenty-six steers were kept in a reserve pasture (5 ha Massai grass) and used as necessary to maintain a post-grazing height of 40-50 cm, as suggested by Euclides *et al.* (2015). The animals all had access to water and mineral salt ad libitum.

Pasture assessments

Pasture evaluations were always performed in the same previously chosen paddock of each module and both the pre- and post-grazing conditions were assessed during each cycle. The sward height was measured with a centimeter ruler at 40 different points distributed along five imaginary straight lines across each paddock. The sward height at each point was determined as the average height of the curvature of the leaves around the ruler. The average of each paddock was calculated based on these 40 points.

The forage mass (FM) was estimated by cutting the forage contained in nine rectangular areas of 1 m^2 distributed randomly across the paddock. The samples were conditioned in paper bags, weighed, and dried in a forced air ventilation oven at 55°C until the weight stabilized, at which point they were again weighed to estimate the forage dry mass.

To evaluate the morphological components, three subsamples were taken and separated into leaf (leaf blade), stem (pseudostem+sheath), and dead material (18). After a manual separation, the components were dried using the same protocol used to evaluate the FM. The leaf to stem ratio was estimated as the product of the mass of leaves and the mass of stems. The net forage accumulation rate (FAR) was calculated by the difference between the FM in the present pre-grazing and after the previous grazing period, considering only the green (leaf and stem) portion, divided by the number of days between the samplings.

The leaf and stem samples obtained in the pre-grazing state were ground using a 1 mm knife mill and later analyzed for the *in vitro* digestibility of organic matter and crude protein, neutral detergent fiber, and acid lignin detergent, using the Near Infrared Reflectance Spectrophotometry (NIRS) system and the methodology proposed by Merten *et al.* (1985).

Animal evaluations

All steers were weighed every 56 days. The stocking rate was calculated by multiplying the mean weight of the test subject and control steers by the number of days they remained in the experimental module (31).

The average daily gain was calculated by subtracting the weight of the steers at the end of the experimental period from that at the beginning and dividing the result by the number of days (196). The body weight gain per area (kg/ha) was obtained by multiplying the average daily gain of the subjects by the number of animals kept in each experimental plot.

Experimental design and statistical analysis

The current study employs a randomized block design with subdivided plots. The data gathered was submitted to an analysis of variance, for the variables related to pasture (forage accumulation, structural characteristics, and nutritive value), the residual effect of the N doses was allocated in the plot and the grazing cycles in the subplot. The following model was used: $Yijk = \mu + Di + Bj + \alpha ij + Ck + (D*C)ik + \beta ijk$; Yijk = value observed at dose i, block j, cycle k; μ = general average effect; Bj = effect of block J; Di = dose effect i, i = 100, 200 and 300; αij = effect of the random error attributed to the plot; Ck = effect of cycle k, k = 1, 2, 3, ...; (DC) ik = effect of interaction between dose and cycle; βijk = random error assigned to the subplot.

For the variables related to the animals (average daily weight, body weight gain per area, and stocking rate) the data was submitted to analysis of variance taking into account the randomized block design. The following model was used: $Yij = \mu + Di + Bj + \alpha ij$, where: YijK = value observed at dose i, repeat j; μ = general average effect; Di = height effect i, i = 100, 200 and 300; Bj = effect of block j; αij = effect of random error attributed to repetition j.

When determined to be significant by the F test, the effects of the sources of variation and their interactions were analyzed using the Tukey test, at 5% significance.

Results and discussion

The interaction between the residual N effect and the grazing cycles was determined not to be significant (P> 0.05) for the structural variables of the sward, in the pre and post-grazing states. The results revealed no effect of residual N fertilization on canopy height and dead material mass (DMM) in pre-grazing conditions (table 2, page 302). The forage canopy did not reach 90 cm of height in the pre-grazing at any residual level of N. This height is significant, because it is the height required for Mombaça grass to intercept 95% of the light, causing competition for incident light at the base of the plant and triggering an increase in the accumulation of stem and losses by senescence (10, 17, 18). Although the recommended management of the grazing (fixed days of occupation and rest) was not properly followed, it is possible to infer that there was no elongation of the stem nor any accumulation of dead material in the forage canopy, since the heights remained close at 80 cm (90% of the light intercept, hereinafter LI).

Adhering to this height therefore seems to be a possible method for successfully managing Mombaça grass (1).

Furthermore, the swards with a residual effect of 300 kg ha^{-1} of N were characterized by a higher forage accumulation, FM, leaf blade mass (LBM), and stem mass (SM) than those with a residual effect of 100 kg ha^{-1} of N. The averages for these variables in pastures under the residual effect of 200 kg ha^{-1} of N were similar to the those under the effect of the other two levels of residual N (table 2). As pastures were managed with fixed days of occupation and rest, the results of FM, LBM, and SM reflected forage accumulation and soil fertility history according to the previous management, pointing out the residual effect of N on the structural characteristics of the pasture. N accelerates plant metabolism and increases morphogenic processes (4).

The use of higher N rates therefore appears to be responsible for an increase in leaf appearance and leaf elongation rates (4, 30). These morphogenic variables directly affect the structural variables of the pasture (12, 14, 20) and increase forage accumulation.

Table 2. Structural variables (average ± standard error) of the canopy in the pre-grazing of the Mombaça grass under residual nitrogen effect.

Tabla 2. Variables estructurales (media ± error estándar) pre-pastoreo en la canopia de la gramínea Mombaça bajo efecto residual de nitrógeno.

Variables	Doses of N (kg ha^{-1} de N)			P Value
	100	200	300	
Canopy height (cm)	67.1 ±2.7	75.2 ± 2.9	75.8 ± 2.5	0.326
Forage accumulation rate (kg ha^{-1} DM day^{-1})	26.7^{b} ± 4.7	36.3^{ab} ± 6.5	43.4^{a} ± 6.7	0.020
Forage mass (kg ha^{-1} DM)	3371.8^{b} ± 86.8	3637.6^{ab} ± 92.4	3853.9^{a} ± 128.2	0.018
Leaf blade mass (kg ha^{-1} DM)	1790.2^{b} ± 77.6	1968.8^{ab} ± 111.2	2181.7^{a} ± 128.4	0.019
Stem mass (kg ha^{-1} DM)	578.6^{b} ± 26.1	761.5^{ab} ± 27.6	856.5^{a} ± 43.6	0.040
Dead material mass (kg ha^{-1} DM)	989.9 ± 77.2	905.3 ± 121.8	815.3 ± 96.3	0.188
Leaf blade:stem rate	3.3 ± 0.2	2.7 ± 0.2	2.7 ± 0.1	0.204

Averages followed by distinct letters in the line differ (P <0.05) by the Tukey test.

Los promedios seguidos de letras distintas en la línea difieren (P <0,05) por la prueba de Tukey.

Forage accumulation rates were 58.1%, 54.3%, and 47.2% lower for the residual doses of 100, 200 and 300 kg ha^{-1} of N, respectively than the values observed by Barbosa (2018) in 2017, when the pastures received the doses of N. The lower FAR can be explained by the direct effect of N fertilization on the forage plant, since the nutrient is responsible for significant increases in forage production (5, 9). Pastures under the residual dose of 100 kg ha^{-1} of N, presented a pre-grazing FM close to the 3000 kg ha^{-1} of DMM observed in a study by Brâncio *et al.* (2003a).

This study examines Mombaça grass in pastures fertilized with 50 kg ha^{-1} of N having undergone grazing management habits, temperatures and average precipitation similar to those adopted in the current experiment and in the same place (latitude 20°27' S and longitude 54°37' W). Therefore, it seems that the residual effect of 100 kg ha^{-1} of N on forage production is equivalent to the direct effect of 50 kg ha^{-1} of N.

The pastures with a residual effect of 200 and 300 kg ha^{-1} of N presented higher masses of leaf and stem, whereas those at 100 kg ha^{-1} of N behaved in opposite ways (table 2). This explains the absence of an effect on the leaf blade to stem ratio, since this ratio is the product of LBM and stem mass.

The SM was similar across the evaluation months (table 3, page 303). Montagner *et al.* (2012) report a stem elongation rate during the rainy season of 0.04 and 0.003 cm/day in the summers of the first and second year of evaluation.

Luna *et al.* (2014) indicate an elongation rate of 0.04 cm/day. These results suggest that the rest period used was adequate to control the sward structure because the low elongation rates and adequate periods of grazing and rest did not increase significantly for stem mass.

The FAR fluctuated during the evaluation months (table 3, page 303). The highest FAR was observed in February, without statistical difference for the months of January and March, although with variation in the values.

The results of December and April were similar to those of January and March, with all presenting higher FAR than observed in May. The values for height of the pasture were higher in December, January, February and March. On the other hand, FM presented its lowest value in March, with no statistical difference for the months of December, April and May, due to the low accumulation of forage caused by a decrease in temperature and a low rate of precipitation. In December, January and February, the water balance was positive (figure 2 and figure 3, page 299). In fact, the rate of forage accumulation fluctuates throughout the year as a consequence of climatic variations (figure 2 and figure 3, page 299).

The largest LBM were observed in December and January, while the lowest were in May, a time with increased precipitation and reduced temperature (figure 2, page 299). The value of DMM was highest in May, accounting for about 50% of the total FM during this period.

Table 3. Canopy height, forage mass and morphological composition (average ± standard error) in pre-grazing of Mombaça grass under residual nitrogen effect during grazing cycles.

Tabla 3. Altura de la canopia, biomasa total de forraje y por componente (media ± error estándar) en el pre-pastoreo de la gramínea Mombaça bajo efecto de nitrógeno residual durante los ciclos de pastoreo.

Variables	Months						P Value
	Dec/17	Jan/18	Feb/18	Mar/18	Apr/18	May/18	
Canopy height (cm)	75.2^{ab} ± 2.2	82.6^{a} ± 2.7	83.2^{a} ± 3.3	79.6^{ab} ± 2.3	61.1^{b} ± 2.6	54.3^{b} ± 1.8	0.005
FAR (kg ha^{-1} day^{-1})	25.2^{b} ± 2.9	44.8^{ab} ± 4.5	62.2^{a} ± 4.5	40.3^{ab} ± 6.4	20.4^{b} ± 1.9	0.5^{c} ± 0.1	0.001
FM (Kg ha^{-1} DM)	3697.8^{ab} ± 142.2	4045.2^{a} ± 68.9	4068.2^{a} ± 87.6	3019.4^{b} ± 111.6	3167.8^{ab} ± 124.9	$3728,0^{ab}$ ± 71.6	0.001
LBM (Kg ha^{-1} DM)	2635.5^{a} ± 101.8	2295.4^{ab} ± 65.9	2170.1^{b} ± 76.4	1924.4^{bc} ± 82.2	1785.48^{c} ± 104.8	$1070,5^{d}$ ± 74.9	0.001
SM (Kg ha^{-1} DM)	668.7 ± 54.2	761.6 ± 33.8	855.9 ± 36.8	628.3 ± 44.5	678.7 ± 70.0	800,0 ± 62.5	0.052
DMM (Kg ha^{-1} DM)	392.9^{c} ± 55.0	988.2^{b} ± 55.1	1011.9^{b} ± 41.3	466.7^{c} ± 33.4	703.6^{bc} ± 67.9	$1857,5^{a}$ ± 98.0	0.001
Leaf blade:stem rate	4.3^{a} ± 0.3	3.2^{b} ± 0.1	2.6^{b} ± 0.1	3.1^{b} ± 0.2	2.7^{b} ± 0.2	$1,4^{c}$ ± 0.1	0.001

Averages followed by distinct letters in the line differ (P <0.05) by the Tukey test. FAR: forage accumulation rate; FM: forage mass; LBM: leaf blade mass; SM: stem mass; DMM: dead material mass.

Los promedios seguidos de letras distintas en la línea difieren (P <0,05) por la prueba de Tukey. FAR: tasa de acumulación de forraje; FM: masa de forraje; LBM: masa de lámina hoja; SM: masa de tallo; DMM: masa de material muerto.

This can be explained in part by the flowering of the cultivar, which occurs in mid-April in Campo Grande (20°250' S). After the emergence of the inflorescence, no more leaves appear, and there is an increase in the accumulation of dead material (17). This could potentially restrict the consumption of forage (15). However, the dead material, in this condition, was concentrated at the base of the plant (17), forage stratum not exploited by the animals due to the post-grazing height adopted.

In the post-grazing condition, there was no effect (P>0.05) of residual N doses on the canopy height (49.4 ± 2.3 cm), FM (2822.8 ± 109.3 kg ha^{-1} DM), LBM (891.9 ± 41.4 Kg ha^{-1} DM), SM (744.5 ± 55.1 kg ha^{-1} DM), DMM (1186.7 ± 82.0 kg ha^{-1} DM), and leaf blade to stem ratio (1.3 ± 0.2). These results are explained by the fact that the stocking rate was adjusted to lower the pastures to 40-50 cm during the period of occupation, as recommended by Euclides *et al.* (2015 and 2017). Even using fixed days of occupancy and rest, the adjustment of the stocking rate was performed weekly based on the control of the target height of the residue. This adjustment was essential to controlling the grazing and maintaining the structural conditions of the canopy. Briske *et al.* (2008) suggest that the adjustment of the stocking rate is the most important tool in managing pastures under intermittent stocking.

The post-grazing height varied over the evaluation months, reaching its highest value in January and February, (table 4, page 304). In turn, January did not differ from the rest of the months. The average height of the plants in the pastures' post-grazing state was above the established target (40-50 cm). This higher height is likely due to the higher accumulations of forage observed during these months (table 3) and indicated that an increase in the number of animals is not sufficient to lowering the pasture to its target height.

The post-grazing FM was highest in February with a similar value to that observed in May, and this was similar to January and December. The higher rainfall (figure 2, page 299) and daily FAR (table 3) in February explain this result. Even though the low FAR was observed in the month of May, the FM results were similar to the months where they had higher TAF. The flowering of Mombaça grass occurs during this period and, because reproductive tillers are larger than vegetative ones, this modifies the structure sward (30, 32, 33). Since reproductive tillers are rejected by animals, this also increases the DMM.

Table 4. Canopy height, forage mass and morphological composition (average ± standard error) in post-grazing of Mombaça grass under residual nitrogen effect during grazing cycles.

Tabla 4. Altura de la canopia, biomasa total de forraje y por componente (media ± error estándar) en post-pastoreo de la gramínea Mombaça bajo efecto de nitrógeno residual durante los ciclos de pastoreo.

Variables	Months						P Value
	Dec/17	Jan/18	Feb/18	Mar/18	Apr/18	May/18	
Canopy height (cm)	46.0^{b} ± 1.6	52.8^{ab} ± 1.2	58.8^{a} ± 0.7	45.7^{b} ± 1.0	47.2^{b} ± 1.4	45.7^{b} ± 0.8	0.009
FM (kg ha^{-1} DM)	2905.1^{b} ± 75.8	3004.6^{b} ± 83.5	3341.3^{a} ± 73.9	2159.4^{c} ± 49.5	2356.2^{c} ± 84.1	3166.9^{ab} ± 57.3	0.001
LBM (Kg ha^{-1} DM)	1073.4^{a} ± 77.7	1213.9^{a} ± 28.7	1065.0^{a} ± 32.9	661.8^{bc} ± 36.2	798.1^{b} ± 72.7	539.1^{c} ± 46.1	0.001
SM (Kg ha^{-1} DM)	684.3^{b} ± 79.6	870.8^{a} ± 39.7	832.6^{a} ± 37.5	683.3^{b} ± 39.8	708.1^{b} ± 43.4	688.1^{b} ± 35.1	0.001
DMM (Kg ha^{-1} DM)	960.9^{cd} ± 71.5	1106.4^{c} ± 41.2	1443.7^{b} ± 48.2	814.3^{d} ± 37.4	855.0^{cd} ± 81.8	1939.6^{a} ± 75.1	0.001

Averages followed by distinct letters in the line differ (P <0.05) by the Tukey test. FM: forage mass; LBM: leaf blade mass; SM: stem mass; DMM: dead material mass.

Los promedios seguidos de letras distintas en la línea difieren (P <0,05) por la prueba de Tukey. FM: masa de forraje; LBM: masa de lámina de hoja; SM: masa de tallo; DMM: masa de material muerto.

The LBM in the post-grazing state decreased over the cycles due to a decrease in the accumulation rate. According to Lopes *et al.* (2011), the amount of leaves remaining indicates the level of grazing pressure exerted by the animal, with a greater mass of foliar leaf in the post-grazing resulting in better regrowth conditions for the plants. Therefore, it impacts subsequent cycles.

The post-grazing SM was highest in January and February due to the higher entry heights recorded in this period, reflecting the higher forage accumulation (table 3, page 303). The post-grazing DMM was highest in May because of higher values for DMM in the pre-grazing state during this period; the animals reject this component, promoting higher accumulation. The increase in dead material and consequent reduction in leaf mass suggest a loss of efficiency in forage production in periods of lower precipitation and temperature (figure 2, page 299).

There was no interaction between the effect of residual N and grazing cycles (P> 0.05) for the nutritive values of leaves and stems. There was no difference (P> 0.05) in the residual N doses for crude protein (9.3 ± 0.2%), neutral detergent fiber (75.0 ± 0.4%), lignin (2.9 ± 0.1%), and *in vitro* digestibility of organic matter (55.3 ± 0.1%) of the leaf. There was also no effect (P> 0.05) of residual N doses for crude protein (4.5 ± 0.2%), neutral detergent fiber (77.2 ± 02%), lignin (4.2 ± 0.1%) and in vitro digestibility of organic matter (51.1 ± 0.4%) of the stem.

These results are probably connected to the short rest period adopted, since the pasture heights observed in pre-grazing conditions were similar (table 2, page 302) and well below 90 cm, a height at which Carnevalli *et al.* (2006) indicate that the canopy intercepts 95% LI. In this context, Sbrissia *et al.* (2013) demonstrate that pastures of Quicuio grass with up to 95% LI present similar nutritive values to those observed in the current study. Alvarenga *et al.* (2020) also present similar nutritive values for Mombaça grass pastures managed with 90 and 95% of the LI. A significant number of studies examine the influence of plant nutrition on the pastures' nutritive value (5). The current study, however, reveals that the suspension of N fertilization for one year is enough to neutralize the effect of soil fertility on the nutritive value of Mombaça grass.

The nutritive value of the leaf varied over the months of evaluation. In December, January, and February, the highest levels of crude protein and the lowest levels of neutral detergent fiber and acid detergent lignin were observed, altering the *in vitro* organic matter digestibility (table 5, page 305), due to the reduction in cellular content with the increase of the fibrous portion. The higher forage accumulation in this period (table 3, page 303) caused by a higher rainfall (figure 2, page 299) explains this response. Machado *et al.* (2008)

highlight that the availability of water in the soil is a determining factor in the chemical composition of the forage plant. Its presence favors a more intense morphogenesis process, which in turn produces new tissues with a higher nutritive value: such plants, consequently, have a better effect on the animals.

The lower rainfall and consequent soil water deficit and the low temperatures (figure 2, page 299) starting in March are responsible for the lower values of crude protein and higher values of the less degradable parts of the fiber in March, April, and May. This period is therefore critical in animal nutrition.

Table 5. Chemical composition (average ± standard error) of the Mombaça grass under residual nitrogen effect during grazing cycles.

Tabla 5. Composición química (media ± error estándar) de la gramínea Mombaça bajo efecto de nitrógeno residual durante los ciclos de pastoreo.

Variables	Months						P Value
	Dec/17	Jan/18	Feb/18	Mar/18	Apr/18	May/18	
------------Leaf-----------							
Crude protein	9.8^{ab} ± 0.2	11.4^{a} ± 0.3	9.3^{ab} ± 0.1	8.6^{b} ± 0.1	8.8^{b} ± 0.1	8.0^{c} ± 0.3	0.001
Neutral detergent fiber	73.9^{cb} ± 0.3	73.3^{c} ± 0.5	75.0^{b} ± 0.2	73.1^{c} ± 0.4	76.8^{a} ± 0.2	78.1^{a} ± 0.2	0.001
Lignin	2.6^{c} ± 0.1	2.6^{c} ± 0.1	2,9^{b} ± 0.1	3.1^{ab} ± 0.1	3.2^{a} ± 0.1	3.2^{a} ± 0.1	0.001
IVOMD	61.0^{a} ± 0.4	58.3^{b} ± 0.7	55.3^{c} ± 0.4	56.1^{c} ± 0.1	51.2^{d} ± 0.1	50.0^{d} ± 0.7	0.001
------------Stem-----------							
Crude protein	5.2^{a} ± 0.2	5.0^{a} ± 0.2	4.5^{b} ± 0.1	4.5^{b} ± 0.1	3.6^{c} ± 0.1	3.9^{c} ± 0.1	0.001
Neutral detergent fiber	74.1^{b} ± 0.2	77.4^{b} ± 0.2	77.2^{ab} ± 0.1	77.4^{ab} ± 0.4	78.3^{a} ± 0.3	78.8^{a} ± 0.2	0.001
Lignin	4.1^{ab} ± 0.1	4.0^{b} ± 0.1	4.1^{ab} ± 0.1	4.1^{ab} ± 0.1	4.1^{ab} ± 0.4	4.3^{a} ± 0.1	0.003
IVOMD	55.5^{a} ± 0.4	50.2^{b} ± 0.7	50.8^{b} ± 0.2	48.7^{b} ± 0.9	49.4^{b} ± 0.1	50.5^{b} ± 0.4	0.001

Averages followed by distinct letters in the line differ from one another by the Tukey test (P <0.05). IVOMD: *in vitro* organic matter digestibility.

Los promedios seguidos de letras distintas en la línea difieren entre sí en la prueba de Tukey (P <0,05). IVOMD: digestibilidad *in vitro* de la materia orgánica.

The chemical composition of the stem was determined to be similar to that of the leaf, with higher crude protein levels and *in vitro* organic matter digestibility observed during the months with a higher precipitation rate, while higher values of the fibrous fractions were present in the dry-water transition months. The highest canopy heights observed during the rainy season could help increase the plants' support structures. The appearance of tissues with a higher nutritive value (tissue renewal) at this time (24) increases the nutritive value of leaves and stems.

The average daily gain of the animals in the pastures under different doses of residual N varied very little (P = 0.765), with an average of 0.490 ± 0.02 kg day^{-1}. This result is like due to the similar nutritive value of the leaves and stems across the pastures. The average daily gain was lower than that found by Araújo (2017) in pastures of Mombaça grass fertilized with three doses of N (100, 200 and 300 kg ha^{-1} of N) during the rainy season of 2014/2015 (0.540 *vs.* 0.490 kg day^{-1}). The highest individual performance recorded by Araújo (2017) was probably due to the higher crude protein levels in the leaves (10.9 *vs.* 9.3%) and the higher rate of in vitro organic matter digestibility (62.1% *vs.* 55.3%), due or direct effect of N that alters the crude protein content and forage digestibility (11).

The stocking density was highest for the pasture under the residual effect of 300 kg ha^{-1} of N and lowest for the one under the effect of 100 kg ha^{-1} of N, with intermediate value for the pastures under a residual effect of 200 kg ha^{-1} (table 6). The stocking density followed the same pattern of FAR variation (table 2, page 302): as the residual level of N increased (table 2, page 302), more animals were needed to maintain the post-grazing height targets (40-50 cm), which consequently increased the stocking density.

Averages followed by distinct letters in the line differ from one another by the Tukey test (P <0.05). *Animal unit (1 AU = 450 kg of live weight). DWG: daily weight gain per hectare.

Los promedios seguidos de letras distintas en la línea difieren entre sí en la prueba de Tukey (P <0,05). * Unidad animal (1 AU = 450 kg de peso vivo). DWG: aumento de peso diario por hectárea.

Table 6. Performance of beef cattle (average ± standard error) in Mombaça pastures under residual nitrogen effect.

Tabla 6. Producción del ganado de carne (media ± error estándar) en pasturas de Mombaça bajo efecto de nitrógeno residual.

Variables	Doses of N (kg ha^{-1} de N)			P Value
	100	200	300	
Stocking rate (AU ha^{-1})*	2.7^{b} ± 0.2	3.1^{ab} ± 0.2	4.0^{a} ± 0.1	0.013
DWG (kg ha^{-1} of live weight)	466.7^{b} ± 28.3	535.9^{ab} ± 31.6	691.5^{a} ± 33.1	0.011

Brâncio *et al.* (2003b) report a stocking density of 2.0 AU/ha and an individual weight gain of 0.500 kg day^{-1} for Nellore cattle fed on Mombaça pasture fertilized with 50 kg ha^{-1} of N, with grazing management similar to that in the current research during the rainy season. Therefore, the residual effect of 100 kg ha^{-1} of N impacts animal production in a way that is equivalent to the direct effect of 50 kg ha^{-1} of N; this repeats the results observed with regard to crop production.

The body weight per area gain was highest in the pastures under the residual effect of 300 kg ha^{-1} of N and lowest in those at 100 kg ha^{-1}, with once again an intermediate value at 200 kg ha^{-1} (table 6). This result can be attributed to the variation in stocking rate values, since pasture productivity is a result of the combination of individual performance and stocking rate (13, 19). Since there was no difference for individual performance between N doses, the observed behavior for stocking rate was repeated for the body weight gain per hectare.

Suspending N fertilization for one year after sequential fertilization over three years in Mombaça grass pastures can be adopted in strategic cases. As the producer cannot fertilize the entire area every year, this can help determine the fertilization scheduling of pastures. Furthermore, this strategy can help balance the high prices of fertilizer.

Finally, suspending N fertilization is a useful option when the objective of the production unit is not to obtain high gains from animals (such as in the case of producing breeding stock). In fact, this study revealed that the residual effect of 100 N is more or less equivalent to the direct effect of an annual application of 50 kg ha^{-1} of N. The residual dose of 100 kg ha^{-1} of produced approximately 25% less than the residual dose of 200 kg ha^{-1} of and 50% less compared to the residual dose of 300 kg ha^{-1} of N.

Conclusion

Suspending the N fertilization of Mombaça grass for one year after three years of sequential fertilization with the same doses leaves a residual effect on the pasture structure and performance of beef cattle. However, this management is not advantageous. It reduces the rate of forage accumulation by an average of 50%, causing a 55% decrease in animal productivity.

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Effect of urea on gas and effluent losses, microbial populations, aerobic stability and chemical composition of corn (*Zea mays* L.) *s*ilage

Efecto de la urea en las pérdidas por gases y efluentes, poblaciones microbianas, estabilidad eróbica y composición química en el ensilaje de maíz (*Zea mays* L.)

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ABSTRACT

We evaluated the effects of urea addition on gas and effluent losses, fermentation profile, microbial populations, aerobic stability and chemical composition of corn silages. A completely randomised design with five levels of urea (0, 0.5, 1.0, 1.5, and 2.0% based on dry matter) and five replicates was used. A decreasing linear effect of urea levels on effluent losses in corn silages was observed. In parallel, an increasing linear effect of urea levels on pH, increasing from 3.49 to 4.12 in silages without urea in relation to silages with the maximum urea level, was also observed. Urea addition improved the aerobic stability of the silages, with 62 h for the silages without urea and from 90 to >96 h for the silages with urea. Based on the results of the principal components, two groups (I and II) could be distinguished. The most discriminating variables in group I were dry matter (-0.9), pH (-1.2) and lactic acid bacteria (-0.9), while in group II, effluent losses (1.0), ethanol (1.0), acetic acid (0.8) and gas losses (0.8) were most important. The use of urea at inclusion levels of around 2% in corn silage reduced gas losses, improved the nutritive value and promote the aerobic stability of silages.

Keywords
lactic acid • lactic acid bacteria • silage additives • yeasts • *Zea mays* L.

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Resumen

El objetivo del presente estudio fue evaluar el efecto de la urea en las pérdidas por gases y efluentes, poblaciones microbianas, estabilidad aeróbica y composición química en el ensilaje de maíz. Se utilizó un diseño completamente al azar con cinco niveles de urea (0, 0,5, 1,0, 1,5 y 2,0% basado en materia seca) y cinco repeticiones. Hubo un efecto lineal decreciente de los niveles de urea en las pérdidas de efluentes en los ensilajes de maíz y en los valores de pH que aumentaron de 3,49 a 4,12 en los ensilajes sin urea en relación con los ensilajes con el nivel máximo de urea. Los ensilajes de urea mejoraron la estabilidad aeróbica de los ensilajes en comparación con aquellos sin la adición de urea, siendo 62 h para los ensilajes sin urea y de 90 a> 96 h para los ensilajes con urea. El análisis de componentes principales permitió formar dos grupos (I y II). Las variables más discriminatorias en el grupo I fueron: materia seca (-0,9), pH (-1,2) y bacterias del ácido láctico (-0,9); mientras que en el grupo II: pérdidas de efluentes (1,0), etanol (1,0), ácido acético (0,8) y pérdidas de gas (0,8). El uso de urea a niveles cercanos al 2% en ensilaje de maíz reduce las pérdidas de gas, mejora el valor nutritivo y promueve una mayor estabilidad aeróbica de los ensilajes.

Palabras clave

ácido láctico • bacterias de ácido láctico • aditivos de ensilaje • levaduras • *Zea mays* L.

Introduction

Ensiling is a preservation technique for moist forage based on anaerobic fermentation, in which lactic acid bacteria metabolise soluble sugars into organic acids, especially lactic acid. Thus, the biomass becomes acidified, and the forage is preserved until the silo is opened (9, 13, 25).

Among the forage species most suitable for silage production, corn (*Zea mays* L.) is highlighted due to its high nutritive value, adequate dry matter content, high concentration of fermentable carbohydrates, low buffering power and high digestibility. However, the high concentration of water-soluble carbohydrates of many cultivars (10, 13, 20) may produce excess of lactic acid and predispose the ensiled mass to the development of yeasts, especially during aerobic exposure throughout the feeding phase (27). According to Pahlow *et al*. (2003), corn plants may have 3 to 5 log colony-forming unit (CFU)/g of yeasts, which may compromise the aerobic stability of the silage (4, 25), reducing its nutritive value (29) and precluding animal performance (6).

Yeasts are the initiating microorganisms of the aerobic deterioration of silages due to lactate assimilation (13). Aerobic instability is associated with molds and yeasts in corn silages, occurring mainly in the upper layers of the silage, which are more prone to deterioration (4, 10). To minimise this effect, additives with antifungal properties are used to reduce yeast populations and increase aerobic stability in silage (4, 25). Previous studies have focused on the reduction of losses during ensiling and after opening the silo, using chemical additives (16, 27). Urea, used as chemical additive, in silages can be converted into ammonia since it undergoes hydrolysis in the presence of moisture and under urease activity (enzyme catalyst) of plants and microorganisms, producing two molecules of ammonia and carbon dioxide (13). Thus, the addition of urea would provide temporary buffering of the ensiled mass, slightly increasing the pH values and mildly reducing the lactic acid content as a function of the heterofermentative lactic acid bacteria in the initial fermentation stage (17). This would result in silage less prone to aerobic deterioration.

Besides reducing aerobic instability, silages ensiled with non-protein nitrogen can show high contents of protein in the silage, meeting ruminant requirements (6).

In this way, the use of urea as a chemical additive in corn silages with a high concentration of water-soluble carbohydrates in experimentally defined proportions can reduce fermentative losses and the toxic effect to the population of yeasts and moulds, improving the nutritional value of silages.

In this respect, the aim was to evaluate the effect of urea on losses by gases and effluents, microbial populations, aerobic stability and chemical composition in corn silage.

Materials and methods

Experimental area and climatic conditions

The experiment was carried out in the Forage Farming Sector of the Department of Animal Science of the Center of Agricultural Sciences, Federal University of Paraíba - UFPB, located in the mesoregion of Agreste and the microregion of Brejo Paraibano, municipality of Areia, at the coordinates of 06°57'46" S and 35°41'31" W and an elevation of 623 m above sea level. The climate in the region, according to the Köppen classification, is As' (hot and humid), with an average annual rainfall of 1,400 mm, an average annual temperature of 24.5°C and an average relative humidity of 80%.

Experimental design and treatments

Corn harvesting was carried out at an age of 97 days, when the grains were in the milky/pasty stage. Whole plants were manually harvested and chopped to a length of 2 cm with a stationary forage machine. Chopped corn was divided into 10-kg piles, and each pile was assigned to one of the following urea levels: 0, 0.5, 1.0, 1.5 and 2.0%, based on dry matter. All treatments were dissolved in 50 mL of deionised water and sprayed on the forages uniformly and under constant mixing.

The material was packed into five polyvinyl chloride (PVC) experimental silos, with a length of 300 mm and a diameter of 150 mm, to achieve a final packing density of 600 ± 20 kg of fresh matter/m^3. The PVC silos were equipped with a Bunsen's valve to allow gas exit and 1.5 kg of sand at the bottom (for effluent drainage). Subsequently, the silos were sealed, weighed and stored for 70 d in a covered area at ambient temperature (25 ± 2°C).

Dry matter losses

The silos were weighed on the silage day and after 70 days of the fermentation process to estimate fermentation losses. To estimate gases, effluent losses and dry matter recovery, the equations described by Zanine *et al.* (2010) were used.

Chemical analysis and aerobic stability

Before ensiling, one entire corn plant was sampled to estimate its chemical composition and microbial populations prior to ensiling (table 1).

Table 1. Chemical composition and microbial populations of a fresh whole corn plant before ensiling.

Tabla 1. Composición química y poblaciones microbianas de toda la planta de maíz antes del ensilaje.

Dry matter (g kg^{-1})	262.49
Crude protein (g kg^{-1} DM)	62.05
Mineral matter (g kg^{-1} DM)	32.34
NDFap[1] (g kg^{-1} MS)	548.34
Total soluble carbohydrates (g kg^{-1} DM)	152.14
Lactic acid bacteria (log cfu/g)[2]	5.50
Yeasts and moulds (log cfu/g)	4.30

NDFap - Neutral detergent fibre corrected for ash and protein; CFU -Colony-forming unit.

NDFap - fibra detergente neutral corregida por cenizas y proteínas; Unidad de entrenamiento de colonia de CFU.

The pH was determined in distilled water in duplicate, using a potentiometer. Briefly, approximately 25 g of the ensiled material from each treatment were mixed with 100 mL of water. After 1h, reading was performed according to the methodology described by Bolsen *et al.* (1992).

A fraction of 10 ml was collected from these solutions, rediluted with 1 mL of distilled water, acidified with 50% H_2SO_4 and filtered through Whatman filter paper for organic acid and ethanol analysis (10).

Subsequently, 2 ml of the filtrate were spiked with 1 mL of 20% metaphosphoric acid and 0.2 mL of 0.1% phenolic acid. The samples were centrifuged, and organic acids (lactic acid, acetic, propionic and butyric) and ethanol were analysed by high-performance liquid chromatography (HPLC), using a SHIMADZU, model SPD detector-10 VP coupled to an ultraviolet (UV) detector at a wavelength of 210 nm.

Microbiological evaluation was performed according to Kung and Ranjit (2001); 25-g fresh silage samples, extracted according to the defined opening periods, were collected. Subsequently, we added 225 mL of sterile Ringer's solution and blended the mixture for approximately 1 min, followed by the removal of 1 mL and appropriate dilution (10^{-1} to 10^{-9}). Plating was performed in duplicate for each culture medium. In addition, the microbial populations of the forages and silages were analysed; LAB were enumerated in triplicate by pour-plating using the de Man, Rogosa and Sharpe MRS agar. Agar plates were incubated anaerobically for 48 h at 39°C, and yeasts and moulds (Y&M) were enumerated in triplicate by pour-plating with potato dextrose agar and anaerobic incubation for 7 days at room temperature. Enterobacteria were determined on violet-red bile and anaerobically incubated for 24 h at 35°C. Plates from the appropriate dilutions were counted when they contained a minimum of 30 and a maximum of 300 colonies. For data analysis, the counts were transformed into log10cfu/g.

Chemical analyses were performed at the Laboratory of Animal Nutrition and Food Evaluation of the CCA/UFPB. The samples were conditioned in paper bags, weighed and kept in an oven at 60°C for 48 h. Afterwards, they were milled in a Willey type mill with a 1-mm mesh sieve and subjected to analyses.

We analysed dry matter (DM -method no. 934·01), ash (method no. 930·05) and crude protein (CP -method no. 981·10) according to the methodologies of the AOAC (1990). Neutral detergent fibre was corrected for ash and protein (NDFap) according to Licitra *et al.* (1996) and Mertens (2002).

Water-soluble carbohydrate (WSC) values were determined via spectrophotometry according to the method described by Dubois *et al.* (1956). For extraction, 100 mg of dry and milled sample were added to 100 mL of 80% ethanol solution and placed in a water bath at 80°C for 30 min. Subsequently, the material was filtered, the solid residue discarded, the volume was completed to 500 mL with distilled water in a volumetric flask and the ethanolic extract was homogenised. We then added 1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid and removed 2-mL aliquots of ethanolic extract. A standard curve was constructed with increasing 0.01% glucose solution concentrations, and the spectrophotometer readings were conducted at 490 nm absorbance. The WSC contents were calculated in g × 100 mL^{-1} based on the solution and then fitted according to the dry matter of each sample.

Corn silage aerobic stability was determined through samples of approximately 2 ± 0.03 kg silage from each silo. The samples were relocated in silos cleaned without compaction, and the internal temperature of the silage mass was verified every 1 h using a digital immersion thermometer. The ambient temperature was recorded through a thermometer suspended in the air. Aerobic stability was calculated as the number of hours before the silage mass temperature reached 2°C above room temperature (25).

Statistical analysis

The data were analysed using the PROC GLM of the SAS 8.2 Software and subjected to analysis of variance and regression (21). The criterion adopted for choosing the regression models was the significance of parameters estimated by the models and the values of the coefficients of determination; P-values less than 0.05 were considered significant.

The significance levels of Pearson's correlation coefficients were estimated for fermentation, microbiological losses and losses of corn silage.

Principal components analysis allows grouping the largest amount of original information contained in p variables (*P*=13, in the present study) into a coordinate system covered by two principal components (1 and 2) (7), allowing to plot the variables in a two-dimensional graph. The multivariate analysis was processed using STATISTICA version 7.0 (23), and the graph was generated via the MVSP tool, trial version 3.2.

Results

Dry matter losses

The addition of urea to corn silage did not significantly affect (P >0.05) dry matter recovery (table 2).

Table 2. Dry matter losses of corn silages treated with urea.
Tabla 2. Pérdidas de materia seca de ensilajes de maíz tratados con urea.

Item	Urea (% of DM)					SEM [1]	P-value	
	0.0	0.5	1.0	1.5	2.0		Lin [2]	Quad [3]
Gas losses (g kg^{-1} DM)	26.7	29.2	21.2	27.0	23.0	1.69	0.4940	0.0121
Effluent losses (kg/ton)	160.0	154.0	109.0	108.0	109.0	0.55	<0.001	0.0060
DMR[4] (g kg^{-1} DM)	946.1	951.0	961.4	951.0	948.6	1.55	0.1895	0.0972

[1] SEM - Standard error mean; [2] Lin - linear; [3] Quad - Quadratic; [4] DMR - Dry Matter Recovery. [1] SEM - Error estándar medio; [2] Lin - lineal; [3] Quad - Cuadrático; [4] DMR - Recuperación de materia seca.

However, there was a decreasing linear effect (P<0.001) of urea levels on effluent losses in corn silages, with a reduction from 160.0 to 109.0 kg/t in silages without urea in relation to silages with the maximum level of urea (2.0%), with an estimated reduction of 29.6kg/t for every 1% of urea added to the ensiled mass. The gas losses presented a positive quadratic effect (P = 0.0121) with regression estimated a maximum point of gas losses close to 0.52% to inclusion of urea.

Fermentation profile and microbial populations of the silages

There was an increasing linear effect of urea level (P<0.05) on pH values in corn silages, increasing from 3.49 to 4.12 in silages without urea in relation to silages with the maximum urea level (table 3).

Table 3. Fermentative profile and microbial populations of corn silages treated with urea.
Tabla 3. Perfil de fermentación y poblaciones microbianas de ensilajes de maíz tratados con urea.

Item	Urea (% of DM)					SEM[1]	*P-value*	
	0	0.5	1.0	1.5	2.0		Lin[2]	Quad[3]
pH	3.49	3.50	4.08	4.06	4.12	0.035	<0.001	0.1689
Lactic acid (g kg^{-1} DM)	46.62	45.79	47.55	38.41	55.83	0.608	0.8010	0.1001
Acetic acid (g kg^{-1} DM)	28.50	15.91	21.38	11.53	18.90	0.206	0.4506	0.7220
Propionic acid (g kg^{-1} DM)	0.66	0.41	0.49	0.37	0.42	0.008	0.4770	0.1384
Butyric acid (g kg^{-1} DM)	0.39	0.38	0.38	0.33	0.29	0.006	0.0131	0.1026
Ethanol (g kg^{-1} DM)	21.95	12.63	5.75	6.01	7.85	0.566	0.2303	0.1107
Lactic acid bacteria[7] (log cfu/g)	7.52	8.01	9.01	9.04	8.58	0.028	0.1400	0.0004
Yeasts and moulds (log cfu/g)	4.86	5.01	3.95	4.25	4.00	0.013	0.3050	0.2261

[1] SEM - Standard error mean; [2] Lin - linear; [3] Quad - Quadratic.
[1] SEM - Error estándar medio; [2] Lin - lineal; [3] Quad - Cuadrático.

Lactic, acetic and propionic acids did not significantly influence the corn silages (P >0.05). However, the butyric acid concentrations of the silages showed a linear decreasing effect (P = 0.0131). There was a quadratic fitting for the concentrations of populations of lactic bacteria (P< 0.001) (table 3), with the maximum point of LAB populations close to a urea inclusion level of 1.5%.

Average lactic acid concentration was 44.59 g/kg DM, showing that the addition of urea maintained the lactic acid contents in the corn silages (P = 0.1001) (table 3). A similar behaviour was found for acetic acid concentrations (P = 0.4506), which showed an average content of 19.24 g/kg DM in corn silages. The urea levels did not alter (P = 0.1384) the concentrations of propionic acid in the silages, averaging 0.47 g/kg DM. The highest concentration of butyric acid (P = 0.0131) was observed in silage without urea (0.39 g/kg DM) and the lowest concentration of butyric acid in silage with the highest level of urea (0.29 g/kg DM) (table 3).

Ethanol concentrations did not affect corn silage (*P*>0.05). For populations of lactic acid bacteria of the silages, the addition level of 1.5% urea resulted in 9.04 log cfu/g in the forage (*P*< 0.001) (table 3, page 313).

Chemical composition

The addition of urea did not significantly influence the dry matter content (*P* = 0.0522) of the silages, averaging close to 28%. There was, however, a linear effect on ash concentrations (P< 0.001) (table 4).

The crude protein content of corn silages increased linearly (*P*< 0.001), and there was a linear decrease (*P*= 0.0150) for the concentrations of NDFap in corn silages (table 4).

Table 4. Chemical composition of corn silage treated with urea.

Tabla 4. Composición química de ensilajes de maíz tratados con urea.

Item	Urea (% DM)					SEM [1]	*P-value*	
	0	0.5	1.0	1.5	2.0		Lin [2]	Quad [3]
DM[4] (g kg^{-1})	270.80	311.20	282.46	296.06	224.93	1.925	0.5273	0.0522
Ash (g kg^{-1} DM)	33.37	33.79	37.86	39.99	50.53	1.256	<0.001	0.3893
CP[5] (g kg^{-1} DM)	82.15	108.98	111.51	139.93	143.18	2.574	<0.001	0.0017
NDFap[6] (g kg^{-1} DM)	494.80	502.72	475.12	444.45	420.60	7.666	0.0150	0.9514
WSC[7](g kg^{-1} DM)	80.42	75.72	87.19	103.32	72.54	1.569	0.2170	0.2600

[1] SEM - Standard error mean; [2] Lin - linear; [3] Quad - Quadratic; [4] DM – Dry matter on a fresh matter basis; [5] CP - Crude protein; [6] NDFap - neutral detergent fibre corrected for ash and protein; [7] WSC - water-soluble carbohydrates.
[1] SEM - Error estándar medio; [2] Lin - lineal; [3] Quad - Cuadrático; [4] DM - Materia seca; [5] CP - proteína cruda; [6] NDFap - fibra detergente neutral corregida para cenizas y proteínas; [7] WSC - carbohidratos solubles en agua.

There was no significant effect of the urea levels in the silages on the water-soluble carbohydrate concentrations (*P* = 0.2170), with average values of 84.89 g/kg.

Aerobic stability of silages

The maximum temperature showed a linear decrease (P = 0.0010) after exposure to air during the 96-h period. The pH values of corn silages after exposure to air during the 96-h period were not affected (*P*= 0.1406), (table 5).

The yeast populations recorded in the corn silages after exposure to air decreased linearly (*P*< 0.001). The average values of yeasts at 96 h were higher in the silages without urea (table 5).

Table 5. Aerobic stability, pH and yeast populations of corn silages treated with urea.

Tabla 5. Estabilidad aeróbica, pH y poblaciones de levaduras de ensilajes de maíz tratados con urea.

	Urea (% DM)					SEM [1]	*P-value*	
	0.0	0.5	1.0	1.5	2.0		Lin [2]	Quad [3]
Maximum temperature (°C)	26.40	25.10	25.0	24.5	24.10	0.110	0.0010	0.12005
Aerobic stability (h)	62	90	>96	>96	>96	0.365	-	-
After 96 h of air exposure								
pH	4.12	4.49	4.24	4.22	4.65	0.072	0.3024	0.1406
Yeasts and moulds (log cfu/g)	9.69	8.10	7.92	7.12	6.84	0.209	<0.001	0.1070

[1] SEM - Standard error mean; [2] Lin - linear; [3] Quad - Quadratic.
[1] SEM - Error estándar medio; [2] Lin - lineal; [3] Quad - Cuadrático.

Urea addition improved the aerobic stability of the silages in relation to those without the addition of urea, being 62 h for the silages without urea and from 90 to >96 h for the silages with urea.

Pearson's correlation coefficient and principal components analysis of fermentative characteristics of silages

Gas losses correlated positively with organic acid concentrations (especially butyric acid). Populations of lactic acid bacteria showed a positive correlation with pH (figure 1, page 315).

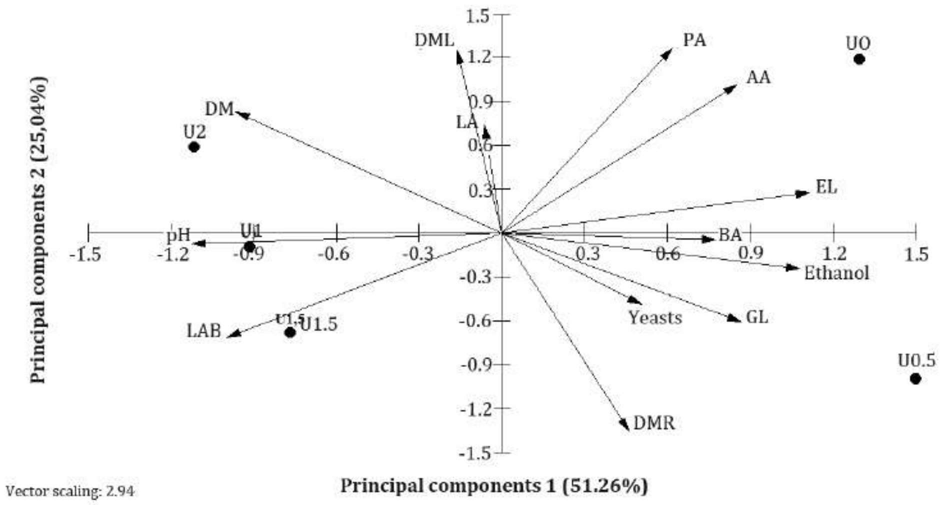

Figure 1. Distribution of dry matter (DM), pH, lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA), ethanol, lactic acid bacteria (LAB), yeasts and moulds (Y&M), dry matter losses (DML), gas losses (GL), effluent losses (EL) and dry matter recovery (DMR) in the coordinate system covered by principal components 1 and 2.

Figura 1. Distribución de materia seca (DM), pH, ácido láctico (LA), ácido acético (AA), ácido propiónico (PA), ácido butírico (BA), etanol, bacterias del ácido láctico (LAB), levaduras y mohos (L&B), pérdidas de materia seca (DML), pérdidas de gas (GL), pérdidas de efluentes (EL) y recuperación de materia seca (DMR) en el sistema de coordenadas cubierto por los componentes principales 1 y 2.

There was a positive correlation between the concentrations of acetic and propionic acids, which in turn had a strong positive correlation with dry matter losses. The ethanol content of the silages was positively correlated with the acetic, butyric and propionic acid contents and strongly correlated with effluent losses and yeast populations (table 6).

Table 6. Pearson's correlation coefficient of the fermentative profile, microbial populations and dry matter losses of corn silages treated with urea.

Tabla 6. Coeficiente de correlación de Pearson del perfil fermentativo, poblaciones microbianas y pérdidas de materia seca de ensilajes de maíz tratados con urea.

	BA [1]	LA [2]	AA [3]	PA [4]	ET [5]	DM [6]	GL [7]	EL [8]	DMR [9]	pH	LAB [10]	Y&M [11]
BA [1]		-0.174	0.442	0.557	0.510	-0.659	0.597	0.583	-0.072	-0.363	-0.432	-0.141
LA [2]			0.156	0.156	0.110	-0.359	0.328	-0.004	0.156	-0.083	-0.203	-0.432
AA [3]				0.850	0.667	-0.526	0.291	0.827	-0.085	-0.769	-0.944	0.008
PA [4]					0.332	-0.585	0.367	0.623	0.307	-0.480	-0.723	-0.154
ET [5]						-0.410	0.264	0.909	0.276	-0.842	-0.803	0.403
DM [6]							-0.738	-0.136	-0.120	0.065	0.327	0.256
GL [7]								0.210	-0.093	-0.253	-0.128	-0.274
EL [8]									0.043	-0.834	-0.918	0.347
DMR [9]										-0.122	0.005	0.504
pH											0.873	-0.413
LAB [10]												-0.299
Y&M [11]												

[1] BA - butyric acid; [2] LA - lactic acid; [3] AA - acetic acid; [4] PA - propionic acid; [5] ET - ethanol; [6] DM - dry matter; [7] GL - gas losses; [8] EL - effluent losses; [9] DMR - dry matter recovery; [10] LAB - lactic acid bacteria; [11] Y&M - yeasts and moulds.

[1] BA - ácido butírico; [2] LA - ácido láctico; [3] AA - ácido acético; [4] PA - ácido propiónico; [5] ET - etanol; [6] DM - materia seca; [7] GL - pérdidas de gas; [8] EL - pérdidas de efluentes; [9] DMR - recuperación de materia seca; [10] LAB - bacterias del ácido láctico; [11] Y& M - levaduras y mohos.

Principal components analysis allowed the formation of two groups (I and II). The most discriminating variables in group I were as follows: dry matter (-0.9), pH (-1.2), and lactic acid bacteria (-0.9), while in group II, they were effluent losses (1.0), ethanol (1.0), acetic acid (0.8) and gas losses (0.8).

Group I, including silages with 1.00, 1.50 and 2.00% urea, determined by the variables pH, dry matter and lactic acid bacterial populations, can be characterised as silages with the main fermentative product (lactic acid) even at higher pH values. Additionally, silage with urea showed a negative correlation with variables such as gas losses, effluent losses and ethanol production.

Discussion

The addition of urea to corn silage increased the pH values by the ammonia released in the hydrolysis of urea, forming a weak base, ammonium hydroxide (NH_4OH) (17). According to McDonald *et al.* (1991), the pH of silage should range from 3.8 - 4.2 to benefit the fermentation process; at pH levels below 3.8, yeast development is facilitated. However, acetic acid and urea may inhibit these microorganisms.

Even with the addition from 1 to 2% urea, the pH of the experimental silages was within the range verified by Weinberg *et al.* (2011), in contrast to the findings of Pinto *et al.* (2012), who verified higher pH values (3.95) in corn silages amended with urea, despite the differences between the values reported in this study. The authors verified that the use of urea in corn silage did not negatively affect the pH value. These lower pH values demonstrate that hybrids with high sugar contents are, in fact, prone to excessive acidification, making the addition of buffering additives, such as urea, a necessity.

The microbial ecosystem of corn silages is generally dominated by homofermentative lactic acid bacteria, especially *Lactobacillus plantarum* (McDonald *et al*., 1991; Pahlow *et al*., 2002), suggesting that the buffering allowed proliferation of heterofermentative lactic acid bacteria in a larger or smaller scale by altering the fermentative profile of silages Storm *et al.* (2010).

The lactic acid concentrations of silages in the present study were similar to those recorded by Pinto *et al.* (2012), who observed concentrations of 44.30 g/kg DM of lactic acid in corn silages. The high concentrations of acetic acid in the present study may be associated with higher activities of heterofermentative lactic acid bacteria. However, it is also possible that a significant production of acetic acid occurred in the initial period of the fermentation process when there was still oxygen in the silo. In an experiment with silages, Li *et al.* (2011) identified species of acetic bacteria in corn silages, mainly in the initial phase of the fermentation process. These acid-tolerant microorganisms convert ethanol to acetic acid under aerobic conditions and are generally associated with aerobic silage deterioration (22).

Considering the acidic pH values of all silages studied, a reduced concentration of butyric acid was expected, since the microorganisms producing this acid were developed in media with a pH close to 6.0. Thus, the low concentration of butyric acid, especially at the highest urea inclusion level, indicates that the addition of urea did not block a desirable fermentation profile of corn silages (3).

According to McDonald *et al*. (1991) and Pahlow *et al*. (2002), the number of lactic bacteria required for a significant reduction in pH of silage is about 8.0 log cfu/g. However, in the present study with the addition of urea, we observed higher numbers. Urea benefited the development of lactic acid bacteria in the silages by maintaining greater pH levels when compared to the silages with urea, besides decreasing the ethanol concentration due to the reduction of yeasts.

The reduction of yeast populations in silages treated with urea may be due to the conversion of urea to ammonia in the ensiled mass, facilitating fermentation (15). Urea is an antifungal substance and impedes the metabolism of yeasts, which are auxotrophic microorganisms and strictly depend on the availability of a specific combination between carbon sources and nitrogen sources (26).

In some studies, the authors observed a decrease in yeast populations when urea was applied to forage silage (27). Urea is a poor source of nitrogen for yeasts, which prefer other nitrogen sources (26). Thus, the addition of urea to silage increases the ammonia concentration in the ensiled mass, delaying the development of yeasts since it unbalances the specific relationship of substrates for yeast growth. Moreover, according to Hess *et al.* (2006), high ammonia concentrations are toxic to yeasts, forcing them to modify the nitrogen metabolism, incorporating ammonium in the formation and excretion of amino acids, such as glutamine or glutamate, in the presence of adequate potassium concentrations.

The contents of dry matter in corn silages are adequate to the fermentation process with low effluent production, thus benefiting nutrient availability to the animal (13). When evaluating the effects of additives on corn silage, Pinto *et al.* (2012) found values of 35.24% DM for urea-added silages. This difference might be due to the harvest period of corn; in their study, corn was in a more advanced physiological stage.

The increase in crude protein in corn silages treated with urea occurred because urea increases the nitrogen compounds of the silages, suggesting partial incorporation of the nitrogen applied (15). Thus, the beneficial effect of the addition of urea to corn silage, besides reducing gas and effluent losses and leading to dry matter recovery due to the improved fermentation profile in the silage, can also be an increase in feed protein value; the increase in urea nitrogen fractions may also benefit rumen fermentation and promote greater synthesis of microbial proteins (15). In the present study, the silages met the protein requirements of the animals, with silage protein contents above 7.0%.

The addition of urea to corn silages resulted in a reduction of the concentrations of NDFap, most likely because urea acted on the fibrous fraction of the corn silage via ureolysis reactions (15), consisting of an enzymatic reaction in which ammonia is released through urea hydrolysis and of ammonolysis between the ammonia and the ester bonds among hemicellulose chains and between the carbohydrate groups or carbohydrate and lignin molecules, resulting in amide formation. Moreover, ammonia shows high affinity with water, thus forming a weak base, ammonium hydroxide (NH_4OH). The high affinity of ammonia with water promotes cell wall expansion and rupture of the fibrous tissue components of the forage ensiled with urea (16).

The degradation of lactic acid in aerobic silages, resulting from the action of acid-tolerant aerobic microorganisms (4, 11), increases the pH of the silages. In the present study, the pH value obtained with a urea inclusion level of 1.5% was ideal (13).

The smaller yeast population at 96 h in corn silages treated with urea may be associated with the ability of urea to increase pH values of the medium, reducing the potential for the development of yeast populations. On the other hand, the positive effect of urea during oxygen exposure may be associated with the lower yeast population at the time of opening, which may have been inhibited by the buffering and antifungal activity of urea during the silage fermentation phase (26). According to McDonald *et al*. (1991), Spoelstra *et al*. (1998), Storm *et al*. (2010) and Pahlow *et al*. (2002), yeasts are associated with silage deterioration and have a fermentative route (pyruvate decarboxylase acetaldehyde and subsequent reduction of acetaldehyde to ethanol), which causes high losses and can consume up to 48.9% of dry matter.

The addition of urea reduced the microbial activity and exothermic reactions in the silo after exposure to air (15). Generally, yeasts are the first microorganisms deleterious to silages exposed to air (Da Silva *et al*., 2010), raising silage temperature and CO_2 production (11). However, as urea has a toxic effect on these microorganisms, it is possible that the higher resistance to heating and changes in the silages exposed to air resulted from the addition of urea to corn silage.

Principal components analysis allowed us to generate the two-dimensional biplot formed by the first two principal components. Experimental variables with positive correlations indicate an association of the variable with the samples located to the right on the horizontal axis (CP1), and those with negative correlations indicate an association with the samples located to the left on the horizontal axis (CP1). Thus, the total amount of information of the original variables retained by the two principal components was 76.3%, with 51.26% for the first principal component and 25.04% for the second principal component.

Determination of the correlation coefficients of the studied variables allowed us to verify that losses are generally associated with the production of ethanol and butyric acid and with yeast populations. Due to the higher amounts of lactic acid bacterial populations in the silages receiving more than 1.0% urea, the losses from the fermentation process were reduced since the production of lactic acid does not generate quantitative losses because of the lack of CO_2 and other undesirable by-products (13).

Group II, consisting of silage without urea or with 0.5% urea, showed discriminations related to losses and the production of ethanol and acetic acid, indicating that the main fermentation of these silages was probably not only lactic fermentation, generating losses characteristic of the performance of other species of microorganisms, such as yeasts and/or acetic and propionic bacteria.

Conclusions

The use of urea at levels close to 2% in corn silage reduces gas losses, improves chemical composition and promotes greater aerobic stability of silages.

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Social impact analysis of cultural tourism in rural areas of Tlaxcala, Mexico

Análisis del impacto social del turismo cultural en espacios rurales de Tlaxcala, México

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Abstract

Alternative tourism, specifically cultural tourism, has gained worldwide importance. This is reflected in the growing number of people preferring this type of leisure activity. However, and even though archaeological and religious contexts represent hubs of attraction for pilgrims and tourists, their development seems to generate social issues. The objective of this research is to analyse the social impact of cultural tourism, from the perspective of tradesmen living in the rural municipality of Tlaxcala, Mexico. Information was collected by surveys. Sample size was calculated using the non-probabilistic method (snowball), and 54 tradesmen owning establishments near tourist attractions, were interviewed. Results evidenced that tradesmen do perceive social problems including traffic congestion, increasing living costs, pollution, street vendors, and augmented competition between businesses. However, they appreciate the benefits of providing tourists with low-cost catering services, considering tourism to be positive or very positive. The conclusion is that economic benefits outweigh the social impacts generated by tourism.

Keywords
cultural tourism • rural spaces • developing • impacts

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Resumen

El turismo alternativo y específicamente, el cultural ha adquirido importancia a escala global y se refleja en el crecimiento del número de personas que prefieren este tipo de esparcimiento. Así los espacios arqueológicos y religiosos, son polos de atracción para peregrinos y turistas, pero su desarrollo está generando problemas sociales. El objetivo de la investigación fue analizar el impacto social del turismo cultural, desde la percepción de los comerciantes en un municipio rural de Tlaxcala, México. Se recabó la información mediante la técnica del cuestionario, se calculó la muestra utilizando el método no probabilístico (bola de nieve), se entrevistaron a 54 comerciantes que poseen establecimientos cerca de los atractivos turísticos. Se encontró que los comerciantes obtienen escasas utilidades, al brindar servicios de restauración de bajo costo a los turistas. También perciben que se están incrementando los problemas sociales incluido el tráfico, incremento del costo de la calidad de vida, contaminación, ambulantaje, y una mayor competencia entre negocios. A pesar de ello, consideraron que la actividad turística es buena o muy buena y contribuye al desarrollo local. Se concluye que aún son más los beneficios económicos que los impactos sociales generados por el turismo y por lo tanto se acepta su desarrollo.

Palabras clave
turismo cultural • espacios rurales • desarrollo • impactos

Introduction

Tourism has become one main promoter of economic growth in many countries, especially in those undeveloped. In Mexico, its advancement is an economic priority. In 2018, this country resulted the seventh most visited destination in the world, with the arrival of 42 million international tourists (37). In recent decades, the tourism industry has faced the challenge of satisfying the individual needs of tourists, rather than addressing the mass market (33). In this context, cultural tourism gains importance, as several cultural resources, converted into touristic attractions, constitute a contributing strategy for economic development.

Cultural tourism constitutes a social phenomenon that rescues the historical aspect of cultural heritage in order to contribute to territorial development (18). The UNWTO (World Tourism Organization) defines this type of tourism as a "social phenomenon" consisting of cultural trips considering folklore, arts and crafts, festivals, sites, monuments, and pilgrimages (12). This organization reaffirmed that cultural tourism represents an important part of international tourism, comprising more than 39% of arriving tourists (34). In Mexico, it included more than 40% of cultural trips (14), representing 5.5% of international travellers and 3% of national travellers (38).

Regarding this business, Mexico is especially competitive, given its artistic and historical value in terms of pre-Hispanic civilizations and colonial history, as well as its traditional cities and cultures (21). In this context, more than 1,200 museums and about 200 archeological sites are open to the public. Mexico has 121 destinations termed as Magical Centers by the Ministry of Tourism. According to the National Institute of Statistics and Geography (2017), in 2016, the state of Tlaxcala received a total of 368,229 national visitors and 20,944 foreigners. Of this total, the archeological zones of Cacaxtla (144,589) and Xochitecatl (119,446) attracted the greatest number of tourists (87.5%, 20).

This intense influx of tourists may negatively or positively disturb the host culture (31). Touristic development causes different social effects, especially evident in developing countries (1). Social behaviour plays a central role in human interactions. Mendoza *et al.* (2011) state that tourism impact assessment is fundamental. In this context, social impacts generated by tourism, principally on host communities, have not been evaluated. Our hypothesis states that social problems are increasing, with great competition between handicraft vendors as a result of the greater influx of tourists in the communities of San Miguel del Milagro, San Miguel Xochitecatitla and San José Atoyatenco, located in the municipality of Nativitas, Tlaxcala.

Bibliographic review

Social impact of tourism in rural areas

Territorial heritage derives from highly proclaimed and socially recognized cultural and natural inherited resources, making them a valuable cultural product (28). Thus, both nature and culture are important, especially if they coexist (9). Its interpretation combines material and immaterial aspects, representing elements with social assigned value and meaning, such as inheritance or collective legacy. This is linked to geographical diversity, as the resources included in the List of World Heritage of the United Nations Educational, Scientific and Cultural Organization (UNESCO), are characterized as universal and exceptional (42). These, in addition to others not included in this organization's list but combining the necessary attributes.

In cultural tourism, the heritage-tourism binomial correlation, stands out. The society *vs.* nature relationship needs to be considered, while analysing the impacts of existing economic, social and cultural affairs. Tourism results from the economic and social changes occurring in the context of societies from which it emerges. Its development focuses on its natural and cultural resources (27). Thus, both positive and negative transformations take place in all contexts, including rural ones. This means that touristic development brings economic benefits in exchange for social and environmental impacts (5, 13). Therefore, to analyse the perception of the local population regarding the effects of tourism, and its impact on socio-economic environments, turns important (35).

Santos (2004) commented that concerning the social aspect, tourism promotes changes in well-being, assimilation and transformation of customs. Likewise, tourism can cause negative changes like increasing insecurity, drug addiction and alcoholism. Social tension is also caused by land purchase for touristic infrastructure or when areas close to cultural heritage, are occupied. Timothy and Gyan (2009), mention that when different social groups claim the same space, event or object as their own, conflict and social discord can arise. These issues represent negative social manifestations of tourism.

Pedersen (2005) argues that, even though rapid touristic growth leads to environmental difficulties, increased property and commodity prices, traffic congestion, decreased life quality, higher taxes and competition in terms of the distribution of benefits, residents still maintain a positive attitude towards tourism, approving its development. This means that, despite the impacts of touristic development on communities, their inhabitants consider it an ensuring survival strategy. Even though tourism by itself, is not sufficient to eliminate poverty, it contributes to its reduction. Many job positions are generated from this activity, benefiting local inhabitants (24).

In this sense, the social exchange theory studies how residents perceive these impacts and how their perceptions may affect their approval or rejection (39). This theory analyses the trending attitudes of residents toward this activity (7). It also helps explain social changes arising from touristic activities, and attempts to provide a general theory of interaction, whose object of study is the group phenomena complying with norms, cohesion, group status and power, among others. Social human behaviour manifests between two or more people interacting spontaneously, expecting to achieve their aspirations (26). In this exchange, power as a positive factor plays an important role in the impacts of tourism involving submission or prohibition. Vargas-Núñez *et al.* (2011) affirmed that power is the capacity to decide on one's own life, but also the competence to decide on other lives. It can be considered a social interaction attribute, providing the link by which rules are established. It also provides procedures leading to unanimous and common agreement. Thus, *status* and power are unilateral relationships, resulting from this exchange. Additionally, the social exchange theory also contributes to understanding inhabitant's perception, assuming they select their transactions once having assessed their costs and benefits. If the benefits - economic or social - are greater than the costs involved, residents will support touristic development, being hospitable and tolerant towards tourism and its negative issues (8, 32).

Ap (1992) stated that when resource exchange turns high for the host, residents consider the impacts of tourism to be positive. But when the exchange is low, whether in a balanced or unbalanced relationship, impacts are perceived as negative. The rural economic framework should also be considered, given that if people are immersed in an agricultural and economic crisis, they will accept these types of activities, despite the low perceived income (15). Over time, social interaction causes change. Morales (2002) mentioned that at first, people adopt a positive view, enabling exchanges in which benefits are obtained. However, over time, differences develop onto unilateral dependence on power. Here, the social exchange relationship goes unnoticed, meaning that the negative aspects of tourism start being noticed. Residents perceive price increases, in addition to negative social impacts such as pollution, drug addiction and road congestion. Harrill (2004) states that people enjoying the greatest economic benefit, will be those who support touristic activities, as they place economic compensation before environmental or cultural costs.

In rural areas, changes in economic activities among inhabitants, bring negative transformations. In many communities, farmers integrated into the economy of tourism, leave agriculture. Others are forced to fabricate and sell low-value souvenirs, or to provide low-cost catering services to tourists, obtaining little profit, while economically generous margins are controlled by large companies (6). This implies that income generated from tourism does not remain in the hands of local people. Instead, it is mostly appropriated by international or regional tour operators.

Substituting agricultural activities for non-agricultural jobs, specifically tourism, has impacted on the social structure of rural areas. Although touristic income is low, some farmers complement their commercial income with this activity (17, 31). Therefore, to identify the social impacts of tourism on the sustainable development of local communities is important. Sites that offer cultural heritage should be analysed in reference to the magnitude of social impact, minimizing conflict between tourists and residents, while optimizing social development (23). Likewise, inhabitants' attitude towards touristic development, needs to be understood, reducing resentfulness, and promoting the touristic destination.

Methodology and characteristics of the study area

This research aimed to determine how salesmen perceive the social impacts of cultural tourism in the communities of San Miguel del Milagro, San Miguel Xochitecatitla and San José Atoyatenco, located in the municipality of Nativitas, Tlaxcala. The municipality is located south of the state of Tlaxcala, at 2,202 meters above sea level and between 19°14' North latitude and 98°18' West longitude (19). The study communities include the San Miguel Arcángel Sanctuary (religious tourism) and the Archeological Zones of Cacaxtla and Xochitecatl (archeological tourism), comprising the most visited attractions in the State (figure 1, page 324).

In 2015, the municipality of Nativitas had a population of 25,053 people, of who (figure 1, page 324), pam, 51.9% were women. Eighty-eight percent of the people, aged 15 or older, was able to read and write. The Economically Active Population reached 9,346 people, of whom, 31.6% worked in the service sector, 26.4% in the primary sector, 25.8% in the secondary sector and 14.7% in commerce (19). Primary economic activities include agriculture, livestock and forestry, being maize, amaranth and vegetables, their main crops.

A bibliographic review of tourism, cultural tourism and the theory of social exchange was undertaken, explaining the objective of this study. A structured questionnaire including 70 variables related to social and economic aspects of tourism was complemented with direct observation techniques, implying that the researcher visited the study place (22). The sample was defined after the non-probabilistic method by applying the snowball technique, given that no register of the existing businesses in the studied communities, was found. 24 of the 54 people interviewed, pertained to the context of religious tourism, while 30 worked at archaeological sites. The owners or representatives of various businesses - shops or stalls, food services, lodging/hotels, and handicraft shops among others- were interviewed. Data were analysed by parametric and non-parametric statistics.

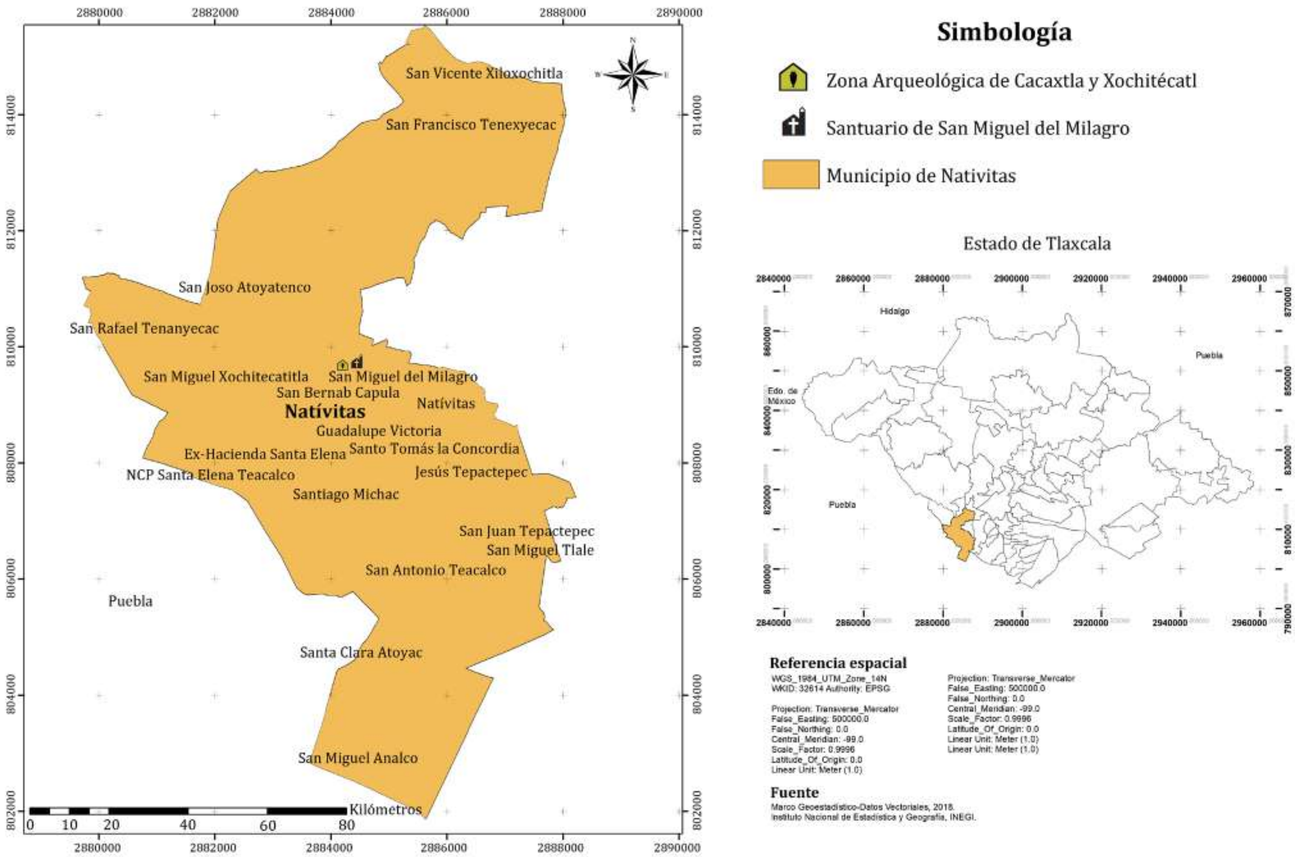


Figure 1. Spatial location of the municipality of Nativitas and tourist places.

Figura 1. Ubicación espacial del municipio de Nativitas y sitios turísticos.

Results and discussion

Analysis of the social impact of cultural tourism.

During the 70s and 80s, the study communities were mainly agricultural. The original inhabitants were Xicalanca Olmecs from the Chontalpa of Tabasco (10), people with significant cultural importance. However, today, agriculture has become a less important activity. Almost 43% of the interviewees stated they were tradesmen - they run a shop, or sell amaranth, clothes, hats and pottery sculptures, among other items. Thirty-seven percent provides some type of tourist service, food sales, lodging, parking and toilets, while the remaining percentage stated to be craftsmen. This means that Non-Agricultural Rural Employment (NARE) is becoming important, transforming the work activity at the studied locations. In this regard, Köbrich and Dirven (16) commented that NARE provides work for approximately 35% of the rural workforce in Latin American countries.

In this study, 64.8% of the interviewees stated to have other activities in addition to working as tradesmen or in tourism services: 48.6% work in agriculture, 22.9% have another occupation and the remaining percentage work for companies or the government. Those involved in religious tourism, chose agriculture (68.8%) over those in archaeological tourism (31.6%). This means that for some interviewees, tourism is a full-time job and not a complement to agriculture, contributing to the fact that, in the study area, agriculture is losing importance. People started up their businesses relying on family inheritance, both in religious (20.8%) and in archaeological tourism (36.7%). In this regard, Carton de Grammont (2015), recognized that this main non-agricultural activity takes place under precarious conditions and low income, where the most deprived households manage to find better paid self-employment. This is the case for most businesses in the study area.

Consequently, cultural tourism may contribute depending on the type of activities and the characteristics of the local economy (30). This result is a consequence of touristic areas evolving over time, given that, as facilities and place awareness grow, the number of visitors also increases (25), partly explaining why tourism had varying impact on business establishment in the study areas.

Given the significant economic benefits, an increasing number of establishments resulted in greater competition. According to 79.2% of the respondents, competition between establishments offering similar assets increased in the religious context, as clearly observed in 2012. A similar phenomenon was observed in archaeological areas, where 83.3% of respondents said that competition between businesses offering similar services increased, while 87.5% involved provision of touristic services, such as food selling. Among those engaged in commerce, 32% stated that competition had increased. These data evidence greater interest in commercial activities in areas with religious tourism, while in the archaeological context, interest seems to be focused on providing services. This can partly be explained by the fact that tourists' perception of valuable monuments is inadequate, as scientific-cultural activities are not properly appreciated (40).

Interviewees indicated that tourism has been positive (64.8%) and moderately positive (31.5%). In terms of positive aspects; transportation (38.9%), public services (16.7%) and lighting (13%) improvements, stood out. Concerning religious tourism, interviewees indicated that it has had a positive impact in terms of transportation improvement (58.4%) and implementation of public infrastructure (12.5%). Public routes were constructed, and public lighting was increased. In this sense, Zhuang *et al.* (2019), commented that tourism can improve local life standards, improving infrastructure, medical and educational care, employment opportunities and income levels.

Regarding conflicts within the population, 22.2% answered that tourism generates problems, to a greater extent as a result of religious tourism (45.8%), compared to archaeological tourism (3.3%). In this context, Almeida *et al.* (2015) mentioned that interactions between local residents and tourists may generate anguish and pressure. At different stages in their lives, residents may feel that their social reality is threatened. In order to understand conflict perception among the population towards tourism, several variables were analysed according to the logistic regression model. Using a variable selection procedure, variables were included and discarded until the appropriate model was obtained. Finally, the presence of street vendors near touristic places and the usage of parking spaces resulted to be significantly associated with conflict (table 1).

Table 1. Estimators of the logistic regression model, using the forward stepwise selection method (Wald).

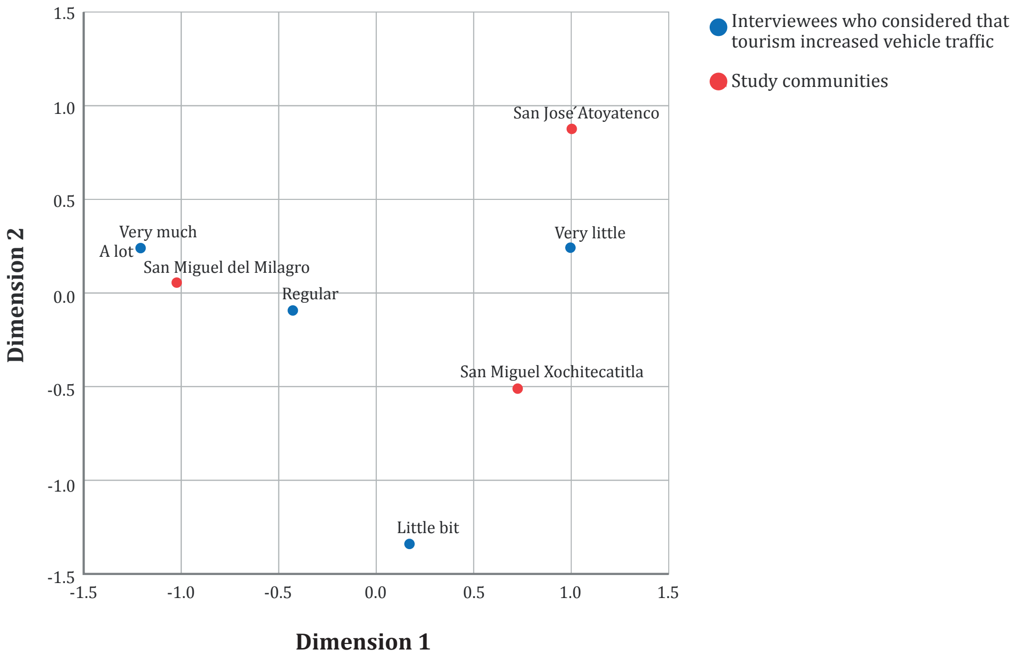
Tabla 1. Estimadores del modelo de regresión logística con el método de selección por pasos hacia adelante (Wald).

Variables	*B*	E.T.	Wald	*P*	Exp(B)
Presence of tradesmen	1.140	.433	6.930	.008	3.125
Parking spaces	-2.214	.835	7.032	.008	0.109
Constant	6.425	3.071	4.378	.036	616.984

Source: survey data, 2017.

Fuente: datos de la encuesta, 2017.

Problem perception after touristic arrival could be related to territorial planning, given that the presence of street vendors had not been regulated and the creation of parking lots meeting the service demand, had not been encouraged. In order to analyse whether the inhabitants of the study communities perceived that tourism caused vehicle traffic congestion in their respective communities, a correspondence analysis was carried out. This technique studies the dependency relationships of a set of categorical variables from the data, in a contingency table. A statistically significant relationship between the variables, study communities and increased traffic congestion (χ^2 = 41,795; $p < 0.001$) was found, suggesting a relationship between traffic and communities. Two groups are defined: 1) In the community of San Miguel del Milagro, religious tourism caused massive traffic increases, 2) In the San Miguel communities of Xochitecatitla and San José Atoyatenco, little or very little traffic resulted of archaeological tourism. Tourist arrivals to archaeological areas are less than in San Miguel del Milagro (figure 2, page 326).



Source: Own elaboration based on survey data, 2017.

Fuente: Elaboración propia con base en datos de encuestas, 2017.

Figure 2. Correspondence analysis of the relationship between study communities and traffic impact.

Figura 2. Análisis de correspondencia entre las comunidades de estudio y el impacto del tráfico vehicular.

When specifically asking about discontent, 16.7% of respondents in the religious tourism area said that traffic had increased greatly, while 45.8% thought it had increased, much. This adversity is aggravated during September, when the San Miguel del Milagro festival begins, and an increased number of pilgrims visit the sanctuary. This issue is partly a consequence of poor planning. In archaeological areas, traffic problems are still not present.

Interviewees (58.3%), from the religious touristic area, complained about a great reduction in the number of available parking lots. However, in archaeological touristic areas, 73.3% of respondents considered almost no increase in traffic due to tourism. In this sense, 76.7% said that tourism didn´t practically reduce the number of parking spaces, 50% said that neither is traffic excessive nor parking insufficient, and only 36.7% complained about insufficient space.

Among other negative aspects of tourism, interviewees mentioned pollution (48.1%) and cultural change (33.3%). In religious touristic areas, 37.5% complained about contamination, 37.5% complained about their community culture being impacted as a consequence of cultural exchange, and 25% observed road congestion negatively impacting society. In the archaeological touristic area, people mainly mentioned pollution (56.7%), and to a lesser extent, cultural impact and water scarcity. These types of social impacts influence life standards at the tourist destination (8).

Tourism has also influenced the increasing cost of living (24.1%), especially for religious touristic areas (41.7%) and to a lesser extent in archaeological areas (10%). In religious touristic areas, house rental (50%), transport (40%) and food (10%) have increased. Archaeological tourism has contributed to rising costs of living, mainly food (66.7%) and housing (33.3%). This increased cost of living is due to new businesses established by extra-community people. In this sense, Pedersen (2005), mentioned research on local concerns in relation to the impact of tourism development. Among many problems that affect the religious touristic area, property value, traffic congestion, life standards, income and price increase, were mostly mentioned.

Conflicts concerning tourism development and the appropriation of premises to establish a business (58.3%), as well as issues concerning lack of communication between the authorities and their inhabitants (33.3%), have arisen. This has led to vertical governmental decisions. In archaeological areas, people stated that this situation is creating social tension. Conflicts in religious touristic areas, concerning the occupation of premises, involved those who provide touristic services (75%) and tradesmen (57.1%).

Another complaint refers to the presence of street vendors and their business facilities, that contribute to the detriment of the area surrounding the tourist centres. Those interviewed about religious (54.2%) and archaeological (10%) tourism, stated that businesses established near the Sanctuary and the archaeological zones, needed to be re-located in specific areas. They also stated that both religious (37.5%) and archaeological (76.7%) tourism, require better facilities. As reflected by percentages, these touristic areas present different problems. In one, quality of facilities is essential, while in the other, business organization is of primary importance. However, the truth is that even though tourism represents a controversial social phenomenon, this activity promotes job positions and increasing income for the inhabitants (11).

Finally, with respect to security, according to respondents, the studied communities are safe or very safe (85.2%) places to visit. Despite this, 50% of respondents added that, in recent years, a regular increase in assaults has been noted while 37.5% mentioned a few acts of aggression. Interviewees who stated that the communities are moderately safe, referred to the increase in assaults during the last two years. This phenomenon is not linked to the arrival of tourists, but to the existence of gangs from these areas and nearby communities, and the arrival of people from other cities, settling in these communities. It can be concluded that the interviewees consider that, despite social problems (traffic, increased life standards cost, pollution and street vendors), (7.4%) tourism is very good and (66.6%) good.

Conclusions

The study communities consist of small rural centres with less than 2,500 inhabitants receiving an important influx of tourists. Almost 130 thousand to 200 thousand tourists arrive annually, in each tourist area. Consequently, tourism is contributing to the transformation of their social environment, meaning that with the arrival of tourists, increasingly rural non-agricultural employment is becoming more important. Almost half of respondents work as tradesmen or in tourism services, indicating that agriculture is losing importance. This type of transformation was more evident in the archaeological zones, where tourism is a full-time employment alternative. Unfortunately, these new activities are carried out under precarious conditions and for fundamentally low salaries.

Tourism is creating conflict in communities, especially in the religious touristic area, where a greater influx of tourists causes a differential increase in the number of businesses, resulting in greater competition. Additionally, tourism also brings more traffic, resulting in less public parking for local cars. Likewise, stalls and consequently more rubbish, are generated. Finally, an increased cost of living completes the disadvantages of touristic development. Perception of Tourism-related problems seems to be associated with poor regional urban planning, as the presence of street vendors and the creation of parking lots have not been regulated.

While promoting tourism is necessary, to ensure inhabitants wellbeing, is also important. In general, tourism-related problems are more pronounced in religious areas than in archaeological ones. If touristic activities are not properly planned, social impacts will increase, causing life quality deterioration with the consequent rejection of this type of economic activity. Thus, to implement strategies that help reduce the negative social impacts of tourism, becomes necessary. Future research should be carried out by incorporating the inhabitants, especially the peasants, and their perception of the social impacts of tourism in their community.

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Review

Factors affecting postpartum ovarian activity of goats in tropical semi-arid region

Factores que afectan la actividad ovárica posparto de las cabras en la región semiárida tropical

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Abstract

There have been few studies on the use of diets and strategies to reduce the postpartum anoestrus in dairy goats, especially in tropical semi-arid regions. This review discusses the factors influencing the return of postpartum ovarian activity in goats. The duration of puerperal anoestrus in goats directly affects the productivity of the herd. During the postpartum period, goats showed a puerperal anoestrus due to physiological changes in their reproductive tract to achieve a new conception. Anoestrus occurs as a result of tissue renewal in the uterus (uterine involution) which is associated with the return of cyclic ovarian activity, and is influenced by factors such as suckling of the offspring, the period of lactation, social interactions, body condition score (BCS) before and after birth, intensity of negative energy balance (NEB) and stress from adverse climatic conditions for example; the heat. The period of anoestrus can be extended, due to the delay in the cyclic reproductive activity in the puerperium. To minimize the negative effects of postpartum anoestrus on productivity, we recommend a mating season and a plan for the kidding period, as well as a program to increase the BCS during pregnancy so that the animals will have a better BCS at parturition and a nutritional plan for the lactation period.

Keywords

Anoestrus • Negative Energy Balance • Puerperium

Resumen

Se han realizado pocos estudios sobre el uso de dietas y estrategias para reducir la duración del anoestro postparto en cabras lecheras, especialmente en regiones semiáridas tropicales. Esta revisión analiza los factores que influyen en el retorno de la actividad ovárica posparto. La duración del anestro puerperal en las cabras afecta directamente la productividad del hato. Durante el período posparto, las cabras presentan un anestro puerperal debido a cambios fisiológicos en su tracto reproductivo para lograr una nueva concepción. El anestro se presenta como consecuencia de la renovación tisular del útero (involución uterina) que está asociada con el retorno de la actividad ovárica cíclica, y está influenciada por factores como la succión de las crías, el período de la lactancia, las interacciones sociales, la condición corporal (CC) antes y después del parto, la intensidad de un balance energético negativo (NEB) y el estrés por las condiciones climáticas adversas como por ejemplo; el calor. El período del anestro se puede extender, debido al retraso en la reanudación de la actividad reproductiva cíclica por el puerperio. Para minimizar los efectos negativos del posparto en la productividad, recomendamos una temporada de apareamiento y una planificación para el período de parición, así como un programa para incrementar la CC durante la gestación para que los animales tengan una mejor CC en el parto y un plan nutricional para el período de lactancia.

Palabras clave

Anestro • Balance Energético Negativo • Puerperio

Introduction

Most goat herds in Brazil (90%) are raised in the north-eastern region, mainly using an extensive management system. This management system shows low productivity indices, low fertility, high mortality from birth to weaning and long kidding intervals. All these factors result in low reproductive efficiency, with herds producing only a small number of kids per year (64).

Low nutrient availability is one of the main causes of deficient productivity indices, as it is strongly related to reproductive parameters and could stop reproductive activity in females. The lack of feed is mainly a problem during the dry season, when substantial reductions in the quality and amount of feed availability reduce the body condition of animals and productive or reproductive capacity in small ruminants (69).

Several studies have shown that the nutritional and metabolic states of animals may affect reproductive functions. While little is known about the mechanisms linking nutrition and the activity of the hypothalamus-pituitary-ovarian axis during postpartum anoestrus, it is known that energy is the main nutrient required by females during reproduction, and that its insufficient supply reduces reproductive efficiency (30, 39).

The main factors influencing postpartum anoestrus are: 1) uterine involution, 2) lack of positive feedback from the hypothalamus-pituitary-ovarian axis causing reduction of ovarian activity after parturition and return to oestrus, 3) lactation period, 4) pre- and post-partum state of nutrition of the mother, 5) body condition and 6) environmental causes, such as heat stress and social interactions (39, 59). This review aims to clarify how these factors are related to reproductive physiology, endocrinology and follicular dynamics and in turn, how they influence the return of postpartum ovarian activity in goats.

Factors influencing postpartum anoestrus

Uterine involution

Goats are seasonal polyestrics, but when reared in tropical regions they may exhibit reproductive activity throughout the year (17). However, during the postpartum period, the ovaries are unable to promote follicles to preovulatory status because of inhibition of the hypothalamic-pituitary axis resulting in a period of anoestrus (puerperal or postpartum) until the goats exhibit oestrous behaviour (46). During this postpartum period, ovarian activity is restored after uterine involution occurs. This process is considered essential to produce a new conception. During this postpartum period, ovarian activity is re-established after the uterine involution occurs. Involution of the uterus is fundamental to producing a new conception and it requires several physiological processes, such as a reduction in the size of the uterus, loss of tissues, repair of the residual tissues and reduction of tissue fluid (55).

Uterine involution is necessary for the return to reproductive activity after parturition. The uterus returns to its pre-pregnancy cellular and spatial conditions via uterine contraction that expels residual material from the placenta, lochia (genital secretion composed of mucus, blood, residual foetal membranes and fluids) (26), preventing possible bacterial infections that can delay the involution and microscopic regeneration of the endometrium (24). Endometrial regeneration is linked to the type of placenta present in each species. For instance, the cotyledonary placenta in ruminants lengthens the time required for regeneration compared with the diffuse placenta in mares (4). According to Olivera *et al.* (2013) the uterus of goats requires 4 to 5 weeks for involution. This period may be extended depending on the degree of contamination, the retention of foetal membranes (26) and oestrogen production. Mirzaei *et al.* (2011) and Takayama *et al.* (2010) reported that uterine involution varies according to breed (size, rusticity, performance), order of parity, type of birth, lactation and nutrition-associated factors and season of birth. These factors are reviewed and discussed below.

Ababneh and Degefa (2005) verified, through ultrasound evaluation and slaughter, that uterine involution was completed in Balady goats and that they were ready for a new pregnancy at 19 days after birth. This can be attributed to the small size of Balady goats, which have a bodyweight of less than 20 kg. Similar results were observed by Degefa *et al.* (2006) for the same breed of goats and by Takayama *et al.* (2010) in Shiba goats, which have a similar size and bodyweight. Salmito-Vanderley and Marques Júnior (2004) reported that uterine involution in animals of undefined genotype is completed by the 30th day after the kid's birth. Greyling and Van Niekerk (1991) studied Boer goats, slaughtering them at different intervals between parturition and 34 days postpartum to observe all macroscopic uterine sizes, and reported that involution ended approximately 28 days after birth. Krajničáková *et al.* (1999) reported this process ending on the 34th day after parturition. These studies show that body size is important in determining the time the animal needs for the uterus to return to its initial size.

The number of parturitions (order of parity) and the number of kids (type of birth) can modify the size of the uterus pre- and postpartum, respectively, and also affect the time to uterus involution. According to Webb *et al.* (2007), primiparous females show longer postpartum anoestrus, because the mother is nursing for the first time and is still growing.

In addition, depending on body condition at first mating, young females may still be completing their body development, which increases the demand for energy and protein. Combined with gestational demands, this promotes additional stress, leading to longer postpartum anoestrus (21, 59).

Eloy *et al.* (2003) stated that after uterine involution, a female might be ready to begin a new pregnancy, but lactation and nutrition-associated factors contribute to extending the postpartum anoestrus, highlighting the importance of these two factors on uterine involution.

Nogueira *et al.* (2011) reported that season of birth (rainy or dry season) interferes with the duration of postpartum anoestrus more than the period of lactation, via modification of feed availability in the environment. Mendoza *et al.* (2020) reported that the use of volatile compounds of the herbal methionine and choline can improve performance and immuno-metabolic status especially in the first days of postpartum.

In summary, studies on uterine involution time remain scarce. They show that this process is affected by many different factors, but generally occurs between 20 and 45 days after parturition (61).

Hormones and oestrous behaviour after birth

The oestrous cycle is interrupted during the puerperium, due to the inhibition of the several levels of the complex interactions between the hypothalamus, pituitary and ovaries that regulate the reproductive cycle. The absence of oestrous behaviour during puerperium is caused by low follicular development, which reduces the production of estradiol and consequently inhibits the hypothalamic-pituitary-gonadal feedback, hence interrupting the oestrous cycle.

In the post-partum anoestrus, GnRH (gonadotropin releasing hormone) release is inhibited and constitutes a limiting factor for the recovery of ovarian activity (33). Consequently, both LH (luteinizing hormone) release and FSH (follicle stimulating hormone) pulses are reduced (29). Schirar *et al.* (1989a) found that the first LH surge in ewes was affected according to whether the offspring was suckling, being faster (up to 10 days) if non-suckling or longer (up to17 days) if suckling.

In sheep, FSH secretion increases progressively after delivery and reaches its peak around 20 days after birth, promoting follicular development (72).

The first ovarian cycle after an anoestrus period in goats is not preceded by the usual oestrous activity and both the duration and concentration of progesterone secretion by the corpus luteum are generally lower than normal (17). In ewes, the first two ovulations are of poor quality, due to the low pulsatility of FSH, due to the fact that the small follicles have a low number of granulosa cells, which produce low levels of estradiol, which may explain the possible absence of sexual receptivity prior to the first ovulation (33), known as "silent ovulation" (59).

During lactation, the influence of prolactin and endogenous opioids, such as β-endorphins, which inhibit the secretion of GnRH as well as FSH- and LH-releasing hormones (FSH-RH and LH-RH), reduces the frequency of LH pulses (13, 25, 44, 76). This reduction in the pulses of LH induces small corpora lutea, which in turn reduce the release of progesterone to levels that may not be enough to maintain gestation (17). The LH release is influenced by body condition, which will be discussed in more detail later.

Shi *et al.* (2015) found that oestrogen receptors (ERα and ERβ) and progesterone receptors (PR) are responsible for modulating follicular responses, and that the expression of ERβ is intrinsically related to the number of granulosa cells in the follicle; for these reasons, ovarian activity is not always associated with oestrous behaviour.

Schirar *et al.* (1989b) found that a faster uterine involution may be associated with increased production of prostaglandin (PGF2α). According to these authors, PGF2α promotes the uterine defence mechanism, with effects on leukocytes, and stimulation of smooth muscle, causing myometrial contractions that help expel the uterine lochia, or other content, in addition to promoting the release of gonadotropins that affect oestrogen production.

Lactation period

Lactation has an important effect on the duration of postpartum anoestrus in ruminants. The duration of lactation and milk production have negative effects on the recovery of reproductive activity after parturition in goats raised in tropical regions (67).

At the start of the postpartum period, lactation is accompanied by high levels of prolactin and endogenous opioids are released in the milk ejection process (25). Prolactins act directly in inhibiting the secretion of other hormones from the anterior pituitary, mainly the LH (13). If LH is absent, ovarian follicles will not develop or will secrete low concentrations of steroids under the influence of FSH (21). The inhibition associated with nursing may be caused by the temporary alteration of this feedback, involving the temporary reduction of GnRH (67).

In addition, endogenous opioids, such as β-endorphin, μ, κ and δ-opioids produced during the lactation period, inhibit the hypothalamic production of GnRH, mainly LH, as a result of the inhibition of the releasing hormones of LH (LHRH) and FSH (FSHRH) (13, 52). Stressed animals (due to other factors, *i.e.* thermal stress) can suffer an increase in the plasma concentrations of endogenous opioids from postpartum and therefore suffer a decrease in reproductive efficiency (42).

Lactation commonly has a suppressive effect on ovarian activity. The lactation period is associated with the production of both oxytocin and prolactin. Oliveira *et al.* (2013) and Gaafar *et al.* (2005) described an inverse correlation between prolactin concentration and oestrus activity in ewes in seasonal anoestrus. During milk production, prolactin concentration is increased due to the stimulus provided by suction from kids. As the lactation period proceeds, prolactin secretion decreases, allowing the recovery of normal ovarian activity (21). Lactation and milk production increase nutritional demands, therefore causing a negative energy balance (NEB) that affects the duration of postpartum anoestrus (53). Lactation also increases the release of oxytocin that promotes milk-ejection from the myoepithelial cell's contraction (16). In addition, the oxytocin causes contraction of the uterus by myometrial contractions stimulating the endogenous release of PGF2α that in the first postpartum days acts by accelerating the expulsion of lochia and causing an earlier uterine involution (16).

Faraz *et al.* (2020) reported some studies that found no deleterious effects of oxytocin on the reproductive health in females due to the oxytocin had a short life span of about 2.5 minutes, however, in this same review it is mentioned that goats increase the concentration of oxytocin at the beginning of milking and concomitantly with the increase in prolactin; it is assumed that in order for lactogenesis to be maintained, milk must be removed from the mammary gland. The sensorial stimulus of teat suction by kids stimulates the production of prolactin, which inhibits the release of dopamine and GnRH-associated peptides. As these are essential for the synthesis of gonadotropins, their inhibition results in a reduction in ovarian activity (36). There is a relationship between the duration of postpartum anoestrus and the degree of teat stimulation. Because of this, management of weaning of the kids affects directly the postpartum anoestrus. Falcão *et al.* (2008) observed that SRD goats in continuous lactation have a longer period of postpartum anoestrus than goats under controlled lactation management in which the kids were isolated from their mothers during the day and suckling occurred only twice a day (table 1, page 335). Similarly, in goats treated with $PGF_{2\alpha}$ (36), the duration of the postpartum anoestrus was shortened in controlled lactation compared to continuous lactation, while $PGF_{2\alpha}$ treatment shorten the anoestrous period even more. However, Maia and Costa (1998) observed that Canindé goats subjected to a controlled lactation management plan showed a longer postpartum anoestrus. This longer postpartum anoestrus could be attributed to the handling stress experienced by the Canindé goats, which might have influenced the anoestrous length. In native breed goats, such as those reported by Maia and Costa (1998), controlled suckling of the offspring can cause elevation of cortisol levels, which may have a deleterious effect on the release of GnRH and consequently increase the anoestrus period. However, in animal breeds that are accustomed to daily handling, such as dairy goats, controlled suckling can reduce the anoestrous period, due to a less teat stimulation. In the other hand, PGF2α treatment can shorten the anoestrous period even more because prostaglandin promotes uterine involution by stimulating the immune system and influencing inflammatory processes in the uterus, thus reducing the time required for uterine involution. In addition, it can stimulate the release of FSH and LH, and consequently, reduce the duration of postpartum anoestrus (63). As shown in table 1 (page 335), the weaning management of kids directly affects postpartum anoestrus.

Table 1. Influence of the suckling of offspring on the duration of postpartum anoestrus (days) in different breeds of goats.

Tabla 1. Influencia del amamantamiento de la descendencia en la duración del anestro posparto (días) en diferentes razas de cabras.

Animals	Continuous[1]	Controlled[2]	Authors
SRD Goats	95.00 ± 4.70a	75.10 ± 3.70b	Falcão, D. P. *et al.* (2008)
[3]SRD Goats treated with $PGF_{2\alpha}$	68.80 ± 8.40a	47.90 ± 3.10b	
[4]SRD Goats treated with PGF2α	69.70± 0.70a	49.20± 3.00b	
Canindé Goats	33.09 ± 3.48a	46.44 ± 3.44b	Maia and Costa (1998)

[1]: The goat kids remained with the mothers; [2]: The kids were isolated from their mothers during the day and suckling occurred only twice a day. [3]: 250 µg of PGF2α in 6th and 12th day postpartum; [4]: 250 µg of PGF2α in 6th, 7th, 8th, 9th and 10th day postpartum. Values with different letters in the same row are significantly different at P < 0.05.

[1]: Los cabritos permanecieron con las madres; [2]: Los cabritos fueron aislados de sus madres durante el día y la succión de la descendencia ocurrió solo dos veces al día. [3]: 250 µg de PGF2α en el sexto y duodécimo día de posparto; [4]: 250 ug de PGF2α en el sexto, séptimo, octavo, noveno y décimo día de posparto. Los valores con letras diferentes en la misma fila son significativamente diferentes en P <0,05.

Other factors influencing the anoestrus postpartum period are type of breed, age and parity order and type of birth (single or multiple) (19). The same authors observed that the interval to the first oestrus after parturition was 78.93 ± 7.61 days in Anglo-Nubian goats, compared with 95.26±11.8 days in Saneen goats, which are better milk producers. In the same study, primiparous animals showed longer postpartum anoestrus than multiparous females ($p<0.05$), in both Anglo-Nubian (89.10 ± 9.73 *vs.* 56.33 ± 7.78) and Saanen goats (133.50 ± 12.71 *vs.* 52.78 ± 5.51). These differences may be attributed to the fact that primiparous females are usually still maturing; in the final third of gestation the foetus occupies a lot of space in the abdominal cavity and the female cannot consume high amounts of feed, even though females with a low body condition score or small size still need to complete their own body development. He *et al.* (2015) suggested the use of feed concentrates starting from 100 days of pregnancy to minimize the drop-in body condition score and thus facilitate the return of postpartum ovarian activity.

The type of birth may also affect the duration of the postpartum anoestrus. Waldron *et al.* (1999) reported that goat kids had a mean age at first estrus close to 7.5 months, and that in twin-born does this age was four months greater than in single-born does. Joshi *et al.* (2018) and Zamuner *et al.* (2020a and 2020b) stated that goats with multiple kids (litter size) showed higher milk production with less fat, increased prolactin and higher mobilization of body reserves resulting in weight and BS loss, associated with plasma elevation of β-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) that act to intensify the NEB, consequently, they may present a more pronounced negative feedback on the hypothalamic-pituitary axis to produce GnRH, FSH and LH and, consequently, a longer period is needed before postpartum ovarian activity reestablishes.

Paez Lama *et al.* (2016) observed that lengths of suckling period significantly modified resumption of ovarian activity in Creole goat, showing that goats weaned at 30 days postpartum presented an anoestrus period of 102 days compared to 155 days for those weaned at 60 days postpartum.

Nutrition

According to Van Knegsel *et al.* (2005), females have high nutritional demands during the last third of gestation due to intense foetal growth. After birth, the demand for energy and protein reaches its highest-level during lactation, causing a negative energy balance (NEB). If these demands are not satisfied, the recovery of ovarian cycles will be delayed both for physiological and nutritional anoestrus (62).

The effects of NEB on fertility are related to metabolic and endocrine changes that affect follicular growth and consequently progesterone production by the corpus luteum (38). According to NRC (2007), postpartum energy intake by goats fluctuates based on the number of kids, milk yield, and body condition at birth. Adequate nutrition is required so that females can deliver their kids with a body condition score between 2.5 and 3.5 (6).

The effects of nutrition on reproduction are mediated by various hormones. Among them, growth hormone (GH), insulin, and insulin-like growth factor (IGF-I) are important mediators of the effect of the energy balance on follicular growth in bovines (57). Insulin and IGF-I act directly on follicle growth, stimulating folliculogenesis and steroid production by stimulating LH production in the theca cells (31). Oliveira *et al.* (2013) reported a positive correlation between IGF-I levels and body condition score in cows. Lucy (2008) stated that after parturition, dairy cows are in a catabolic state, with high blood levels of GH, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (54) and low levels of IGF-I, insulin and GnRH.

Changes may occur in the serum biochemical parameters as aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), triglyceride (TG) whose values can be increased in the postpartum period, signalling the possibility of liver TG infiltration and fatty liver disease (3). He *et al.* (2015) observed that body reserve mobilization in goats started from the 125th day of pregnancy and caused changes in serum levels with a reduction in glucose levels and increased NEFA and triglycerides; these changes were related to the body condition score. On the other hand, Shi *et al.* (2015) found no correlation between these parameters and the length of time until the first dominant follicle developed after parturition in JiNing Grey goats.

Wettemann *et al.* (2014) reported that a lack of glucose reduces the release of hypothalamic GnRH. In contrast, an increase in available glucose causes an increase in insulin levels, stimulating GnRH release. The increase of insulin associated with lower GH levels is important in relation to nutritional impacts on reproduction (32). The functional relation between insulin and GH seems to be anabolic in nature. Growth hormone seems to be associated with the central mediation of metabolic status (34). These authors explain in their review that ovarian follicles do not have receptors for GH, although this hormone operates directly upon luteal cells. GH interacts with insulin to control the hepatic production of IGF-I (42), having opposing actions. When GH is increased the animal is in a catabolic state, and insulin and IGF-I are decreased, because GH promotes the increase of insulin-like growth factor-binding protein (IGFBP), which impairs the synthesis of IGF-I in the liver (31). GH also promotes high levels of NEFA, β-hydroxybutyrate and adiponectin and low levels of leptin and neuropeptide Y, which are signs of lipolysis and, consequently, catabolism (6, 31, 54). The effects of nutrition on the oestrous cycle are directly mediated by GnRH and gonadotropic hormones, and indirectly through the GH-IGF I-insulin axis (12).

Figure 1 (page 337) shows the possible mechanisms through which nutrition can act upon follicular growth in females with a normal oestrous cycle. The supply of nutrients above maintenance requirements promotes an increase in the plasmatic glucose concentration that in turn has an inhibitory effect on the release of neuropeptide Y. The inhibition of neuropeptide Y promotes the release of GnRH from the hypothalamus, which in turn promotes FSH and LH pulses (9). FSH acts upon ovarian cells, stimulating follicular growth. The increase in LH pulses, together with IGF-I, GH and insulin, stimulates the growth and maturation of follicles. Follicle growth stimulates an increase in metabolism and production of progesterone (P_4) and estradiol (E_2). Estradiol acts on the hippocampus, promoting positive feedback, which stimulates the release of GnRH (9). This mechanism is typical in female ruminants with adequate nutrition under favourable environmental conditions.

Small ruminants, in particular goats, which are generally a more prolific species, show some adaptations mainly with regard to follicular dominance *e.g.* Ginther and Kot (1995) observed that there may be more than one dominant follicle, known as codominance. This phenomenon occurs due to the expression of genes linked to Toll-like receptors, such as WNT4, during follicular selection (14). The codominance is due to the higher FSH pulse which enables the recruitment of a larger number of follicles and growth of more than one follicle, as well as the gene expression action (14); after that the largest LH pulse promotes maturation and ovulation of more than one follicle, these larger pulses of FSH and LH are possible by adequate nutrition under favorable environmental conditions.

Freitas-de-Melo and Ungerfield (2016), in their review, explained that females subjected to unfavourable environmental conditions can present acute or chronic responses that generally occur simultaneously, with the sympathetic response being displayed a few seconds or even minutes after the animal is exposed and the endocrine response takes a little longer to display and it lasts longer (minutes to hours). These responses are physiological and behavioural changes that help the adaptation to the unfavourable environmental conditions, the response intensity may depend on factors of the animal such as previous experience, or adaptations to the adverse environmental conditions from some breeds (20).

Source: Adapted from Diskin *et al.* (2003).

Fuente: Adaptado de Diskin *et al.* (2003).

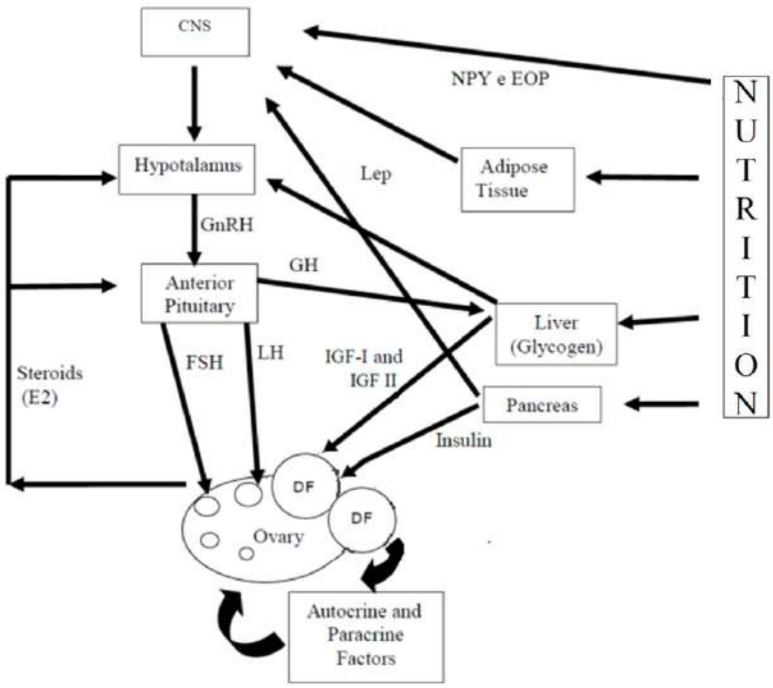

Figure 1. Diagram representing the mechanisms by which nutrition has an impact on ovarian follicular function. CNS: central nervous system, NPY: neuropeptide Y, EOP: endogenous opioid peptide, Lep: leptin; GnRH: gonadotropin releasing hormone; FSH follicle stimulating hormone; LH: luteinizing hormone; DF: dominant follicle; GH: growth hormone; IGF-I: insulin-like growth factor I; IGF-II: insulin-like growth factor II; E2: oestrogen.

Figura 1. Diagrama que representa los mecanismos por los cuales la nutrición incide sobre la función folicular ovárica. SNC: sistema nervioso central, NPY: neuropéptido Y, EOP: péptido opioide endógeno, Lep: leptina; GnRH: hormona liberadora de gonadotropina; Hormona estimulante del folículo FSH; LH: hormona luteinizante; DF: folículo dominante; GH: hormona del crecimiento; IGF-I: factor de crecimiento similar a la insulina I; IGF-II: factor de crecimiento similar a la insulina II; E2: estrógeno.

The sympathetic response releases the neurotransmitters adrenaline and noradrenaline that increase heart and respiratory rates, temperature and blood glucose concentrations (20). Endocrine response from hypothalamus-pituitary-adrenal axis releases corticotrophin-releasing corticosterone hormone (CRH), adrenocorticotrophic hormone (ACTH) and vasopressin which stimulate the secretion of glucocorticoids, mainly cortisol. This hormone, during long periods of time (from days to weeks), promotes a chronic stress response, generally stimulating high concentrations of glucocorticoids that may negatively affect the immune system and sexual behaviour and appetite (20).

High plasma concentrations of glucose stimulate the pancreas to produce insulin and the liver to produce IGF-I and IGF-II. Glucose is stored as glycogen in the liver and as fat in adipose tissue (9). Energy stored in the adipose tissue increases the metabolism and production of leptin, which also inhibits the release of endogenous opioids and neuropeptide Y (9).

Endogenous opioids and the dopaminergic and serotoninergic systems are associated with nutrition mediate LH pulses (78). Some authors observed that an increase in plasma glucose availability increased GH and insulin secretion, acting directly on the follicular dynamics (68, 75). During the initial period of anoestrus, the release of GH and IGF-I is reduced due to low circulating plasma glucose and high levels of NEFA and ketone bodies from lipolysis, especially 3-beta-hydroxybutyrate, which inhibits the pulses of LH (78, 80).

Thomas and Williams (1996) confirmed that the ingestion of vegetable oils may increase the bioavailability of cholesterol, a precursor of steroid hormones. Other authors reported that oils are important not only because they are precursors of cholesterol, the base molecule for the production of steroid hormones, but also because of their high energy density, which would increase the energy balance and reduce the mobilization of energy reserves (46). Ovarian luteal cells use cholesterol as a precursor for the synthesis of progesterone (75). According to Bao *et al.* (1995), diets with high concentrations of lipids lead to increased serum concentrations of high-density lipoproteins (HDL). High concentrations of HDL stimulate the production of IGF-I by luteal granulosa cells and may interfere with cyclic ovarian function by modifying the quantity of HDL used for the synthesis of ovarian steroids. Muñoz-Gutiérrez *et al.* (2002) observed that energy supplements stimulated the recruitment (2-3 mm and 3-4 mm) and selection (>6 mm) of follicles in ewes. Although these authors did not find any relation between supplements (glucose, glucosamine or lupins) and FSH concentration, they reported that nutritional supplementation might have a direct effect upon follicle production. The addition of fat to the diet stimulates the programmed growth and size of pre-ovulatory follicles and increases the number of follicles (45). Zarazaga *et al.* (2011) studied the influence of nutrition on LH release and compared two groups of goats with different levels of concentrate in the diet. The high diet group received 700 g and the low diet group received 350 g of concentrate, both with 500 g of barley straw. These diets corresponded to 110% and 70% of the daily requirements for maintenance, respectively. They described that goats fed with low diet showed lower LH concentrations, which highlights a positive correlation between diet and LH release.

To reduce the length of postpartum anoestrus in goats, attention should be given to the nutritional requirements during this period, providing supplements with high levels of energy in the first days postpartum, as female goats are unable to ingest a large volume of feed. Supplementary feeding with digestible energy levels above 2.0 Mcal/day/goat weighing 40 kg is recommended for goats raised in the semiarid region of Brazil (46).

Nogueira *et al.* (2016) evaluated the reproductive response of goats in seasonal anoestrus that were supplemented with maize (10.6 MJ of Metabolic Energy/day) and/or hormonally synchronized and observed that maize supplementation for only 9 days increased plasma insulin levels, leptin and IGF-I, and that dietary supplementation associated with oestrus synchronization increased the ovulatory rate of goats by 43%. Nutritionally induced changes in metabolic hormones may have improved the responsiveness of growing follicles to gonadotropins and reduced the rate of atresia, thereby influencing ovulation rate. Supplementation with a relatively small quantity of maize has the potential to increase the ovulation rate in anoestrus when treated with conventional protocol for oestrus synchronization by exogenous hormones, although further investigation with larger groups of animals in seasonally anoestrus goats is needed to verify this conclusion (48).

In a similar study, Nogueira *et al.* (2017) observed that the ovulatory rate in synchronized goats can be increased when corn supplementation is offered to reach requirements 1.5 times above maintenance (10 MJ of Metabolic Energy/day) when compared to a control diet, without supplementation. Therefore, the addition of 220 g maize/goat/day for 9 days may promote an increase in ovulatory rate and, consequently, an increase in the number of offspring born. Energy supplementation with maize can provide a readily available source of energy for the microorganisms in the rumen. In summary, Nogueira *et al.* (2016 and 2017) reported that supplementation with maize (10.6 MJ of Metabolic Energy/day) may improve the responsiveness of growing follicles to gonadotrophins and stimulate ovulation. The positive relationship of corn supplementation on follicle responsiveness as well as on ovulation stimulation may reduce postpartum anoestrus. Nogueira *et al.* (2017) reported that the nutrition of goats (metabolic status or energetic balance) is the main factor affecting ovulation and corpora lutea and follicular development, by modulating gonadotropin secretion (FSH and LH). These authors demonstrated that undernutrition with a negative energy balance can reduce the follicular recruitment efficiency associated with low ovulation rates in the goat ovary. De Santiago-Miramontes *et al.* (2008) observed that goats grazing natural vegetation plus supplementation with 1.4 kg of mixture (69% of alfalfa hay, 21% of rolled corn and 10% of soybean meal) increased the ratio of goats that ovulated (25/25 and 21/25 with and without supplementation, respectively), but not the ratio of females in oestrus (24/25 and 22/25, respectively), which was consistent with Mani *et al.* (1996).

Body condition score

The body condition score (BSC) is a subjective measurement that aims to classify the animals' body energy reserves through their accumulation of fat in some points of the body such as in the chest, ribs, rump and loin, the animals are classified in a fat scale ranging from 1 = very thin to 5 = very fat (10, 12).

The BSC is the main index representing metabolic status and is important both during gestation and at birth. During the first two thirds of gestation the goat needs to store enough energy for the final third of gestation, during which body reserves are used due to an increase in energy demands from the foetus; during this period, body development is associated to initial milk production (28).

Females must have a good BSC at the end of gestation to enable more rapid postpartum ovarian activity in order to reduce the kidding interval (60). Zarazaga *et al.* (2005) reported a negative correlation between body condition score or nutritional level, and postpartum anoestrus, such that a low body condition score or low nutritional level extended postpartum anoestrus. The negative effects caused by stress and puerperal NEB can be reduced when the females have a good body condition and those receiving nutritional supplementation (79).

De Santiago-Miramontes *et al.* (2008) reported that goats with a BSC < 2 have more abnormal oestrous cycles with a longer time between oestrus and ovulation and fewer oestrous behaviours and ovulations than those with a body condition score > 2.5, which can be attributed to the reduction in frequency of episodic LH release, which compromises oestrous behaviour, ovarian folliculogenesis and ovulation. These authors explained that the lower body condition score indicates a low energy reserve, and because of this the animals tend to reduce energy expenditure on the maintenance of reproductive activity as an evolutionary characteristic for the survival of the female, a striking characteristic in goats.

He *et al.* (2015) emphasized that from the 125th day of gestation the mobilization of body reserves is accentuated by the large size of the foetus and the increase in the female's energy demand for both body maintenance and milk production. For these reasons these authors recommended that the body condition score of the female should be monitored and supervised following confirmation of pregnancy to minimize the deleterious effects of the mobilization of energy reserves.

Heat stress

In the semi-arid region of Brazil a decrease in reproductive activity of small ruminants is commonly associated with the low availability of feeds during the dry season. Garcia-Ispierto *et al.* (2006) reported that heat stress can also affect the reproduction of dairy cattle by increasing the secretion of cortisol, which decreases the synthesis of gonadotropic hormones (20). The stress degree can be measured by the level of cortisol (20, 53, 69). Heat stress produces acute and chronic alterations in the plasmatic concentrations of estradiol and progesterone, causing physiological and behavioural alterations to the oestrous cycle, such as the duration of the oestrous cycle, timing of ovulation and clinical signs of oestrus (20, 44).

Several authors have reported longer postpartum anoestrus in goats during the dry season when compared to animals giving birth during the rainy season (17, 37). During the dry season, animals grazing on natural pasture can experience a decline in LH secretion due to the severe reduction in feed availability (figure 2, page 340).

Several authors have reported that the duration of postpartum anoestrus during the dry or rainy season is associated only with the availability of feed. Torreão *et al.* (2008) worked with Morada Nova ewes and observed that in rainy season the ratio roughage:concentrated 80:20 (2.2 Mcal ME) compared to 60:40 (2.8 Mcal ME) and 40:60 (3.4 Mcal ME) did not decrease the period of return to cycling activity (ewes fed 2.2 and 3.4 Mcal ME have 34 *vs* 42 days for ewes fed 2.8 Mcal ME). However in the dry season, these authors observed that animals fed with higher energy levels showed shorter period of return to cycling activity than (58 days in ewes fed 3.4 Mcal ME compared to more than 70 days in ewes fed 2.2 and 2.8 Mcal ME), and showed better reproductive performance suggesting an interaction between seasonal and nutritional factors.

However, researchers in the Brazilian semi-arid region are still searching for further explanations as to how the rainy season has a positive effect on goats' ovarian activity (52). Freitas *et al.* (2004) associated it with the stress caused by the dry period because of high environmental temperature that promotes heat stress, related with severe reduction in feed availability and quality increasing the secretion of cortisol (20, 41, 49, 68).

Source: Adapted from Nogueira *et al.* (2011).
Fuente: Adaptado de Nogueira *et al.* (2011).

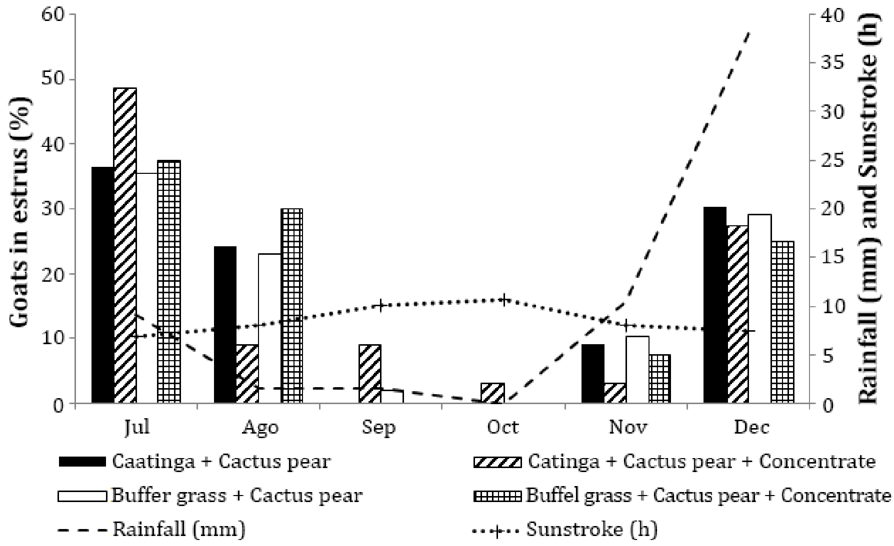

Figure 2. Percentage of goats in oestrus between July and December in 2007 in relation to rainfall, sunstroke and pasture growth.

Figura 2. Porcentaje de cabras en celo entre julio y diciembre de 2007 en relación con las precipitaciones, el fotoperíodo y el crecimiento de las pasturas.

Nogueira *et al.* (2011) reported a higher concentration of oestrus at the start of the rainy season, probably associated with the cooler temperature, and with the higher quantity and better quality of feed available during this period. According to these authors, the reproductive behaviour of native or crossbred goats of the semiarid region of Brazil was not affected by the different diets offered to the animals. The start of the reproductive activity of goats was more affected by the beginning of the rainy season than the inclusion of concentrated supplement which increased the weight gain of the animals (figure 2). A possible explanation for the rain effect may be related to the reduction in body temperature and thermal sensation of the goats, possibly reducing the effect of thermal stress, therefore decreasing the cortisol levels (41, 49, 68).

Nogueira *et al.* (2011) also showed that different diets did not influence oestrus activity during the dry season. This may be associated with the poor quantity and quality of feed offered with the different diets during the study, which may have caused a nutritional deficit during the most critical season of the year. Nascimento *et al.* (2014a) reported that goats are adapted to heat stress and yet under moderate stress at a wet bulb globe temperature (WBGT) above 85, they can show sexual behaviour when receiving energy supplementation. Carmona *et al.* (2020) reported that in arid or semi-arid environments the presence of agro-ecological productive systems can collaborate to minimize the heat stress in the herds.

Despite differences in authors´ studies regarding the importance of nutrition on postpartum anoestrus, there is sufficient evidence that it is possible to shorten the anoestrus period through strategic supplementation. Nascimento *et al.* (2014b) reported that postpartum anoestrus in goats during the dry season can be reduced by the addition of concentrate with high levels of energy. These authors observed a negative linear effect: the higher the level of energy in the diet, the shorter time for uterine involution and postpartum anoestrus. Further evidence is referenced in different sections of this review to support the relationship between postpartum anoestrus and nutrition.

Social interactions

Paez Lama *et al.* (2016) agreed that the nutritional and endocrinological factors discussed in this review are important but attributed the length of postpartum anoestrus to the doe–kid relation in the suckling period. They found that the suckling period promoted a statistically significant change in the anoestrus period independent of the energy supplementation and pointed out that the weaning time determined the duration of the anoestrus period.

The use of the male effect to reduce the anoestrus period in small ruminants is mainly reported in places with latitudes above 25°. The "male effect" is a cheap and common technique used to stimulate female's sexual activity and, according to the literature, exposure of females to sexually active bucks can induce the secretion of LH and thereby promote ovulation; in seasonal anovulatory goats just 15 minutes of female-male contact was enough to reactivate GnRH-LH secretion and consequently promote oestrous behaviour and ovulation (58). The use of the male effect is a resource widely used in the different species of domestic ruminants in sheep (2, 5, 8, 18), cattle (43, 66), goats (8, 71).

Cattle showed an interaction between the BSC and the male effect, females of high BSC (fat) tend to be less responsive to the male effect than cows with moderate BSC (66). Monje *et al.* (1992) observed that cows feeding diets with nutritional intake above requirements were more responsive to the male effect, but when they receive a diet with nutritional intake below requirements, the presence of bulls showed no influence on the length of postpartum anoestrus.

Delgadillo *et al.* (2009) pointed out that social interactions are complex, and possible, there is an effect of the combination of social interactions, i.e. ram effect combined with temporary weaning, corroborating data from other authors (18).

Recommendations

To minimize the negative effects of postpartum anoestrus, we recommend that the mating season and the kidding period should be planned according to the wet season, and that body condition during pregnancy should be monitored in order to implement a nutritional supplementation with levels of energy above the requirements for maintenance so that females present an acceptable body condition at parturition.

Conclusion

The duration of postpartum anoestrus in goats directly conditions the productivity of the herd and is influenced by different factors, mainly nutrition, lactation period and, in the north-eastern region of Brazil, by heat stress.

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Acknowledgements and conflict of interest

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