Revista de la
Facultad de Ciencias Agrarias
Universidad Nacional de Cuyo

Tomo 52 - Nº 1 Año 2020
ISSN on-line 1853-8665
Mendoza - Argentina

Evaluadores 2020

Federico Agrain | IADIZA
María Pia Aleandri | Universidad della Tuscia- Italia
Soledad Alessandro | INTA
Sandra Aliscioni | UBA
Liliana Inés Allegretti | CONICET
Erika Banchio | CONICET
Gonzalo Berhongaray | UBA
Federico Berli | FCA|UNCuyo
Gabriel Bernardello | UNCórdoba
Joana Boiteux | FCA|UNCuyo
Carlos Bouzo | UNLitoral
Gustavo Bretschneider | INTA
Andres Buffoni | INTA
Nicolás Cara | UNCuyo
Maria Esperanza Ceron Cucchi | INTA
Ramón Cieza | UNLP
Guillermo Claps | Instituto Superior de Entomología "Dr. Abraham Willink"
Ana Cohen | UNCuyo
Victoria Coll Araoz | PROIMI-CONICET
Verónica Conti | INTA
Santiago Cotroneo | FAUBA
Graciela Cuesta | INTA
José Sebastián Dambolena | UNCórdoba
Adriana de Caro | UBA
Susana del Toro | UNCuyo
Inés de Rosas | UNCuyo
Maria Jose Dieguez | IGEAF-CICVyA-INTA
Inés Dinolfo | UNICEN
Andrea Duplancic | FCEN
Mónica Duque | UniRemington
Luciana Elizalde | INIBIOMA-CONICET
Guillermo Eyherabide | INTA
Juan Carlos Favaro | UNLitoral
Claudia Faverin | INTA
María Gabriela Fernández Pepi | UBA
Jorge Frana | INTA
Andres Gambini | UBA
Alejandro Gibbons | INTA
Mariel Gonzalez | UBA
Nancy Greco | CEPAVE
Silvina Greco | FCA-UNCuyo
Fernando Hernandez | CONICET
Michel Hick | UCatólica de Córdoba
Jorge Hilbert | INTA
Leandra Ibarguren | FCA-UNCuyo
Adriana Kantolic | UBA
Carolina Kozub | FCA-UNCuyo
Dolly Lanfranco Leverton | UAustral de Chile
Pedro Leiva | INTA
María Valeria Longone | INTA
Gabriel Lorenzo | UBA
Marcelo Lovisolo | UNLomas de Zamora
Gabriela Lucero | FCA-UNCuyo
Matias Maggi | CONICET
Laura Magrí | INTA
Gisela Marcoppido | INTA
Laura Martínez | INTA
Angelo Mazzaglia | Università della Tuscia-Italia
Guillermo Meier | INTA
Diego Meloni | UNSE
Massimo Menenti | UTécnica de Delft-Países Bajos
Carolina Merlo | UNCórdoba
Marcos Montoya | INTA
Paula Mussetta | CONICET
Juan Nieto Nafria | Ude León-España
Raul Novello | INTA
Diego Ortiz | INTA
Sebastián Paez Lama | CONICET-UNCuyo
Carlos Passera | FCA-UNCuyo
Adriana Pazos | INTA
Martín Pérez | INTA
Eduardo Pérez Centeno | IPAF Patagonia
Juan Andrés Pina | DGI Mendoza
Aníbal Pordomingo | INTA
Daniel Alberto Presello | INTA
Mariela Quiroga | USan Luis
Geraldine Ramallo | Fundación Miguel Lillo
Fernando Ramos Gourcy | UAutónoma de Aguascalientes
Alejandra Ricca | INTA
Margarita Rocca | CEPAVE-La Plata
Adriana Rodriguez | UBA
Rosana Rotondo | UNRosario
Roberto Rubio | UNICEN
Valeria Santa | UNRío Cuarto
Carmen Sartor | FCA-UNCuyo
Javier Schapiro | INTA

Berta Schnettler | Universidad de la Frontera-Chile
Liliana Scoponi | UNSur
Gabriel Sevilla | INTA
Fidelina Silva | INTA
Verónica Soto | FCA-UNCuyo
Mario Steinberg | UNCórdoba
Hector Svartz | FAUBA
Natalia Szerman | INTA
Edith Taleisnik | INTA
Mauricio Tcach | INTA
Paula Toro|Mujica | Ude O`Higgins-Chile
Samuele Trestini | UNIPD-Italia
María Paula Turiello | UNRío Cuarto
Martín Uliarte | INTA
Rodrigo Valdes Salazar | PUCV
Silvia Van den Bosch | FCA-UNCuyo
María Eugenia Van den Bosch | INTA
Laura Varone | FUEDEI
Damian Vega | FAUBA
Mario Vigna | INTA
Marcos Yanniccari | INTA
Bernardita Zeballos | INTA
Alex Zilli | UNNE

Revista de la Facultad de Ciencias Agrarias
Universidad Nacional de Cuyo

Tomo 52(1) - Junio 2020

Índice

Genética y mejoramiento vegetal

Ecofisiología y manejo de cultivos

Recursos Naturales y ambiente

Economía y política agraria

Protección vegetal

Prducción y sanidad animal

Tecnologías agroindustriales

Revisión

Evaluation of a lentil collection (*Lens culinaris* Medik) using morphological traits and digital phenotyping

Evaluación de una colección de lentejas (*Lens culinaris* Medik) utilizando caracteres morfológicos y fenotipado digital

María Andrea Espósito [1, 2], Ileana Gatti [2, 3], Carolina Julieta Bermejo [2], Enrique Luis Cointry [2]

Originales: *Recepción:* 04/10/2017 - Aceptación: 11/10/2018

Abstract

The objective of this work was to evaluate 81 lentil cultivars using morphological traits and seed characteristics by digital phenotyping. Caliber (C) and the color traits luminosity (L), color coordinates a and b, and color index (CI) were measured and analyzed with appropriate software. Additionally, also yield (Y), plant height (PH) and days to flowering (DF) were measured. Highly significant differences between cultivars were found for all traits, while high broad sense heritability (H^2B) for C (97%), CI (94%), a (93%) and L and b (83%) were found, indicating high genetic variability for these traits. Digital phenotyping showed to be a powerful tool for germplasm characterization along with field evaluation of agronomical traits. Principal Component Analysis and Cluster Analysis allows de identification of differentiated groups of cultivars with similar characteristics, leading to a more efficient use of the germplasm available as commercial cultivars or as parents in a breeding program. Among these groups, group 1 had 32 cultivars with highest C and group 2 had 21 cultivars with higher Y.

Keywords
lentil • digital phenotyping • morphological characterization

1 EEA INTA Oliveros. Ruta Nacional 11. Km 353. C. P. 2206. mesposi@unr.edu.ar
2 Universidad Nacional de Rosario. Facultad Ciencias Agrarias. Cátedra Mejoramiento Vegetal y Producción de Semillas. IICAR-CONICET. Campo Experimental. Villarino s/n. Zavalla. C.C. 14 (S2125ZAA).
3 Consejo de Investigaciones UNR (CIUNR). Universidad Nacional de Rosario.

Resumen

El objetivo de este trabajo fue evaluar 81 cultivares de lenteja usando caracteres morfológicos y características de semilla utilizando fenotipado digital. El Calibre (C) y los caracteres Luminosidad (L), las coordenadas de color a y b, y el índice de color (IC) fueron medidos y analizados con un software apropiado; también fueron medidos el rendimiento (Y), altura de planta (PH) y los días a floración (DF). Se encontraron diferencias altamente significativas entre cultivares para todos los caracteres y se obtuvieron elevados valores de heredabilidad en sentido amplio (H^2B) para las variables C (97%), IC (94%), a (93%) y L y b (83%) indicando la presencia de alta variabilidad genética. El fenotipado digital mostró ser una poderosa herramienta para la caracterización de germoplasma junto con la evaluación a campo de caracteres agronómicos. El Análisis de Componentes Principales y el análisis de agrupamiento permitieron la identificación de diferentes grupos de cultivares con características similares lo que conduce a un uso más eficiente del germoplasma disponible como cultivares comerciales o como parentales en un programa de mejoramiento genético. Entre estos grupos, el grupo 1 tuvo 32 cultivares con mayor C y el grupo 2 tuvo 21 cultivares con mayor Y.

Palabras clave

lenteja • fenotipado digital • caracterización morfológica

Introduction

Lentil (*Lens culinaris Medik. ssp. culinaris*) is one of the most ancient crops in history (McVicar *et al.*, 2005). This cool season pulse, used in human nutrition as whole grain or flour, is an excellent source of dietary fiber, protein, healthy fat, carbohydrates and a range of micronutrients (Thavarajah *et al.*, 2011). Its high levels of low digestible carbohydrates reduces glycemic response in humans (Siva *et al.*, 2017) and its high fiber content gives it strong satiating properties, resulting in lower food intake (Faris *et al.*, 2013). This pulse is also a significant dietary source of a plethora of vitamins including folate, thiamin (B1) and riboflavin (B2) and relatively high levels of Mg, P, Ca and S (16).

All these characteristics make lentil a fundamental dietary component in low-income population and developing countries, as it is a substitute to proteins from livestock and fisheries (7) and have beneficial effects on human health (9).

The main consumer countries are those from Asia, north of Africa, Western Europe and part of Latin America. There are several market classes based on consumer preference, seed size and color. According to seed size the classes can be extra small (29-32 g/1000 seeds), small (33-45 g/1000 seeds), medium (51-52 g/1000 seeds) and large (55-73 g/1000 seeds). According to seed coat color they can be classified in green, brown, gray and purple or black; with cotyledon colors ranging from yellow to red and green (8).

In Argentina, lentil is cultivated mostly in the central area (south of the province of Santa Fe and north of the province of Buenos Aires), where is an important rainfed crop during the winter season (2). The main problem for growers is the lack of available cultivars, as only two commercial varieties are used in the present.

To solve this inconvenient, a breeding program is being carried out in the National University of Rosario, with the objective of obtaining new cultivars with higher yield and suitability for the different export markets.

A first step in any breeding program is to evaluate the variability of the germplasm available in the working collection using traits with agronomical importance. The characterization of traits whit high heritability and the evaluation of traits with low heritability and highly influenced by the environment can determine the utilization of this germplasm (3, 4).

The aim of the present work was to evaluate the genetic variability of a lentil collection for agronomical traits and seed characteristics (size and color) for later selection of accessions for commercial use or as parents in the breeding program. Seed traits where evaluated using digital phenotyping, performed by non-destructive, automated and image-based technology that offers an objective and quantitative method for estimation of morphological parameters as color and size.

Materials and Methods

Plant material and experimental design

Eighty one accessions of a working collection (table 1, page 4) were sowed in July of 2016, in plots of 3 m long and 3 rows 0.25 apart (approximately 200 plants) at the Experimental Field of the College of Agricultural Sciences, Rosario National University, located in Zavalla (33°1' S and 60°53' W) in a complete randomized design with three replications. The harvest was done manually.

Traits analyzed

The analyzed variables were days to 50% of flowering (DF); plant height (PH), (cm from the root, in 20 plants per plot) and yield (Y grams per plot). Color traits and seed caliber (C) were measured on two-dimensional digital images of 600 dpi taken on a Samsung CLX 3300 scanner of samples of 50 seeds per repetition and analyzed using Tomato Analyzer (TA) software (12).

The color traits were the coordinates *a* and *b*, and the psychometric index of lightness L from the CieLab system of color where:

- Coordinate *a* indicates the greenness-redness of the color (-*a* is green, +*a* is red) and varies between -128 and 128.
- Coordinate *b* indicates blueness-yellowness of the color (-*b* is blue, +*b* is yellow) and varies between -128 and 128.
- Parameter L is an approximate measurement of luminosity, the property according to which each color can be considered as equivalent to a member of the greyscale between black and white.

With these color parameters, a color index (CI) was calculated as: CI = (1.000 x a) / (L x b).

Statistical analysis

An ANOVA between cultivars and a comparison of means using the Fisher's least significant difference test (LSD) (14) were performed.

Table 1. Name and country of origin of the evaluated cultivars.

Tabla 1. Nombre y país de origen de los cultivares evaluados.

Cultivar	Country	Cultivar	Country
1A	Lebanon	25A	Lebanon
1R	Lebanon	26A	Lebanon
2A	Lebanon	26R	Lebanon
3A	Lebanon	27A	Lebanon
3R	Lebanon	27R	Lebanon
4A	Lebanon	28A	Lebanon
4R	Lebanon	28R	Lebanon
5A	Lebanon	29A	Lebanon
6R	Lebanon	29R	Lebanon
7A	Lebanon	30A	Lebanon
7R	Lebanon	30R	Lebanon
8A	Lebanon	31R	Lebanon
9A	Lebanon	32A	Lebanon
9R	Lebanon	32R	Lebanon
10A	Lebanon	33A	Lebanon
10R	Lebanon	33R	Lebanon
11A	Lebanon	34A	Lebanon
12A	Lebanon	34R	Lebanon
12R	Lebanon	35A	Lebanon
13R	Lebanon	35R	Lebanon
14A	Lebanon	A1062	Argentina
14R	Lebanon	A1145	Argentina
15A	Lebanon	B1051	Argentina
15R	Lebanon	B1052	Argentina
16A	Lebanon	B1053	Argentina
16R	Lebanon	B1054	Argentina
17A	Lebanon	B1055	Argentina
17R	Lebanon	B1056	Argentina
18A	Lebanon	B1151	Argentina
18R	Lebanon	B1153	Argentina
19A	Lebanon	B1156	Argentina
19R	Lebanon	B1157	Argentina
20A	Lebanon	B1181	Argentina
20R	Lebanon	B1182	Argentina
21A	Lebanon	NAILE	Canada
22A	Lebanon	PARDINA	Spain
22R	Lebanon	ROSE TOWN	Canada
23A	Lebanon	SEL 133	Argentina
23R	Lebanon	SEL 205	Argentina
24A	Lebanon	SILVINA	Argentina
24R	Lebanon		

Broad sense heritability was calculated as

$$\mathbf{H^2B = \sigma^2 g/(\sigma^2 g+\sigma^2 e)}$$

where:
$\sigma^2 g$ = represents genotypic variance
$\sigma^2 e$ = represents the environmental variance.

Finally, a Principal Component analysis (PC) and a Cluster analysis using average linkage method with Euclidean distances were performed in order to identify groups of cultivars with similar characteristics.

All statistical analysis were made using the software InfoStat for Windows (1).

Results and discussion

Genetic variability

Mean value, standard deviation (SD), F value for the ANOVA analysis, Fisher's least significant difference value (LSD) and broad sense heritability (H^2B) for all the traits are shown in table 2, page 5-6; table 3, page 7-8. All traits presented highly significant differences between cultivars ($p<0.001$), and broad sense heritability varied between 0.33 for DF to 0.97 for C, demonstrating the existence of genetic variability suitable for selection purposes. Bermejo *et al.* (2012) in the evaluation of 28 lentil RIL's; Lázaro *et al.* (2001) in a working collection of Spanish materials and Erskine *et al.* (1998) in collections from ICARDA (International Center for Agricultural Research in the Dry Areas) found similar values of H^2B.

Regarding mean values, for DF, cultivars 7A, Rose Town and Pardina were late (104 and 105 DF) while cultivar 34A was the earliest (57 DF).

Table 2. Mean, standard deviation (SD), LSD value, F value and broad sense heritability (H^2B) for days to flowering (DF), plant height (PH), yield (Y) and seed caliber (C).

Tabla 2. Media, desviación estándar (SD), valor de LSD, valor de F y Heredabilidad en sentido amplio (H^2B) para los caracteres días a floración (DF), altura de planta (PH), rendimiento (Y) y calibre de grano (C).

	DF		PH (cm)		Y (g)		C (mm)	
Cultivar	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
10A	99.5	6.4	29.0	4.2	123.0	38.2	7	0.00
10R	98.0	8.5	28.0	5.7	72.5	29.0	5	0.01
11A	98.0	8.5	29.0	9.9	173.5	13.4	7	0.01
12A	98.0	8.5	33.0	5.7	116.5	72.8	7	0.00
12R	98.0	8.5	25.0	2.8	185.0	131.5	5	0.01
13R	94.0	2.8	34.5	6.4	86.0	48.1	6	0.01
14A	101.0	7.1	36.0	5.7	97.0	1.4	7	0.01
14R	95.5	0.7	26.0	2.8	54.5	57.3	6	0.00
15A	93.5	3.5	31.5	5.0	138.5	14.9	7	0.00
15R	92.5	5.0	28.5	2.1	98.5	12.0	6	0.01
16A	88.0	11.3	24.5	0.7	206.5	41.7	6	0.00
16R	98.0	2.8	21.0	4.2	66.0	33.9	6	0.00
17A	82.0	2.8	28.5	5.0	88.0	46.7	8	0.01
17R	88.0	5.7	26.0	1.4	58.5	23.3	6	0.01
18A	98.0	2.8	25.5	3.5	100.5	14.9	6	0.01
18R	86.5	3.5	32.0	9.9	66.0	18.4	5	0.01
19A	95.0	4.2	34.0	5.7	64.5	47.4	7	0.01
19R	90.0	2.8	29.5	3.5	67.5	24.8	5	0.01
1A	101.5	0.7	29.5	0.7	69.0	12.7	7	0.00
1R	87.5	0.7	29.5	0.7	43.5	5.0	6	0.02
20A	98.5	2.1	30.0	4.2	49.0	26.9	7	0.01
20R	94.5	0.7	31.5	0.7	81.0	49.5	6	0.01
21A	97.5	0.7	34.5	0.7	126.5	2.1	7	0.00
22A	87.5	20.5	32.5	0.7	101.0	1.4	7	0.04
22R	95.5	0.7	29.5	0.7	90.5	5.0	6	0.01
23A	87.5	0.7	29.5	0.7	105.0	9.9	7	0.01
23R	91.5	0.7	39.5	0.7	117.5	6.4	6	0.02
24A	93.5	0.7	26.5	2.1	104.0	19.8	7	0.01
24R	99.0	1.4	32.0	11.3	77.5	3.5	6	0.01
25A	97.5	0.7	28.0	1.4	136.5	44.6	7	0.04
26A	94.0	1.4	26.0	1.4	92.5	20.5	7	0.01
26R	87.5	0.7	27.5	0.7	114.5	2.1	6	0.02
27A	100.5	0.7	29.0	7.1	143.0	72.1	7	0.00
27R	90.5	0.7	31.0	1.4	80.5	30.4	6	0.03
28A	91.5	0.7	37.0	2.8	31.5	31.8	6	0.01
28R	94.5	0.7	28.5	0.7	94.5	5.0	4	0.00
29A	102.0	5.7	31.0	7.1	156.5	9.2	7	0.00
29R	92.0	1.4	28.5	5.0	99.5	47.4	6	0.00
2A	102.5	0.7	31.0	1.4	162.0	45.3	7	0.01
30A	92.0	1.4	32.5	3.5	86.0	0.0	7	0.01
30R	91.5	0.7	30.5	3.5	175.5	21.9	6	0.00
31R	76.5	29.0	28.0	2.8	175.0	56.6	6	0.02

Table 2 (cont.). Mean, standard deviation (SD), LSD value, F value and broad sense heritability (H^2B) for days to flowering (DF), plant height (PH), yield (Y) and seed caliber (C).

Tabla 2 (cont.). Media, desviación estándar (SD), valor de LSD, valor de F y Heredabilidad en sentido amplio (H^2B) para los caracteres días a floración (DF), altura de planta (PH), rendimiento (Y) y calibre de grano (C).

	DF		PH (cm)		Y (g)		C (mm)	
Cultivar	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
10A	99.5	6.4	29.0	4.2	123.0	38.2	7	0.00
10R	98.0	8.5	28.0	5.7	72.5	29.0	5	0.01
11A	98.0	8.5	29.0	9.9	173.5	13.4	7	0.01
12A	98.0	8.5	33.0	5.7	116.5	72.8	7	0.00
12R	98.0	8.5	25.0	2.8	185.0	131.5	5	0.01
13R	94.0	2.8	34.5	6.4	86.0	48.1	6	0.01
14A	101.0	7.1	36.0	5.7	97.0	1.4	7	0.01
14R	95.5	0.7	26.0	2.8	54.5	57.3	6	0.00
15A	93.5	3.5	31.5	5.0	138.5	14.9	7	0.00
15R	92.5	5.0	28.5	2.1	98.5	12.0	6	0.01
16A	88.0	11.3	24.5	0.7	206.5	41.7	6	0.00
16R	98.0	2.8	21.0	4.2	66.0	33.9	6	0.00
17A	82.0	2.8	28.5	5.0	88.0	46.7	8	0.01
17R	88.0	5.7	26.0	1.4	58.5	23.3	6	0.01
18A	98.0	2.8	25.5	3.5	100.5	14.9	6	0.01
18R	86.5	3.5	32.0	9.9	66.0	18.4	5	0.01
19A	95.0	4.2	34.0	5.7	64.5	47.4	7	0.01
19R	90.0	2.8	29.5	3.5	67.5	24.8	5	0.01
1A	101.5	0.7	29.5	0.7	69.0	12.7	7	0.00
1R	87.5	0.7	29.5	0.7	43.5	5.0	6	0.02
20A	98.5	2.1	30.0	4.2	49.0	26.9	7	0.01
20R	94.5	0.7	31.5	0.7	81.0	49.5	6	0.01
21A	97.5	0.7	34.5	0.7	126.5	2.1	7	0.00
22A	87.5	20.5	32.5	0.7	101.0	1.4	7	0.04
22R	95.5	0.7	29.5	0.7	90.5	5.0	6	0.01
23A	87.5	0.7	29.5	0.7	105.0	9.9	7	0.01
23R	91.5	0.7	39.5	0.7	117.5	6.4	6	0.02
24A	93.5	0.7	26.5	2.1	104.0	19.8	7	0.01
24R	99.0	1.4	32.0	11.3	77.5	3.5	6	0.01
25A	97.5	0.7	28.0	1.4	136.5	44.6	7	0.04
26A	94.0	1.4	26.0	1.4	92.5	20.5	7	0.01
26R	87.5	0.7	27.5	0.7	114.5	2.1	6	0.02
27A	100.5	0.7	29.0	7.1	143.0	72.1	7	0.00
27R	90.5	0.7	31.0	1.4	80.5	30.4	6	0.03
28A	91.5	0.7	37.0	2.8	31.5	31.8	6	0.01
28R	94.5	0.7	28.5	0.7	94.5	5.0	4	0.00
29A	102.0	5.7	31.0	7.1	156.5	9.2	7	0.00
29R	92.0	1.4	28.5	5.0	99.5	47.4	6	0.00
2A	102.5	0.7	31.0	1.4	162.0	45.3	7	0.01
30A	92.0	1.4	32.5	3.5	86.0	0.0	7	0.01
30R	91.5	0.7	30.5	3.5	175.5	21.9	6	0.00
31R	76.5	29.0	28.0	2.8	175.0	56.6	6	0.02

*** significant with p<0.001. / *** diferencias significativas con p<0,001.

Table 3. Mean, standard deviation (SD), LSD value, F value and broad sense heritability (H^2B) for L, *a* and *b* coordinates of color and color index (CI).

Tabla 3. Media, desviación estándar (SD), valor de LSD, valor de F y Heredabilidad en sentido amplio (H^2B) para los caracteres L, las coordenadas de color a y b y el índice de color (CI).

	L		**a**		**b**		**CI**	
Cultivar	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
10A	55.2	1.1	9.6	0.6	20.7	0.6	8.4	0.1
10R	53.4	1.6	15.0	0.2	23.2	0.0	12.2	0.6
11A	52.3	1.4	8.6	0.7	19.2	0.1	8.6	1.0
12A	52.9	1.5	10.2	0.5	20.8	0.3	9.3	0.6
12R	58.2	1.3	10.0	0.8	22.5	0.8	7.7	1.1
13R	50.4	1.4	16.5	0.2	22.0	0.6	14.8	0.6
14A	54.7	0.9	10.0	0.6	21.1	0.5	8.6	0.5
14R	47.1	0.7	11.3	0.5	17.3	0.1	13.9	0.9
15A	46.1	0.8	11.2	0.3	17.7	0.1	13.8	0.5
15R	47.8	0.2	11.8	0.3	20.5	0.6	12.1	0.0
16A	54.3	0.1	8.0	0.8	19.6	0.1	7.6	0.7
16R	54.3	0.1	8.0	0.8	19.6	0.1	7.6	0.7
17A	51.7	2.3	9.9	0.9	19.0	0.3	10.1	1.2
17R	44.8	2.4	12.8	1.0	19.6	0.4	15.4	3.0
18A	51.3	0.3	3.8	0.2	16.7	0.2	4.4	0.3
18R	63.4	18.1	7.0	3.3	15.1	7.1	7.6	2.2
19A	53.1	1.0	10.3	0.1	20.5	0.1	9.4	0.2
19R	40.9	0.8	15.1	0.6	21.7	0.1	17.1	1.0
1A	53.8	2.1	9.2	0.8	20.4	0.4	8.4	0.9
1R	46.8	0.7	9.1	0.2	13.6	0.2	14.4	0.7
20A	53.3	0.4	10.5	0.1	20.1	0.3	9.8	0.0
20R	55.9	0.8	5.3	0.2	18.3	0.2	5.2	0.0
21A	53.0	0.3	8.6	0.4	20.0	0.3	8.1	0.2
22A	49.0	0.8	11.2	0.8	19.6	0.6	11.7	0.6
22R	44.7	2.2	12.6	1.1	19.5	0.4	14.5	1.7
23A	54.8	1.1	7.4	0.0	19.7	0.1	6.9	0.2
23R	54.6	1.5	8.1	0.3	18.2	0.5	8.1	0.7
24A	55.8	0.8	7.2	0.8	19.5	0.3	6.6	0.7
24R	45.9	0.7	9.3	0.2	14.2	0.1	14.5	0.6
25A	52.8	0.8	8.2	0.2	19.4	0.3	8.0	0.2
26A	55.0	1.0	6.3	0.3	18.6	0.5	6.1	0.0
26R	44.6	0.9	12.4	0.1	19.9	0.3	14.0	0.3
27A	53.9	0.7	9.1	0.3	18.7	0.2	9.0	0.1
27R	54.0	1.6	8.5	1.6	19.1	0.7	8.2	1.5
28A	47.1	1.1	12.1	1.0	19.4	0.7	13.2	0.9
28R	45.2	0.5	8.6	0.1	15.3	0.1	12.5	0.3
29A	48.7	1.7	11.6	0.9	19.5	0.1	12.3	1.3
29R	49.1	1.6	11.5	1.2	20.9	0.4	11.2	1.3
2A	50.1	0.7	3.7	0.2	16.0	0.1	4.6	0.2
30A	49.0	0.2	11.3	0.2	19.3	0.1	11.9	0.1
30R	44.5	1.2	11.5	0.6	19.3	0.9	13.5	1.6
31R	53.3	1.4	8.7	0.7	20.3	0.1	8.1	0.9

*** significant with $p<0.001$. / *** diferencias significativas con $p<0,001$.

Table 3. (cont.). Mean, standard deviation (SD), LSD value, F value and broad sense heritability (H^2B) for L, *a* and *b* coordinates of color and color index (CI).

Tabla 3. (cont.). Media, desviación estándar (SD), valor de LSD, valor de F y Heredabilidad en sentido amplio (H^2B) para los caracteres L, las coordenadas de color a y b y el índice de color (CI).

	L		a		b		CI	
Cultivar	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
10A	55.2	1.1	9.6	0.6	20.7	0.6	8.4	0.1
10R	53.4	1.6	15.0	0.2	23.2	0.0	12.2	0.6
11A	52.3	1.4	8.6	0.7	19.2	0.1	8.6	1.0
12A	52.9	1.5	10.2	0.5	20.8	0.3	9.3	0.6
12R	58.2	1.3	10.0	0.8	22.5	0.8	7.7	1.1
13R	50.4	1.4	16.5	0.2	22.0	0.6	14.8	0.6
14A	54.7	0.9	10.0	0.6	21.1	0.5	8.6	0.5
14R	47.1	0.7	11.3	0.5	17.3	0.1	13.9	0.9
15A	46.1	0.8	11.2	0.3	17.7	0.1	13.8	0.5
15R	47.8	0.2	11.8	0.3	20.5	0.6	12.1	0.0
16A	54.3	0.1	8.0	0.8	19.6	0.1	7.6	0.7
16R	54.3	0.1	8.0	0.8	19.6	0.1	7.6	0.7
17A	51.7	2.3	9.9	0.9	19.0	0.3	10.1	1.2
17R	44.8	2.4	12.8	1.0	19.6	0.4	15.4	3.0
18A	51.3	0.3	3.8	0.2	16.7	0.2	4.4	0.3
18R	63.4	18.1	7.0	3.3	15.1	7.1	7.6	2.2
19A	53.1	1.0	10.3	0.1	20.5	0.1	9.4	0.2
19R	40.9	0.8	15.1	0.6	21.7	0.1	17.1	1.0
1A	53.8	2.1	9.2	0.8	20.4	0.4	8.4	0.9
1R	46.8	0.7	9.1	0.2	13.6	0.2	14.4	0.7
20A	53.3	0.4	10.5	0.1	20.1	0.3	9.8	0.0
20R	55.9	0.8	5.3	0.2	18.3	0.2	5.2	0.0
21A	53.0	0.3	8.6	0.4	20.0	0.3	8.1	0.2
22A	49.0	0.8	11.2	0.8	19.6	0.6	11.7	0.6
22R	44.7	2.2	12.6	1.1	19.5	0.4	14.5	1.7
23A	54.8	1.1	7.4	0.0	19.7	0.1	6.9	0.2
23R	54.6	1.5	8.1	0.3	18.2	0.5	8.1	0.7
24A	55.8	0.8	7.2	0.8	19.5	0.3	6.6	0.7
24R	45.9	0.7	9.3	0.2	14.2	0.1	14.5	0.6
25A	52.8	0.8	8.2	0.2	19.4	0.3	8.0	0.2
26A	55.0	1.0	6.3	0.3	18.6	0.5	6.1	0.0
26R	44.6	0.9	12.4	0.1	19.9	0.3	14.0	0.3
27A	53.9	0.7	9.1	0.3	18.7	0.2	9.0	0.1
27R	54.0	1.6	8.5	1.6	19.1	0.7	8.2	1.5
28A	47.1	1.1	12.1	1.0	19.4	0.7	13.2	0.9
28R	45.2	0.5	8.6	0.1	15.3	0.1	12.5	0.3
29A	48.7	1.7	11.6	0.9	19.5	0.1	12.3	1.3
29R	49.1	1.6	11.5	1.2	20.9	0.4	11.2	1.3
2A	50.1	0.7	3.7	0.2	16.0	0.1	4.6	0.2
30A	49.0	0.2	11.3	0.2	19.3	0.1	11.9	0.1
30R	44.5	1.2	11.5	0.6	19.3	0.9	13.5	1.6
31R	53.3	1.4	8.7	0.7	20.3	0.1	8.1	0.9

*** significant with p<0.001. / *** diferencias significativas con p<0,001.

In PH, cultivar 4A was the tallest (44 cm) while B1053 was the shortest, with only 20.5 cm of plant height. Cultivars B1157 and B1051 had the best yielding with 363 g plot^{-1}and 317 g plot^{-1} respectively, and cultivar SEL was the poorest with 15.5 g plot^{-1}.

Digital phenotyping showed that cultivars 17A, 22A, 19A and 30A had larger seeds with calibers of 7.5 mm, 7.4 mm, 7.4 mm and 7.3 mm respectively, while B1181, B1182 and 28R had the smallest seeds, with calibers ranging from 0.44 to 0.45. Color parameter L was high for cultivar 18R (63.43) and low for B1182 (36.40).

The *a* coordinate of color showed the highest values for 13R and 19R (16.45 and 15.10 respectively) meaning that these two cultivars are material for greater reddish color, while 35A, 18A and 2A showed the least (4.81, 3.79 and 3.70 respectively).

The *b* coordinate denotes the greenish color and was high for 10R and 12R, (23.22 and 22.45 respectively) and low for Pardina (13.26). When the color index (CI) was analyzed, cultivars B1182 (24.11) and B1181 (23.53) had the highest values, while 18A (4.43) was the lowest.

Cluster Analysis

Cluster analysis (figure 1, page 10), showed that cultivars conformed six groups with differential traits. This analysis allows the identification of cultivars with convenient characteristics, as seed size. A comparison of mean values of each group (table 4, page 11) using the Fisher's least significant difference test (LSD) showed that Group 1 had 32 cultivars with high C; group 2 had 21 cultivars with higher Y but lower CI. Group 3 had 17 cultivars with high values for coordinates *a* and *b*; group 4 included only one cultivar (18R) with the highest L; group 5 had 4 cultivars with lower C and coordinate *b*; and group 6, with 6 cultivars, had the cultivars with higher DF and CI but lower C and L.

Principal Component Analysis

Principal component analysis showed that two principal components explained 58% of the variation in the data set (PC1, 40% and PC2, 18%) and with the addition of a third component the proportion of variation explained reached 73% (PC3, 15%).

However, when the first two components are plotted against each other (figure 2, page 11) the cultivars conform 4 clearly differentiated groups. PC1 was associated with C, L, *a*, *b* and CI while PC2 was associated with DF, PH and Y.

In figure 2 (page 11), points represent lentil cultivars and vectors represent analyzed traits.

The perpendicular projection of the points on the vectors indicates the relative position of that cultivar against the others for that particular trait, having the highest values those cultivars in the positive direction of the vector, while the angle between vectors shows the correlation among traits. In this case, cultivars B1181 and B1182 had the highest values of CI and cultivar B1051 had the highest yield. Correlations shows that traits CI and *a*, DF and PH, C and L, and Y and *b* have positive correlations, while CI and a have *a* negative correlation with L.

There is a clear concordance between groups obtained by Cluster Analysis and by Principal Component Analysis. Groups 2 and 6 are separated groups, and were conformed with the same cultivars in both analyses. Groups 1 and 2 in one hand, and groups 3 and 5 on the other, conform two different groups in the Principal Component analysis. The selected materials, as parents for a breeding program, were those from group 2, given their higher yields and shorter cycle, and those from group 1, with higher caliber.

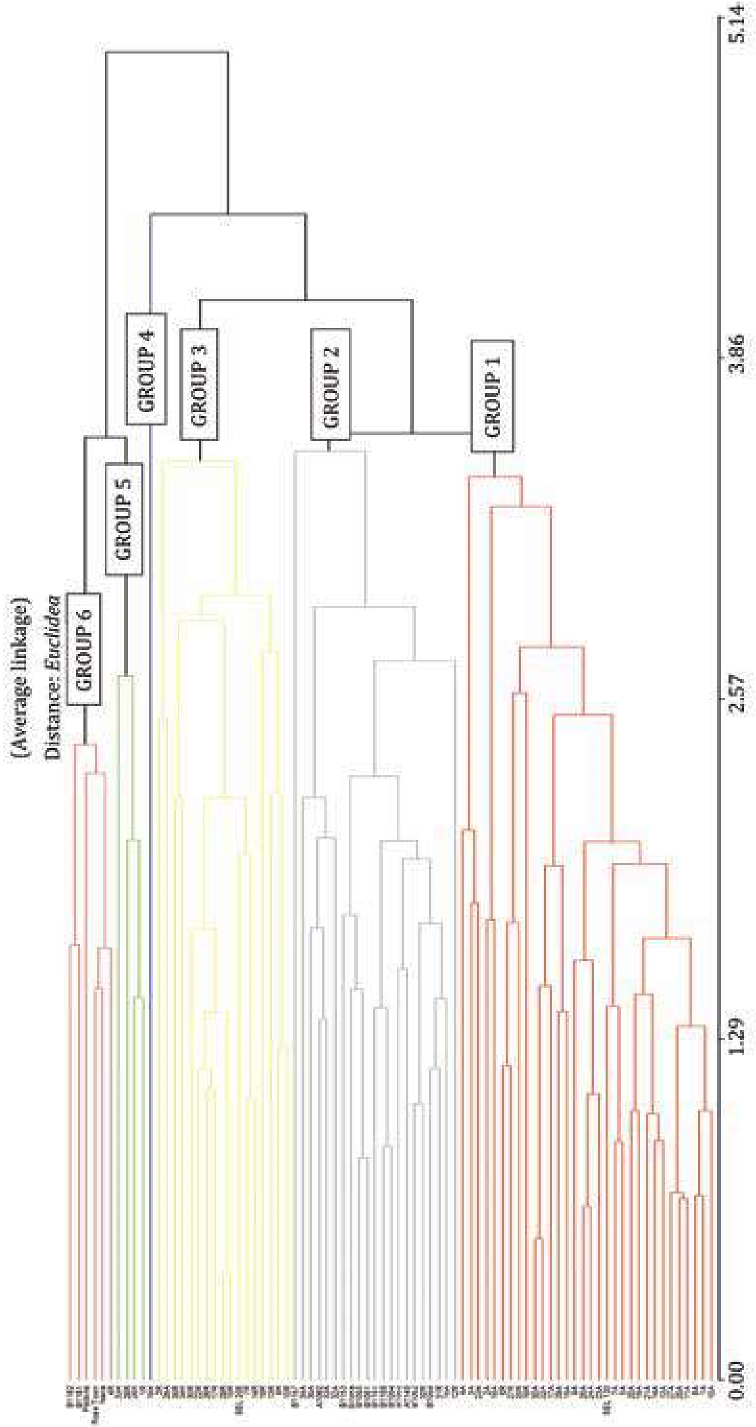


Figure 1. Cluster Analysis.
Figura 1. Análisis de conglomerados.

Table 4. Fisher's least significant difference test between group's means in the Cluster Analysis.
Tabla 4. Prueba de la mínima diferencia significativa de Fisher entre las medias de los grupos del Análisis de conglomerados.

	Traits							
Group	**DF**	**PH**	**Y**	**C**	**L**	**a**	**b**	**CI**
1	95.84a	20.83a	100.95b	0.67a	53.21c	8.42b	19.28b	8.23c
2	80.6b	32.0a	189.83a	0.63b	54.61b	6.97b	20.47a	7.35d
3	101.83a	29.06a	66.0b	0.56c	46.86d	12.86a	20.23a	13.08b
4	86.5ab	28.75a	84.97b	0.54cd	63.43a	8.68b	15.43c	7.60cd
5	90.91a	25.14a	76.5b	0.52d	46.10d	8.08b	15.06c	13.54b
6	88.88a	27.5a	124.50b	0.50d	38.93e	12.05a	14.65c	20.52a

Means with a common letter don't differ ($p<0.05$). / Medias con una letra común no son significativamente diferentes ($p>0,05$).

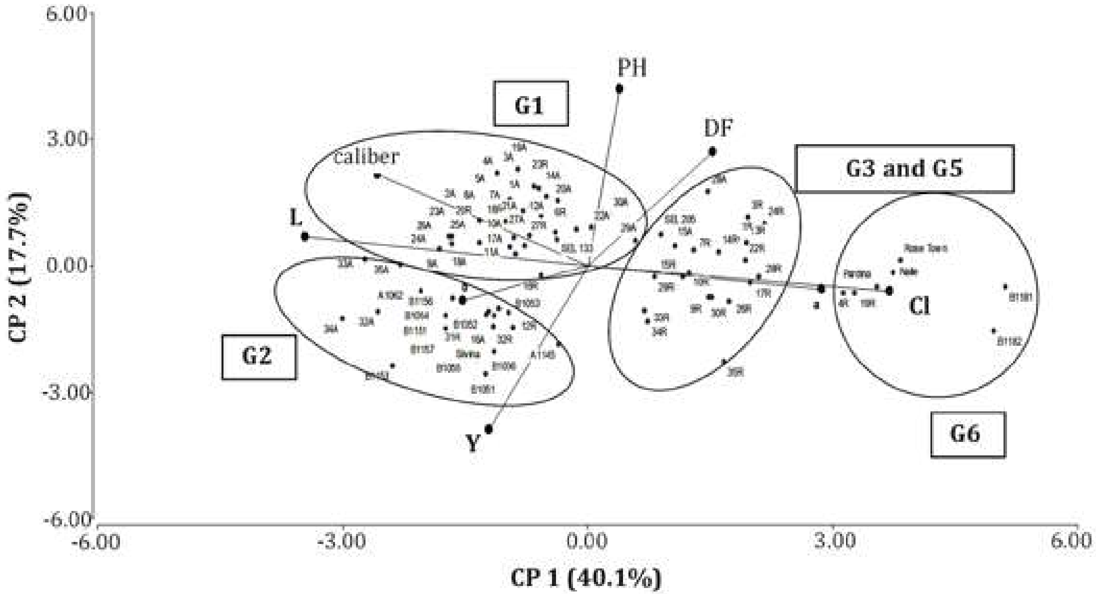


Figure 2. Biplot of the first two Principal Components. / **Figura 2.** Biplot de las dos primeras Componentes Principales.

Conclusions

Digital phenotyping showed to be a powerful tool for germplasm characterization along with field evaluation of agronomical traits. Principal Component Analysis and Cluster Analysis the identification of differentiated groups of cultivars with similar characteristics, leading to a more efficient use of the germplasm available.

Preliminary evaluation of the set of cultivars presented in this study demonstrate the existence of high phenotypic and genotypic diversity for different traits, showing their potential commercial or breeding value.

References

1. Balzarini, M.; Di Renzo, A.; González, L.; Tablada, M.; Guzmán; W. R. 2012. InfoStat software estadístico InfoStat versión 2008. Manual de usuario. Grupo InfoStat. FCA. Universidad Nacional de Córdoba, Argentina.
2. Barreiro, E. 2010. Cadenas Alimentarias: Producción de lentejas [en línea] Edición No. 49. Argentina: Alimentos Argentinos. [Consultado: 13/09/2013]. Available in: <http://www.alimentosargentinos.gov.ar/contenido/revista/ediciones/49/productos/r49_10_Lentejas.pdf>.
3. Bermejo, C.; López Anido, F. S.; Cointry, E. L. 2012. Descripción de líneas recombinantes de lenteja (RIL) mediante caracteres morfológicos. Horticultura Argentina. 31: 74.
4. Carloni, E.; López Colomba, E.; Ribotta, A.; Quiroga, M.; Tommasino, E.; Griffa, S.; Grunberg, K. 2018. Analysis of genetic variability *in vitro* regenerated buffelgrass plants through ISSR molecular markers. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 1-13.
5. Erskine, W.; Chandra, S.; Chaudhry, M.; Malik, I. A.; Sarker, A.; Sharma, B.; Tufail, M.; Tyagi, M. C. 1998. Abottleneck in lentil: widening its genetic base in South Asia. Euphytica. 101: 207-211.
6. Faris, M.; Takruri, H. R.; Issa, A. Y. 2013. Role of lentils (*Lens culinaris* L.) in human health and nutrition: a review Mediterr J Nutr Metab. 6: 3-16 DOI 10.1007/s12349-012-0109-8.
7. Financiera Rural. Monografía de la Lenteja. 2010. [en línea]. Dirección Ejecutiva de Análisis Sectorial. México. [Consultado: 07/05/2013]. Available in: <http://www.financierarural.gob.mx/informacionsectorrural/Documents/Monografias/Monograf%C3%ADa%20Lenteja_Mayo-2010.pdf>.
8. Government of Saskatchewan. 2016. Varieties of Grain Crops. Available in: http://www.saskseed.ca/images/varieties2016.pdf Accessed 26.06.2017.
9. Jenkins, D. J.; Kendall, W.; Augustin, L. S.; Mitchell, S.; Sahye-Pudaruth, S.; Blanco, M. S.; Chiavaroli, L.; Mirrahimi, A.; Ireland, C.; Bashyam, B.; Vidgen, E.; de Souza, R. J.; Sievenpiper, J. L.; Coveney, J.; Leiter, L. A.; Josse, R. G. 2012. Effect of legumes as part of a low glycemic index diet on glycemic control and cardiovascular risk factors in type 2 diabetes mellitus. Archives of Internal Medicine. 172(21): 1663-1660.
10. Lázaro, A.; Ruiz, M.; De la Rosa, L. & Martin, I. 2001. Relationship between agro/morphological characters and climatic parameters in Spanish landraces of lentil (*Lens culinaris*Medik.). Genetics Resources and Crop Evolution 48: 239-249.
11. McVicar, R.; Pearse, P.; Brenzil, C.; Hartley, S.; Panchuk, K.; Mooleki, P. 2005. Lentil in Saskatchewan. Saskatchewan Agriculture and Food. A. Vandenberg, S. Banniza, University of Saskatchewan. [(accessed August 2017)]. Available online: http://publications.gov.sk.ca/documents/20/86381-Lentils in Saskatchewan.pdf.

12. Rodríguez, G.; Moyseenko, J.; Robbins, M.; Huarachi Morejón, N.; Francis, D.; Van der Knaap, E. 2010. Tomato Analyzer: A useful software application to collect accurate and detailed morphological and colorimetric data from two-dimensional objects. Journal of Visualized Experiment. 37: 1856.
13. Siva, N.; Thavarajah, D.; Johnson, C.; Duckett, S.; Jesch, E.; Thavarajah, P. 2017. Can lentil (*Lens culinaris Medikus*) reduce the risk of obesity? Journal of Functional Foods. Available in: http://dx.doi.org/10.1016/j.jff.2017.02.01. Accessed August 2017).
14. Steel, R.; Torrie, J. 1993. Comparaciones múltiples. En Bioestadística. Principios y Procedimientos. McGraw and Hill. Eds. Segunda Edición. 622 p.
15. Thavarajah, D.; Thavarajah, P.; Sarker, A.; Materne, M.; Vandemark, G.; Shrestha, R.; Idrissi, O.; Hacikamiloglu, O.; Bucak, B.; Vandenberg, A. 2011. A global survey of effects of genotype and environment on selenium concentration in lentils (*Lens culinaris* L.): implications for nutritional fortification strategies. Food Chem. 125: 72-76.
16. United States Department of Agriculture (USDA). 2011. USDA National Nutrient Database for Standard Reference. Release 23. Retrievable from http://www.ars.usda.gov/research/publications/ publications.htm? seq_no_115=243584 (accessed Jan 2011).

Protein content and quality of seeds in central mexican maize (*Zea mays*) accessions

Contenido de proteína y calidad de semilla en accesiones de maíz (*Zea mays*) del centro de México

César Leobardo Aguirre-Mancilla [1 *], Rosario Yarandín Godínez-Galán [1], Juan Carlos Raya-Pérez [1], Glenda Margarita Gutiérrez-Benicio [1, 2], Juan Gabriel Ramírez-Pimentel [1], Jorge Covarrubias-Prieto [1], J. Guadalupe García-Rodríguez [1]

Originales: *Recepción:* 25/03/2019 - *Aceptación:* 30/11/2019

Abstract

Mexico is the center of origin, domestication and diversity of maize. This cereal is the main constituent of the Mexican diet, especially for low-income families. In this research, 10 maize accessions derived from a large landrace collection, with the 'INIFAP-QPM' accession and a 'regional landrace' as controls, were studied to identify the accessions with the best biochemical and physiological characteristics showing good adaptation to 'El Bajío' (regional center in Mexico) conditions. The accessions were statistically superior to the two controls in the germination and accelerated aging tests. In the assessment of variable plumule length, variability was observed among the accessions, but the controls showed the lowest values. Protein contents in different fractions (albumins, globulins, prolamins and glutelins) showed variability as did oil and fiber contents. The 'HRH2015' accession showed high contents of albumins and globulins and low contents of prolamins and glutelins. The 'regional landrace' accession exhibited the highest contents of glutelins and prolamins but the lowests content of globulins and albumins. The total percentage of proteins showed variability among the accessions, but the values were within those reported in the literature. The 'RQ2015' accession presented the highest oil content (5.25%). The electrophoretic patterns of prolamins were obtained, and some differences were observed between them. The 'regional landrace' presented the lowest protein content, which was significantly different from those of the other evaluated accessions. This research demonstrates biochemical, germination and vigor variability among the studied maize accessions.

Keywords

Zea mays • corn • protein fractions • vigor • quality protein

1 Tecnológico Nacional de México/I. T. Roque. km 8 Carretera Celaya-Juventino Rosas. CP. 38110. Roque. Celaya. Guanajuato. México. * ceaguirre@itroque.edu.mx

2 Universidad de Guanajuato. Campus Celaya-Salvatierra. Sede Mutualismo. Celaya. Guanajuato. México.

Resumen

México es el centro de origen, domesticación y diversidad del maíz. Este cereal ocupa el primer lugar como constituyente de la dieta de los mexicanos, sobre todo de las familias de bajos recursos. En esta investigación se estudiaron 10 accesiones de maíz derivadas de una colecta amplia de Criollos y como testigos INIFAP-QPM y un criollo de la región, con el objetivo de identificar las accesiones con mejores características bioquímicas y fisiológicas con buena adaptación a las condiciones de El Bajío. Las accesiones fueron estadísticamente superiores a los dos testigos en las pruebas de germinación y envejecimiento acelerado; en la variable longitud de plúmula se observó variabilidad entre las accesiones, pero los testigos siguieron mostrando los valores más bajos. El contenido de proteína en sus diferentes fracciones (albúminas, globulinas, prolaminas y glutelinas) mostró variabilidad, así como el contenido de aceite y fibra. Se encontró una accesión (HRH2015) con alto contenido de albúminas y globulinas y bajo contenido de prolaminas y glutelinas; el 'genotipo criollo' presentó el mayor contenido de glutelinas y prolaminas pero el menor en globulinas y albúminas. El porcentaje total de proteínas presentó variabilidad entre las accesiones, pero los valores están dentro de los reportados en la literatura. El genotipo 'RQ2015' presentó el más alto contenido de aceite (5,25%). Se obtuvieron los patrones electroforéticos de las prolaminas y se observaron algunas diferencias entre ellas. El criollo de la región, tomado como referencia, presentó el menor contenido de proteína estadísticamente diferente al resto de las accesiones evaluadas. Esta investigación demuestra la variabilidad existente a nivel bioquímico, germinación y vigor entre las accesiones de maíz estudiadas.

Palabras clave

Zea mays • maíz • fracciones proteicas • vigor • calidad proteica

Introduction

Maize is the main grain cultivated in the "El Bajío" region of Mexico and is the principal ingredient in the Mexican population's diet (15, 30). Information on the chemical composition of corn grain is abundant and clearly states that the variability of each component is broad as a result of both genetic and environmental factors (20, 28). These factors can influence the chemical composition of different parts of the grain (11).

The prolamin fraction constitutes the highest proportion of the protein present in cereals, followed by glutelins and in smaller amounts albumins and globulins. The nutritional quality of the protein as well as its physicochemical and functional characteristics, depend on the proportion of each protein fraction in the grains (37). Cereal proteins present low biological or nutritional value because they are deficient in some of the essential amino acids, such as methionine and lysine (19, 38). These proteins include wheat gliadins, barley hordeins, corn zeins (prolamins), wheat glutelins, and rice orizein.

High-quality-protein materials contain higher amounts of the albumin and globulin fractions and lower amounts of prolamins, as indicated by Vivas-Rodriguez *et al.* (1990) and Yang *et al.* (2018). The expression of the physicochemical characteristics of maize is influenced by the environment and depends on genotype-environment interaction (2). Agricultural practices have allowed the characteristics of the grain to be improved according to Zepeda-Bautista *et al.* (2009). Prolamins are stored in the starchy endosperm, and albumins and globulins are concentrated in the embryo and aleurone layer (19). The distribution of these proteins could alter some properties of the grain, depending on their relative abundance. Nitrogen application affects grain protein content. Its effect is greater in the prolamin fraction because approximately 60% of the grain protein consists of prolamins (zeins). The albumin and globulin fractions represent 22% of the protein content and are concentrated in the embryo. Glutelins are found in both the germ and the endosperm and account for up to 25% of the grain protein (26, 38).

Ten gene families encode prolamin proteins in maize, with 3-10 genes in each family (19). Corn contains 7-13% protein, which can be differentiated into three types: 1) storage proteins, which are the most abundant type, constituting a reserve of amino acids deposited during the development of the seeds; 2) enzymes involved in metabolism; and 3) structural proteins (23, 39).

Although studies have been conducted on this topic, more research on the protein quality of maize landraces cultivated in Mexico is required (27). Evaluation of the performance of different accessions in different environments and the influence of agronomic practices on the composition of essential amino acids, which determine the quality of grain proteins, is also required (37). Finally, it is vital to keep in mind that, for breeding purposes, to consider seed vigor is also necessary. This characteristic is usually tested with the accelerated aging test (17). With this objective, maize accessions were evaluated to characterize them biochemically and physiologically. They were integrated from a broad genetic base population (3). The 'INIFAP-QPM' accession and a maize landrace were used as controls.

The objective of this research was to identify accessions with good protein and physiological quality among accessions that are well adapted to the environmental conditions of El Bajío, Mexico, under the hypothesis that the accessions show good seed quality.

Materials and methods

Ten maize accessions derived from the Celaya, Tuxpeño, Cónico Norteño and Bofo races were used (14): '12015', '22015', 'A2015', 'ROQUE2', 'POL2015', 'FVR12015', 'ERH2015', 'MRH2015', 'RQ2015' and 'HRH2015' (12, 27), along with 'INIFAP-QPM' (accession with high protein quality from the "Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias") and a 'regional landrace' harvested in the SS 2015 growing season. The analyzed accessions were derived from collections from the Mexican states of Michoacan, Hidalgo, Estado de México and Puebla. Each collection was developed from 200 plants. Standard germination (18) was evaluated; a sample of 200 seeds from each accession was obtained, and each sample was divided into four replicates of 50 seeds (12) and incubated in a germination chamber (hel-La HC30R®) at 25°C with 80% relative humidity. Two sets of records were obtained: the first was obtained on the fourth day and the second on the seventh day. Germination was registered as the percentage of seeds with emerged radicles (18, 21). The plumule length test (21) was carried out. In the accelerated aging tests, a sample of 40 seeds collected at random were disinfected with 1% hypochlorite in water (v/v) and then placed in a beaker with a grid. Water was poured into the beaker to a height that did not reach the grid, and the beaker was covered with aluminum foil and placed at 42°C with 80% relative humidity for 72 h. Thereafter, the seeds were removed, and the standard germination test was performed (17).

Protein and oil extraction was performed by grinding the seeds (20 g each accession) in a mill (Techmark ® model A 10), followed by sieving through a No. 60 mesh. The samples were placed in paper bags and stored at 4°C until use. Oil extraction was performed in Soxtec® equipment; the flour was placed in filter paper cartridges at a ratio of 4:1 solvent - sample (hexane) (V/W) in the cup which was then stored in a desiccator for 24 h. Thereafter, the cup was mounted in the equipment. Protein extraction was carried out according to a previously described methodology (12, 25) using 1 g of flour and 4 mL of distilled water. After incubation for 1 h with shaking, the extraction solution was centrifuged for 1 h at 13,200 rpm (in a microfuge). The supernatant was then stored, and the pellet was re-extracted with a solution of 0.3 M NaCl in 50 mM Tris-HCl, pH 8, followed by centrifugation under the conditions described above, this second supernatant was stored. Supernatant was stored. The pellet was extracted two more times: once with 55% isopropanol and then with 50 mM sodium borate solution and 0.1% SDS (sodium dodecyl sulfate), pH 9. Protein quantification was carried out via the Bradford method (4). Electrophoretic patterns were obtained under denaturing conditions in 10% polyacrylamide gels (PAGE-SDS) following the technique of Schagger and von Jagow (1987).

Statistical analysis of physical and physiological quality and protein content traits was performed with the statistical package SAS version 9.0. Statistical tests of the homogeneity of variance assumptions and normality of the data distribution were carried out. Comparisons of means were conducted with Tukey's test ($\alpha = 0.05$).

Results and discussion

The analysis of variance (data not shown) showed significant differences between the accessions for germination percentage, with a coefficient of variation of 1.77%. The experiment was conducted properly, and the degree of precision in the comparison of the accessions was good (13). The germination percentage trait was statistically equal for all accessions (table 1, page 18) but the 'INIFAP-QPM' and 'regional landrace' controls, showed germination percentages of 82 and 87.3%, respectively. Similar results were obtained in the accelerated aging test. The results showed that the evaluated accessions exhibited good adaptation to the environmental conditions of El Bajío, while the controls were statistically inferior in the mentioned tests. This result is in agreement with the findings of Gutiérrez-Hernández *et al.* (2011), who observed differences in tolerance to artificial aging among accessions of blue corn in different maize accessions. Another previous study (12) of 22 accessions, showed 4 accessions with 99% germination after being subjected to accelerated aging. Artificial aging allows low-vigor seeds with a high germination percentage that lose germinative ability when subjected to this treatment, to be distinguished. These previous results are in agreement with those obtained in the present investigation, in which the evaluated accessions showed higher tolerance to accelerated aging, indicating higher seed vigor (8).

Table 1. Means comparison by the Tukey test for the germination percentage, accelerated aging test and plumule length traits in the evaluation of 10 maize accessions, Roque, Guanajuato. Spring-Summer 2015.

Tabla 1. Comparación de medias mediante la prueba de Tukey para las variables porcentaje de germinación, envejecimiento acelerado y longitud de plúmula en la evaluación de 10 accesiones de maíz, Roque, Guanajuato. Primavera-Verano 2015.

Accession	GER[1] (%)	AA[2] (%)	PL[3] (cm)
12015	96.6 a	94.0 a	5.6 f
22015	98.6 a	97.3 a	6.9 ef
A2015	99.3 a	98.0 a	12.6 a
ROQUE2	98.0 a	96.6 a	10.4 c
POL2015	99.3 a	99.3 a	11.3 abc
FVR12015	98.6 a	97.3 a	11.9 ab
ERH2015	98.6 a	98.6 a	10.9 bc
HRH2015	95.3 a	94.0 a	8.4 d
MRH2015	97.3 a	96.6 a	9.1 d
RQ2015	98.6 a	97.3 a	7.9 de
INIFAP-QPM	82.0 c	40.0 c	4.3 f
Regional landrace	87.3 b	83.3 b	6.6 f
Mean	95.83	91.05	8.85

Means with the same letter for each trait are statistically equal, Tukey's test, P≤0.05.
[1] Germination percentage; [2] Accelerated aging; [3]Plumule length.

Medias con la misma letra dentro de cada variable son estadísticamente iguales, Tukey, P≤ 0,05.
[1] Germinación; [2] Envejecimiento acelerado; [3]Longitud de plúmula.

Many factors participate in seed physiological behavior, such as oligosaccharides, which play an important role in seed longevity (16). Methionine sulfoxide reductase activity, tocopherol content, late embryogenesis abundant (LEA) protein accumulation and heat shock proteins (HSPs) (6) as well as changes in membrane permeability (34) are important factors affecting seed vigor.

Plumule length showed higher variation than the two previous traits. Accessions A2015 and FRV12015 exhibited the highest, however statistically similar, seed vigor. This variable best indicates physiological quality. Once more, INIFAP-QPM and Regional landrace, both controls, presented the lowest values together with 12015 and 22015 accessions. This result indicates that these accessions exhibited little vigor, since they obtained the lowest values in these three traits. In this research, plumule length was more effective in evaluating seed vigor, given that it allowed discrimination among them.

Vigor traits and accelerated aging

Analysis of variance (not shown) of the vigor traits indicated significant differences between the accessions with a coefficient of variation of 2.43%, which indicates reliable results. In the means comparisons (table 1), the 'POL2015', 'A2015' and 'FVR12015' accessions showed the highest values for the three traits, indicating that they are vigorous accessions, whereas the 'INIFAP-QPM' accession showed the lowest vigor value. The storage of seeds under adverse conditions causes aging, which results in a variety of symptoms ranging from a reduction in viability or a decreased ability to germinate, to poor seedling development (24). Stress causing aging due to conditions to which seeds are subjected, provokes higher reserve depletion, reducing seed vigor. The accumulation of proteins such as late embryogenesis-abundant (LEA) proteins, proteases and amylases in the embryo, may confer higher tolerance in breeding (19).

Analysis of variance (not shown) of the protein fraction content showed highly significant differences between the accessions, indicating that the accessions have contrasting characteristics. The coefficient of variation for total protein was good (CV 2.98) (13). Tandag-Silvas *et al.* (2011) noted that the classification of extracted proteins is a convenient approach for studying seed storage proteins due to the relative ease of protein extraction.

The results of the means comparisons (table 2) showed that accession 'HRH2015' presented the highest total soluble protein content of 3.48 g per 100 g flour, which was significantly different from that of 'FVR12015' (3.05 g). The accession with the lowest soluble protein content was the 'regional landrace' (1.75 g of protein) (9).

The QPM accession showed the highest protein quality because its endosperm content includes high levels of albumins and globulins (rich in lysine and tryptophan) with a decreased zein content. These proteins constitute the major fraction of storage proteins, accounting for 50 to 70% of the proteins (33).

Table 2. Means comparison by the Tukey test for the soluble protein contents of maize accessions. Roque, Guanajuato Spring-Summer 2015.

Tabla 2. Comparación de medias mediante la prueba de Tukey para contenido de proteína soluble de los genotipos de maíz. Roque, Guanajuato Primavera-Verano 2015.

Accession	ALB[1]	GBL[2]	PRL[3]	GLT[4]	TOTAL[5]
12015	1.28def	1.11bc	0.26bcd	0.03e	2.69cde
22015	1.31cde	1.19ab	0.26bcd	0.16c	2.93bc
A2015	1.36bc	1.12bc	0.35ab	0.05de	2.90bcd
ROQUE2	1.18f	1.32ab	0.17d	0.04de	2.71cde
POL2015	1.22ef	1.16bc	0.19cd	0.05de	2.63e
FVR12015	1.42bc	1.28ab	0.26bcd	0.083d	3.05b
ERH2015	1.44b	0.95c	0.21cd	0.05de	2.67de
HRH2015	1.61a	1.41a	0.38a	0.07de	3.48ª
MRH2015	1.22ef	1.21ab	0.39ª	0.06de	2.88bcd
INIFAP-QPM	1.22ef	1.21ab	0.39ª	0.06de	2.88bcd
Regional landrace	0.37h	0.24e	0.37ab	0.75ª	1.75g
RQ2015	0.95g	0.57d	0.29abc	0.39b	2.20f
MEAN	0.76	0.512	0.945	0.329	2.55
LSD	0.0706	0.0388	0.0883	0.0412	0.16

Means with the same letter for each trait are statistically equal, Tukey's test, P≤0.05.
[1] Albumins; [2] Globulins; [3] Prolamins; [4] Glutelins; [5] Total soluble protein content (g P / 100 g of flour).

Medias con la misma letra dentro de cada variable son estadísticamente iguales, Tukey, P≤ 0,05.
[1] Albúminas; [2] Globulinas; [3] Prolaminas; [4] Glutelinas; [5] Contenido total de proteína soluble (g P/100 g de harina).

For the albumin fraction, the accessions with the highest content were 'HRH2015', with 1.61 g of protein / 100 g of flour, followed by 'ERH2015', with 1.44 g, and 'FVR12015', with 1.42 g. The accessions with the lowest contents were the 'regional landrace', with 0.37 g, followed by 'RQ2015', with 0.95 g. Similar values were found in a previous study (12).

The accessions with the highest globulin fraction contents were 'HRH2015' (1.41 g), 'ROQUE2' (1.32 g), 'FRV2015' (1.28 g) and '22015' (1.19 g); the accession with the lowest globulin protein content was the 'regional landrace' (control; 0.24 g).

The 'HRH2015' accession contained high percentages of the albumin, globulin and prolamin fractions, but a lower percentage of the glutelin fraction, making it suitable material for breeding. The two first fractions were of better protein quality (32). The 'MRH2015' accession exhibited high percentages of globulins and prolamins. The 'HRH2015', 'INIFAP-QPM', 'MRH2015', 'ROQUE2', '22015' and 'FVR2015' accessions contained high percentages of the globulin fraction. Literature indicates that high percentages of albumins and globulins and a low percentage of prolamins are indicators of the protein quality of accessions, since the first two fractions are rich in essential amino acids such as lysine and tryptophan.

In OPACO-2, maize genes have been introduced to restore grain hardness and even low levels of α. Introduced quantitative trait loci increase the expression of γ-zeins, which appears to restore grain hardness (37).

Figure 1 shows the electrophoretic pattern of the prolamin fraction (zein), which is of interest for determining the possible genetic differences between the accessions. This fraction presented a similar pattern among the obtained bands.

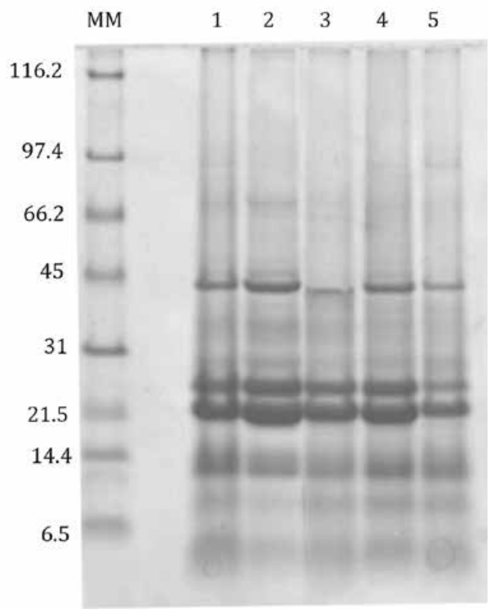


Figure 1. Electrophoretic pattern of the prolamin fraction. MM: Molecular weight marker (kDa); 1: '12013', 2: 2. '22013', 3. 'A2013', 4. 'ROQUE2', 5. 'POL2015'. At the 21 kDa marker position, two prominent bands characteristic of this fraction are shown.

Figura 1. Patrón electroforético de la fracción de prolaminas. MM: Marcador molecular (kDa) 1. Genotipo '12013', 2. '22013', 3. 'A2013', 4. 'ROQUE 2', 5. 'POL2015'. A la altura del marcador de 21 kDa se muestra dos bandas prominentes características de esta fracción.

The 'POL2015' accession showed a band at 95.6 kDa, and the '12013' and '22013' accessions showed a band at 77.8 kDa. All accessions presented bands at 43.6, 39.2, 29.1, 23.7, 19.8 and 13.2 kDa, which are characteristic of maize germplasms.

Prolamins are small globular proteins with a high cysteine content. High percentage accumulation of zeins confers a poor protein quality (37).

Figure 1 (page 20) shows some of the differences between the accessions, such as a missing prominent band in the middle of the gel (45 kDa) in the '12013' accession (third lane). Apparently, the molecular weight of this band changes. This characteristic could help to differentiate the accessions.

Figure 2 shows the electrophoretic pattern of the prolamin fraction; a similar pattern is observed among the bands of accessions 'FVR2015', 'ERH2015', 'HRH2015' and "MRH2015". The 'INIFAP-QPM' accession presents a band at 33.1 kDa that the other materials do not present. Accessions 'FVR12015', 'ERH2015' and 'HRH2015' present a very intense band at 20.1 kDa. In general, the pattern includes bands at 67, 45, 25, 20, 16 and 13.2 kDa. According to Wu *et al.* (2012), the 22 and 19 kDa zein bands are the most prominent storage proteins. Additionally, high accumulation of zeins results in a poor protein quality according to these researchers.

The analysis of variance (not shown) of the bromatological data (moisture, ash, proteins, oil, fiber and carbohydrates) showed significant differences for all traits, indicating that at least one accession presented different characteristics for some of the evaluated traits. These results indicate that the accessions are not related. The coefficients of variation were good for the evaluated traits (13).

The average moisture trait value was 12.40%; the highest values were obtained for accessions 'INIFAP-QPM' and '12015' (13.11%), while the values for '22015' (12.86%) and 'HRH2015' (12.45%) were statistically equal.

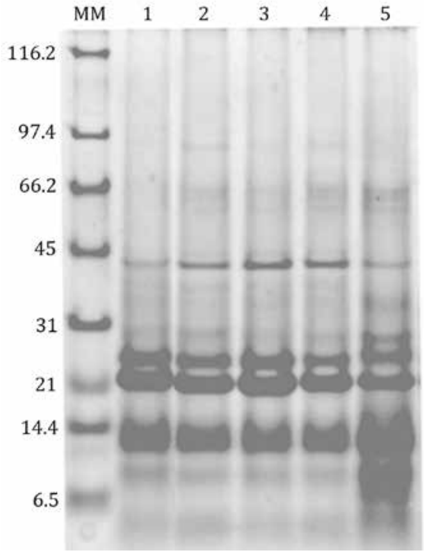


Figure 2. Electrophoretic pattern of the prolamin fraction of 5 maize accessions. MM: Molecular marker 1.-FVR12015, 2.-ERH22015, 3.-HRH2015 4.- MRH2015, 5.- INIFAP-QPM. At the 21.5 kDa marker position, prominent bands of prolamins are visible.

Figura 2. Patrón electroforético de la fracción de prolaminas de 5 genotipos de maíz. Donde: MM: Marcador molecular 1.-FVR12015 ,2.-ERH22015, 3.-HRH2015 4.- MRH2015, 5.- INIFAP-QPM. A la altura del marcador de 21.5 kDa se ven las bandas prominentes de las prolaminas.

The average ash trait value was 1.27%; the accessions with the highest values (statistically equal) were '12015' (1.42%), 'ERH2015' (1.36%), 'RQ2015' (1.33%), 'INIFAP-QPM' (1.32%), 'ROQUE2' (1.3%), the 'regional landrace' (1.29%) and 'HRH2015' (1.26%). For the protein trait, the average value was 8.55%; the highest value was obtained for the '12015' accession (10.44%), which exhibited an excellent protein percentage that was

20.55% higher than that of 'INIFAP-QPM' and 35.8% higher than that of the 'regional landrace'. In addition, it also showed high ash and carbohydrate contents, constituiting good material for breeding.

Accessions 'ROQUE2', 'PLO2015', '22015', 'ERH2015' and the 'regional landrace' showed a low protein content (7.64%), similar to those of non-breeding maize varieties (21). The mean oil percentage trait value was 2.7%; accessions 'RQ2015' (5.2%), 'ERH2015' (4.2%) and 'ROQUE2' (4.28%), showed high oil content, while the accession with the lowest oil content was '12015' (0.66%). The mean fiber percentage trait value was 2.22%; the 'INIFAP-QPM' variety showed the highest value for this trait (3.96%), followed by '22015' (3.21%).

The observed ash contents were consistent with the findings of Cázares-Sánchez *et al.* (2015), who evaluated 41 native maize accessions from central-northern Yucatán, México, and found values of 1.12 to 1.83%. These findings are consistent with those of Agama-Acevedo *et al.* (2005), who evaluated pigmented maize and found ash values of 1.1 to 1.6%. These results are within the range established by the Codex Alimentarius Commission, with a maximum of 3% (10).

Protein content ranged from 7.23 to 10.44%; this range has been reported for different genotypes by others, such as Narváez-González *et al.* (2006), who observed contents between 6.8 and 14.2%, and Díaz-Coronel *et al.* (2009), who found protein values ranging from 10.6 to 12.2% in five corn hybrids. These results indicate that the protein content has a genetic basis. Vidal-Martínez *et al.* (2008) evaluated 45 collections of maize landraces from "Sierra de Nayarit", Mexico, where the highest protein value was 12%. Vázquez-Carrillo *et al.* (2010) observed 12.5% protein as a maximum value in 26 maize landraces from the Mezquital Valley, Hidalgo, Mexico, similar to the percentage found in this research. A high protein quality in maize is caused by the synthesis of larger albumin and globulin fractions, which consist of lysine- and tryptophan-rich proteins, while the zein content is decreased. This means that the total protein content does not vary quantitatively (33). Zeins are the main fraction (up to 60%) of the total endosperm protein (22). These results show that some of the evaluated accessions presented a good protein quality and seed quality, supporting the hypothesis.

Conclusions

The results of the germination test were superior to 80% for all accessions. Accession "INIFAP-QPM" showed susceptibility to the accelerated aging test. The 'HRH2015' accession exhibited the highest amount of soluble protein, and the 'regional landrace' presented the lowest. Accession 'HRH2015' showed high albumin, globulin and prolamin contents; the 'regional landrace' showed the highest content of glutelins plus prolamins. The '12015' accession showed the highest total protein content, followed by 'HRH2015'; the lowest total protein content was presented by 'RQ2015' and the 'regional landrace'. Accession 'RQ2015', on the other hand, presented the highest oil content and '12015' the lowest. In terms of fiber content, the 'INIFAP-QPM' accession presented the highest value. The average ash content was 1.27%. The electrophoretic patterns of prolamins showed some differences between accessions. These maize accessions could be used for breeding purposes. The 'HRH2015' accession showed high performance in most of the evaluations conducted in this study, except for glutelin content, and this fraction does not provide essential amino acids. Accessions 'HRH2015' and 'FVR12015' were superior in protein quality, and accessions 'A2015', 'POL2015' and 'FVR12015' were superior in seed quality; therefore, these accessions could be used in a future breeding program.

References

1. Agama-Acevedo, E.; Ottenhof, M. A.; Farhat, I. A.; Paredes-López, O.; Ortíz-Cereceres, J.; Bello-Pérez, L. A. 2005. Aislamiento y caracterización del almidón de maíces pigmentados. Agrociencia. 39: 419-429.
2. Alcalá-Rico, J. S. G. J.; Espinoza-Velázquez, J.; López-Benítez, A.; Borrego-Escalante, F.; Rodriguez-Herrera, R.; Hernández-Martínez, R. 2019. Agronomic performance of maize (*Zea mays* L.) populations segregating the polyembryony mutant. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 1-1.
3. Alvarado-Beltrán, G.; López-Sánchez, H.; Santacruz-Varela, A.; Muñoz-Orozco, A.; Valadez-Moctezuma, E.; Gutiérrez-Espinosa, Ma. A.; López, P. A.; Gil-Muñoz, A.; Guerrero-Rodríguez, J. de D.; Taboada-Gaytán, O. R. 2019. Morphological variability of native maize (*Zea mays* L.) of the west highland of Puebla and east highland of Tlaxcala, Mexico. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(2): 217-23.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry. 72: 248-252.
5. Cázares-Sánchez, E.; Chávez-Servia, J. L.; Salinas-Moreno, Y.; Castillo-González F.; Ramírez-Vallejo, P. 2015. Variación en la composición del grano entre poblaciones de maíz (*Zea mays* L.) nativas de Yucatán, México. Agrociencia. 49:15-30.
6. Chätelain, E.; Satou, P.; Laugier, E.; Ly Vu, B.; Payet, N.; Rey, P.; Montrichard, F. 2013. Evidence for participation of the methionine sulfoxide reductase repair system in plant seed longevity. Proceedings of the National Academy of Science of the U. S. A. 110:3633-3638.
7. Díaz-Coronel, G. T.; Sabando-Avila, F. A.; Zambrano-Montes, S.; Vásconez-Montúfar, G. H. 2009. Evaluación productiva y calidad del grano de cinco híbridos de maíz (*Zea mays* L.) en dos localidades de la provincia de los ríos. Ciencia y Tecnología. 3:15-23.
8. Durán-Hernández, D.; Gutiérrez-Hernández, G. F.; Arellano-Vázquez, J. L.; García-Ramírez, E.; Virgen-Vargas, J. 2011. Caracterización molecular y germinación de semillas de maíces criollos azules con envejecimiento acelerado. Agronomía Mesoamericana. 22: 11-20.
9. Faleiros, R. R. S.; Seebauer, J. R.; Below, F. E. 1996. Nutritionally induced changesi n endospermo f shrunken-1 and brittle-2 Maize Kernels Grown In Vitro. Crop Science. 36:947-954.
10. FAO. 2005. Codex Alimentarius. Available in: http://www.fao.org/3/a-a0369s.pdf (Fecha de consulta: 23/02/2019).
11. FAO. 2008. El maíz en la nutrición humana. Disponible en: http://www.fao.org/docrep/t0395s/T0395S00.htm#Contents (Fecha de consulta:23/02/2019).
12. Godínez-Galán, R. Y.; García-Rodríguez, J. G.; Raya-Pérez, J. C.; Morales-Rodríguez, I.; Ramírez-Pimentel, J. G.; Torres-Castillo, J. A.; Aguirre-Mancilla, C. L. 2014. Caracterización física, fisiológica y de las proteínas de semillas de maíz (*Zea mays* L.) de Guanajuato e Hidalgo, México. Ciencia y Tecnología Agropecuaria de México. 2: 17-23.

13. Gómez, A. K.; Gómez, A. A. 1984. Statistical procedures for agricultural research. Second edition John-Wiley and Sons. New York. N. Y. 680 p.
14. Goodman, M. M.; Brown, W. L. 1988. Races of corn In: GF Sprague & JW Dudley (eds) Corn and Corn Improvement ASA Monograph18 ASA Madison, Wisconsin. p: 33-79.
15. Guevara-Hernández, F.; Hernández-Ramos, M. A.; Basterrechea-Bermejo, J. L.; Pinto-Ruiz, R.; Venegas-Venegas, J. A.; Rodríguez-Larramendi, L. A.; Cadena-Iñiguez, P. 2019. Maíces locales; una contextualización de identidad tradicional. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 369-381.
16. Gurusinghe, S.; Bradford, K. J. 2001. Galactosyl-sucrose oligosaccharides and potential longevity of primed seed. Seed Science Research. 11: 121-134.
17. Gutiérrez-Hernández, G. F.; Vázquez-Ramos, J. M.; García-Ramírez, E.; Franco-Hernández, M. O.; Arellano-Vázquez, J. L.; Durán-Hernández, D. 2011. Efecto del envejecimiento artificial de semillas de maíces criollos azules en su germinación y huella genómica. Revista Fitotecnia Mexicana. 34: 77-84.
18. ISTA, International Seed Testing Association. 2005. International rules for seed testing. Ed. Baessersdorf. CH-Switzerland. 243 p.
19. Mandal, R. K.; Mandal, S. 2000. Seed storage protein and approaches improvement of their nutritional quality by genetic engineering. Current Science. 79: 576-589.
20. McCann, M. C.; Trujillo, W. A.; Riordan, S. G.; Sorbet, R.; Bogdanova, N. N.; Sidhu, R. S. 2007. Comparison of the forage and grain composition from insect-protected and glyphosate-tolerant MON 88017. Corn to Conventional Corn (*Zea mays* L.). Journal of Agricultural and Food Chemistry. 55: 4034-4042.
21. Moreno-Martínez, E. 1996. Análisis físico y biológico de semillas agrícolas. 3ª. Edición. Instituto de Biología. UNAM. México. D. F. 393 p.
22. Mu-Forster, C.; Wasserman, B. P. 1998. Surface localization of zein storage proteins in starch granules from maize endosperm. Plant Physiology. 116: 1563-1571.
23. Narváez-González, E. D.; Figueroa-Cárdenas, J. D.; Taba, S.; Castaño-Tostado, E.; Martínez-Peniche, R. A.; Rincón-Sánchez, F. 2006. Relationships between the microstructure, physical features, and chemical composition of different maize accessions from Latin America. Cereal Chemistry. 83:595-604.
24. Perry, D. A. 1984. Manual de métodos de ensayos de vigor. Instituto Nacional de Semillas y plantas de vivero. Ministerio de agricultura, pesca y alimentación. Madrid. España. p. 56.
25. Raya-Pérez, J. C.; Gutiérrez-Benicio, G. M.; Ramírez-Pimentel, J. G.; Covarrubias-Prieto, J.; Aguirre-Mancilla, C. L. 2014. Caracterización de proteínas y contenido mineral de dos variedades nativas de frijol de México. Agronomía Mesoamericana. 25: 1-11.
26. Salazar-Martínez, J.; Rivera-Figueroa, C. H.; Arévalo-Gallegos, S.; Guevara-Escobar, A.; Malda-Barrera, G.; Rascón-Cruz, Q. 2015. Calidad del nixtamal y su relación con el ambiente de cultivo de maíz. Revista Fitotecnia Mexicana. 38: 67-73.
27. Salinas-Jiménez, V.; Raya-Pérez, J. C.; Aguirre-Mancilla, C. L.; Chablé-Moreno, F.; Ramírez-Pimentel, J. G.; García-Rodríguez, G.; Covarrubias-Prieto, J. 2016. Prueba temprana en líneas S1 de maíz. Phyton Revista Internacional de Botánica Experimental. 85: 203-209.
28. Sánchez-Toledano, B. I.; Kallas, Z.; Gil, J. M. 2017. Importancia de los objetivos sociales, ambientales y económicos de los agricultores en la adopción de maíz mejorado en Chiapas, México. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 49(2): 269-287.
29. Schagger, H.; von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-pilyacrylamide gel eletrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry. 166: 368-379.
30. Tandang-Silvas, M. R. G.; Tecson-Mendoza, E. M.; Mikami, B.; Utsumi, S.; Murayama, N. 2011. Molecular design of seed storage proteins for enhanced food physicochemical properties. Annual Review of Food Science and Technology. 2: 59-73.
31. Valdes Salazar, R. 2018. Measuring market integration and pricing efficiency along regional maize-tortilla chains of Mexico. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 279-292.

32. Vázquez-Carrillo, M. G.; Pérez-Camarillo, J. P.; Hernández-Casillas, J. M.; Luz-Marrufo, M. L.; Martínez-Ruiz, E. 2010. Calidad de grano y de tortillas de maíces criollos del altiplano y valle del mezquital, México. Revista Fitotecnia Mexicana. 33: 49-56.
33. Vázquez-Carrilo, M. G.; Mejía-Andrade, H.; Tut-Couoch, C.; Gómez-Montiel, N. 2012. Características de granos y tortillas de maíces de alta calidad proteínica desarrollados para los Valles Altos Centrales de México. Revista Fitotecnia Mexicana. 35: 23-31.
34. Ventura, L.; Donà, M.; Macovei, A.; Carbonera, D.; Buttafara, A.; Mondoni, A.; Rossi, G.; Balestrazzi A. 2012. Understanding the molecular pathways associated with seed vigor. Plant Physiology and Biochemistry. 60: 196-206.
35. Vidal-Martínez, V. A.; Vázquez-Carrillo, G; Coutiño-Estrada, B; Ortega-Corona, A.; Ramírez-Díaz, J. L.; Valdivia-Bernal, R.; Guerrero-Herrera, M. J.; Caro-Velarde, F. J.; Cota-Agramont, O. 2008. Calidad proteínica en colectas de maíces criollos de la Sierra de Nayarit, México. Revista Fitotecnia Mexicana Vol. 31 (Número especial 3): 15-21.
36. Vivas-Rodriguez, N. E.; Serna-Saldivar, S. O.; Waniska, R. D.; Rooney, L. W. 1990. Effect of tortilla chip preparation on the protein fractions of quality protein maize, regular maize and sorghum. Journal of Cereal Science. 12:289-296.
37. Wu, Y.; Wang, W.; Messing, J. 2012. Balancing of sulfur storage in maize seed. BMC plant Biology. 12: 77.
38. Yang, J.; Fu, M.; Ji, C.; Huang, Y.; Wu, Y. 2018. Maize Oxalyl-CoA Decarboxylase1 Degrades Oxalate and Affects the Seed Metabolome and Nutritional Quality. The Plant Cell. 30: 2447-2462.
39. Zarkadas, C. G.; Hamilton, R. I.; Yu, Z. R.; Choi, V. K.; Khanizadeh, S.; Rose, N. G.; Pattison, P. L. 2000. Assessment of the quality protein of 15 new northern adapted cultivars of quality protein maize using amino acid analysis. Journal of Agricultural and Food Chemistry. 48: 5351-5361.
40. Zepeda-Bautista, R.; Carballo-Carballo, A.; Muñoz-Orozco, A.; Mejía-Contreras, J. A.; Figueroa-Sandoval, B.; González-Cossio, F. V.; Hernández-Aguilar, C. 2009. Proteína, triptófano y componentes estructurales del grano en híbridos de maíz (*Zea Mays* L.) producidos bajo fertirrigación. Agrociencia. 43: 143-153.

SSR markers linked to stem canker resistance in soybean *(Glycine max)*

Marcadores SSRs ligados a la resistencia al cancro del tallo en soja *(Glycine max)*

Javier Ramón Gilli [1*], Gabriel Ricardo Vellicce [2], Clarisa Noelia Bernardi [1]

Originales: *Recepción:* 28/09/2018 - *Aceptación:* 14/09/2019

Abstract

This work studied 40 samples of *Diaporthe phaseolorum* var. *meridionalis* (Dpm), causal agent of stem canker in soybeans (SCS). In the susceptible genotype Golondrina65, the isolate RSF12 showed the highest percentage of dead plant index (DP = 85.7 %) and was used to characterize all known sources of resistance to Dpm. The soybean MJ19RR experimental line showed, the best behaviour against this isolate with a DP = 2.4 % and was used to develop a segregating population with the susceptible cultivar FT-2001. In the F_2 generation, a chi-square test determined a 3:1 ratio of resistant plants against susceptible plants, as expected for a dominant gene. In order to advance in our study, we proposed as objective, to map the resistance to *Diaporthe phaseolorum* var. *meridionalis*. The Bulked Segregant Analyses and the genetic linkage study identified a region on chromosome 6 of the genetic map of soybean, located at 13.3 cM from the Satt433 *locus* associated with resistance to SCS. The soybean experimental line MJ19RR was selected as the best source of resistance, available in the active bank of soybean germplasm of INTA, for the genetic control of this disease. The results obtained in this work represent a first approximation for the understanding of the genetic basis of resistance to SCS.

Keywords
Diaporthe phaseolorum var. *meridionalis* • molecular markers • fungal resistance • *Glycine max*

1 EEA INTA Marcos Juárez. Laboratorio de Biotecnología. Ruta provincial N° 12. km 2. C.P. 2580. Marcos Juárez. Córdoba. Argentina. * gilli.javier@inta.gob.ar
2 Estación Experimental Agroindustrial Obispo Colombres (EEAOC). Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA). Av. William Cross 3150. C. P. T4101XAC. Las Talitas. Tucumán. Argentina.

Resumen

En este trabajo estudiamos 40 muestras de *Diaporthe phaseolorum* var. *meridionalis* (Dpm), agente causal del cancro del tallo en soja (CTS). En el control susceptible Golondrina65, el aislado RSF12 presentó el mayor porcentaje del índice de plantas muertas (DP = 85,7 %) y fue utilizado para caracterizar las fuentes de resistencia conocidas al Dpm. La línea experimental de soja MJ19RR mostró el mejor comportamiento frente a este aislado, con un valor de DP= 2,4 %, y fue utilizada para desarrollar una población segregante con el cultivar susceptible FT-2001. En la generación F_2 la prueba de chi-cuadrado determinó una proporción 3:1 de plantas resistentes *versus* plantas susceptibles, como se espera para un gen dominante. Para avanzar en nuestro estudio, proponemos como objetivo localizar en el mapa genético de soja la resistencia a *Diaporthe phaseolorum* var. *meridionalis*. El Bulked Segregant Analyses y el estudio de ligamiento genético identificaron una región del cromosoma 6 del mapa genético de soja, a 13,3 cM del *locus* Satt433, asociada a la resistencia al CTS. Además la línea experimental de soja MJ19RR fue seleccionada como la mejor fuente de resistencia disponible en el banco activo de germoplasma de soja de INTA para el control genético de esta enfermedad. Los resultados obtenidos en este trabajo representan una primera aproximación para la comprensión de las bases genéticas de la resistencia al CTS.

Palabras claves

Diaporthe phaseolorum var. *meridionalis* • marcadores moleculares • resistencia a hongos • *Glycine max*

Introduction

Soybean stem canker (SSC) is caused by *Diaporthe phaseolorum*. First reported in USA in 1940s, it was one of the pathogens with major impact on soybean yield. A variant named meridionalis was identified in 1973 in southern USA with two different stages: the asexual one as *Phomopsis phaseoli* var. *meridionalis* in infected plant tissue, and the sexual phase, as *Diaporthe phaseolorum* var. *meridionalis* (Dpm) on plant detritus (14). In Argentina, *D. phaseolorum* v ar. *meridionalis* was first reported in 1992 (7). It is currently distributed all over the soybean production areas with four different physiological breeds identified according to response to inoculation of different resistant cultivars (10).

SSC resistance is controlled by five major, dominant, nonallelic genes: *Rdm1* and *Rdm2* in cv. Tracy-M (11); *Rdm3* in cv. Crockett, *Rdm4* in cv. Dowling and cv. Hutcheson (2, 3) and *Rdm5* in cv. Hutcheson (20). The pyramiding of these resistance genes could be the better strategy for achieving control of all physiological breeds causing SSC. In this sense, marker assisted selection is a tool that is currently available in most breeding programs, however information about mapped markers associated with resistance to SSC is scarse.

With the objective of locating the genetic resistance to *Diaporthe phaseolorum* var. *meridionalis* in the genetic soybean map, it was used a Bulked

Segregant Analysis (BSA) strategy (13) was used in a F2 population derived from a simple cross between the MJ19RR x FT-2001 genotypes.

BSA is a simple strategy used as first approach to locate genomic regions associated with important agronomic traits. It is based on segregation disequilibrium caused by genetic linkage and consists in of comparing two DNA bulks from plants of a segregating population (generation F2) derived from a simple cross or a backcross (generation BC1F2).

Materials and Methods

Fungal isolation

D. phaseolorum var. *meridionalis* were obtained from infected plants showing typical SSC symptoms from soybean production fields located in Córdoba and Santa Fe regions of Argentina, during the 2013/2014 harvest season. Isolation was conducted by the method of Keeling (9). *D. phaseolorum* var. *meridionalis* were cultivated on potato dextrose agar (PDA) plates at 27 ± 2°C for 5 days, and then maintained at room temperature for 45 days in order to induce perithecium fructification. Afterwards, the cultures were maintained at 4°C. Morphologic characterization considered the aspect of the colony, the perithecium, the pycnidium and whether α or β conidia were present (5). Isolates fitting to *D. phaseolorum* var. *meridionalis* description were subcultured on new PDA media. Finally virulence studies of each isolate were performed by inoculation of the susceptible control Golondrina65 under greenhouse conditions.

Plant Materials

The chosen genotypes included: the susceptible control Golondrina65 and the cultivars Tracy-M, Crockett, Dowling, Hutcheson, MJ19RR, Hartwig, Pickett71, FT-2001 and Peking. In addition, 147 F_2 plants were obtained after crossing the contrasting parents FT-2001 (susceptible) and MJ19RR (resistant). This cross was performed in INTA Marcos Juarez in January 2015, 147 F_2 seeds were obtained from a single F_1 plant.

Phenotypic screening

The toothpick method (9) was chosen to screen response to SSC under greenhouse conditions. For rating resistance of cultivars against SSC, a random blocks design was used with three replications of 15 plants each. In order to rate the 147 F_2 plants, a complete randomized design was performed. Three replicates with 15 Golondrina65 plants randomly distributed, of Golondrina65 were included as positive controls to the inoculation. Seven days after emergence, plants were inoculated with Dpm mycelium and kept at a 25-30°C temperature with 100% of relative humidity (RH) for 48 h. Subsequently, the plants were maintained in a greenhouse for 25 days before rate disease severity was recorded. A longitudinal section of the stem was taken to measure pathogen penetration into plant tissue (photo 1, page 29). Genotype resistance was rated as the average value of the percentage of dead plants index (% DP) in three replicates using Equation 1:

$$\%DP = \frac{(DP + \frac{IP}{2})100}{TP} * 100 \qquad (1)$$

where:
DP = number of dead plants
IP = number of infected plants
TP = total number of plants

Four levels of disease severity were established to rank cultivars response to Dpm, according to percentage of death plant index (% DP): resistant R = 0 to 14.9%, moderately susceptible MS = 15 to 49.9 %, susceptible S = 50 to 84.9% and highly susceptible HS = 85 to 100% (11), F_2 individuals were scored in two levels: as resistant when no disease symptoms had developed, and susceptible when disease symptoms were present (photo 1).

DNA extraction

DNA was purified from leaf tissue (15) and suspended in TE buffer (Tris-HCl 10 mM, EDTA 1 mM pH = 8). The concentration was determined by means of electrophoresis on 0.8% agarose gel and comparison with standard samples. For bulk segregant analysis, equimolar solutions were obtained from 15 resistant plants (resistant bulk: RB), and the 15 susceptible plants (susceptible bulk: SB).

PCR amplification

Genetic analysis was performed by PCR amplification of 84 SSR markers covering the 20 chromosomes of the soybean genome (table 1, page 30). The selection of the SSR was based on the location of the clusters of disease resistance genes previously reported (SoyBase, available in: http://www.soybase.com, accessed, September 2015).

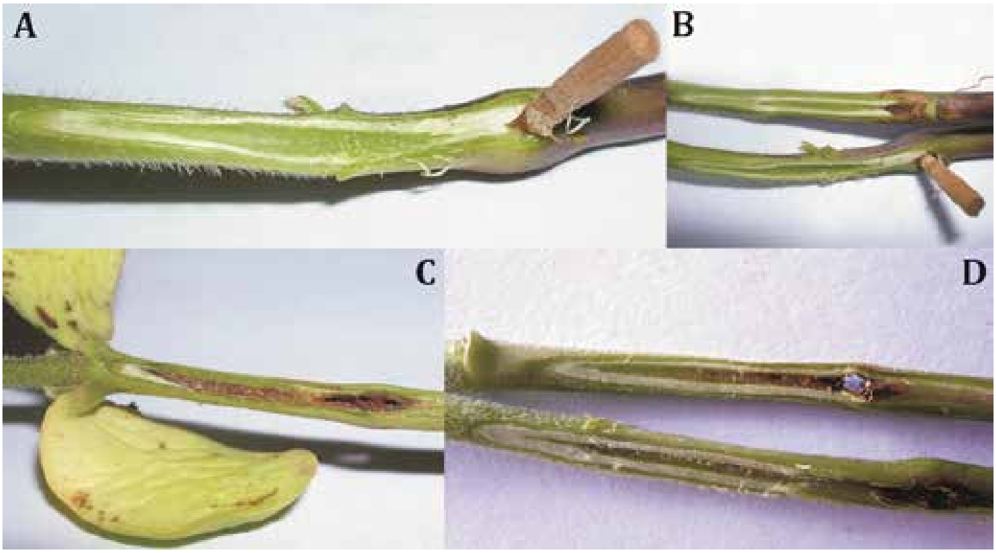


Photo 1. Reaction of soybean genotypes 25 days after inoculation with *D. phaseolorum* var *meridionalis*. A and B, resistant reaction in MJ19RR; C and D, susceptible reaction in FT-2001, showing necrosis caused by fungal growth on hypocotyl plant.

Foto 1. Reacción de genotipos de soja luego de 25 días de la inoculación con *D. phaseolorum* var *meridionalis*. A y B, reacción de resistencia en MJ19RR. C y D, reacción susceptible en FT-2001, se observa necrosis causada por el hongo en el hipocotilo de las plántulas.

Table 1. Chromosome (Chr) and SSR markers used in the genetic characterization of the parents MJ19RR and FT-2001.

Tabla 1. Cromosomas (Chr) y marcadores SSRs usados en la caracterización de los parentales MJ19RR y FT-2001.

Chr	SSR markers
1	Sat_036; Satt184.
2	Sat_069; Satt005; Sat_135.
3	**Sat_125**; **Satt154**; Satt584; Satt393.
4	Sat_042; **Satt139.**
5	**Satt382**; Satt545; Satt174.
6	**Satt281**; **Satt079**; Satt202; Satt316; Satt371; **Satt307**; **Satt433**; S**att460**; Sct028; **Satt277**; Satt286; Satt319; Satt357.
7	Satt245; Satt590.
8	Satt409; Sat_162; Sat_157; Satt632.
9	Sat_020; Sat_119; **Satt518**; Satt337.
10	Sat_108; Satt445.
11	**Satt509**: **Satt415**; Satt332.
12	Sat_118; Satt541.
13	Satt030; **Satt516**; Satt334; Satt657.
14	Satt063; Satt168; Satt416.
15	Sat_107; Sat_112; **Satt384**; Satt602; Satt369.
16	Satt596; Satt244; Sat_396; Satt285; **Satt547**.
17	Sat_001; Satt458; Satt301; Satt574.
18	Satt131; **Satt309**; Satt288; Sat_141; Sat_163; Satt505; Satt012; Satt472; **Satt191**; Satt517; **Satt038**; **Satt130**; Satt610; Satt503.
19	**Satt182**; Satt462; Satt652.
20	**Satt440**; Satt127.

Bold letters indicate polymorphic markers among both parents.

Letras en negrita indican los marcadores polimórficos entre parentales.

SSR amplification was conducted with a GeneAmp PCR System 9700 (Applied Biosystems, Framingham, MA, USA), using a final volume of 15µl containing 50 ng of DNA of the resistant bulk, the susceptible bulk or the population parents, 1x GoTaq Buffer Green (1.5mM of Cl_2Mg), 0.2 mM of each dNTPs, 1U of GoTaq polymerase (PROMEGA, Madison, US), and 0.5 µm of each primer. Amplification conditions were as follows: 94°C for 120 s; 35 cycles of 92°C for 45 s; 47°C for 45 s; 68°C for 45 s; and 68°C for 60 s. PCR products were separated by electrophoresis on 12% polyacrylamide gels, stained with ethidium bromide solution (10 mg/ml) and visualized under UV light. The correct size of amplicons was analyzed by comparison with the reference genotype Williams82 (SoyBase, available in: http://www.soybase.com, accessed June 2016).

Linkage analysis

Linkage analysis was performed with 147 F_2 plants derived from the MJ19RR x FT-2001 cross. Map construction was accomplished with GQMol software (4), using distance unit of Kosambi with 3.0 LOD score and maximum recombination distance of 50 cM. Graphics were obtained with GGT 2.0 software (21).

Results

Fungal isolation

Plants from soybean fields, putatively infected with Dpm were collected. Thirty seven out of 40 samples, produced isolates fitting Dpm description. These were used to inoculate the susceptible control Golondrina65 in order to confirm their identity and measure their virulence on soybean. The isolates that produced % DP values from 80% to 100% were considered highly virulent. The isolate RSF12 obtained in Roman (29°30'49" S, 59°46'40" W), Santa Fe, Argentina, produced the highest % DP values and was selected for further analysis.

Reaction of MJ19RR to RSF12 isolate

The responses of susceptible control Golondrina65 and nine soybean cultivars (Tracy-M, Crockett, Dowling, Hutcheson, MJ19RR, Hartwig, Pickett71, FT-2001, Peking) to inoculation with RSF12 are presented in table 2 (page 32). Eighty five plants of Golondrina65 in six replicates were inoculated. All these plants presented typical SSC symptoms corresponding to a highly susceptible reaction (HS) with values % DP of 85.7 ± 4.5. A sub-set of four genotypes produced a susceptible reaction (S) in terms of % DP: Hartwig (69.3 ± 3.4%), Pickett71 (67.7 ± 4.2%), FT-2001 (67.3 ± 2.1%) and Peking (53.1 ± 1.6%), while Dowling, Crockett and Hutcheson produced a moderately susceptible reaction (MS) with % DP values of 23.4 ± 0.8%, 20.5 ± 1.1% and 15.6 ± 2.9%, respectively. On the other hand, Tracy-M and MJ19RR had a resistant reaction (R) with % DP values of 12.5 ± 2.5% and 2.4 ± 1.6%, respectively. It is noteworthy that the MJ19RR was the only genotype that presented no dead plants by inoculation with SSC.

Inheritance of the resistance

The phenotypic analysis of 147 F_2 plants by inoculation with Dpm isolate RSF12 produced 113 and 34 plants showing resistant and susceptible reactions, respectively. The chi-square value $\chi2 = 0.28 < 3.86$ at a $p \leq 0.05$ confirmed a 3:1 mendelian segregation that fitted in with a frequency of a single dominant gene (table 3, page 32).

Table 2. Percentage of dead plant index (% DP) of soybean genotypes inoculated with isolate RSF12 of *Diaporthe phaseolorum* var. *meridionalis*.

Tabla 2. Porcentaje del índice de plantas muertas (% DP) de genotipos de soja inoculados con el aislado RS12 de *Diaporthe phaseolorum* var. *meridionalis.*

Cultivars	Number of Plants	% DP	Reaction (11)
Golondrina65[a]	85	85.7 ± 4.5	Highly Susceptible
Hartwig	41	69.3 ± 3.4	Susceptible
Pickett71	41	67.7 ± 4.2	Susceptible
FT-2001	45	67.3 ± 2.1	Susceptible
Peking	38	53.1 ± 1.6	Susceptible
Dowling	41	23.4 ± 0.8	Moderately Susceptible
Crockett	40	20.5 ± 1.1	Moderately Susceptible
Hutchenson	45	15.6 ± 2.9	Moderately Susceptible
Tracy–M	42	12.5 ± 2.5	Resistant
MJ19RR	40	2.4 ± 1.6	Resistant

[a] The susceptible control Golondrina65 was tested in six repetitions randomly distributed among the soybean cultivars and the 147 plants of the mapping population.

[a] El control susceptible Golondrina65 fue incluido en seis repeticiones distribuidas al azar entre los cultivares y las 147 plantas de la población de mapeo.

Table 3. Chi-square for the resistance *locus* Rdm_{MJ19RR} and the SSR markers in F_2 generation of MJ19RR x FT-2001 and its positions (MP) in the soybean molecular map (19).

Tabla 3. Chi-cuadrado para el *locus* de resistente Rdm_{MJ19RR} y los marcadores SSRs en la generación F_2 de MJ19RR x FT-2001 y sus posiciones (MP) en el mapa molecular de soja (19).

Locus	MP (cM)	Hypothesis	Expected	Observed	X^2
Satt079	117.8	1:2:1	36.75:73.50:36.75	39:66:42	1.66
Satt307	121.30	1:2:1	36.75:73.50:36.75	35:61:44	3.64
Satt433	128.30	1:2:1	36.75:73.50:36.75	39:82:26	4.26
Rdm_{MJ19RR}		3:1	110.25:36.75	113:34	0.28

Polymorphism detection

All the amplified fragments showed the expected size as reported for the reference genotype Williams 82. Out of all 84 SSR, 22 were polymorphic between the parental cultivars MJ19RR and FT-2001 (table 1, page 30).

In order to detect the SSR that were close to the resistance gene, we considered PCR sensitivity as reported for bulk segregant analysis (13). The markers Satt382 from Chr 5, Satt433 from Chr 6, Satt182 from Chr 19, and Satt152 from Chr 3 were selected as candidates considering low intensity amplification the resistant allele in susceptible bulk (SB) as a sign of low recombination between the markers and the resistance gene.

Mapping SSC resistance in MJ19RR

Out of the four markers (Satt382, Satt433, Satt182 and Satt152) analyzed in the 34 susceptible F_2 plants, only in Satt433, the susceptible allele (a band of 200 bp), was observed in 26 of the 34 plants; whereas in the remaining eight plants, the resistant allele (290 bp) was amplified. These latter plants represented recombination events between the marker and the resistance gene (photo 2 and figure 2, page 35).

Eleven additional SSR were selected from the Satt433 genomic region covering about 40 cM, five of which produced polymorphic bands in the parental genotypes (table 1, page 30). Satt433, Satt079 and Satt307 were included in the analysis and the chi-square test confirmed the segregation of theses markers with mendelian ratio (table 3, page 32). Using GQMOL the Satt433 marker was positioned at 25.1 cM from Satt307 and at 34.2 cM from Satt079, whereas resistance to SSC (Rdm_{MJ19RR}) was located at 13.3 cM from Satt433 (figure 1, page 34). This region has not been previously reported as associated with SSC resistance.

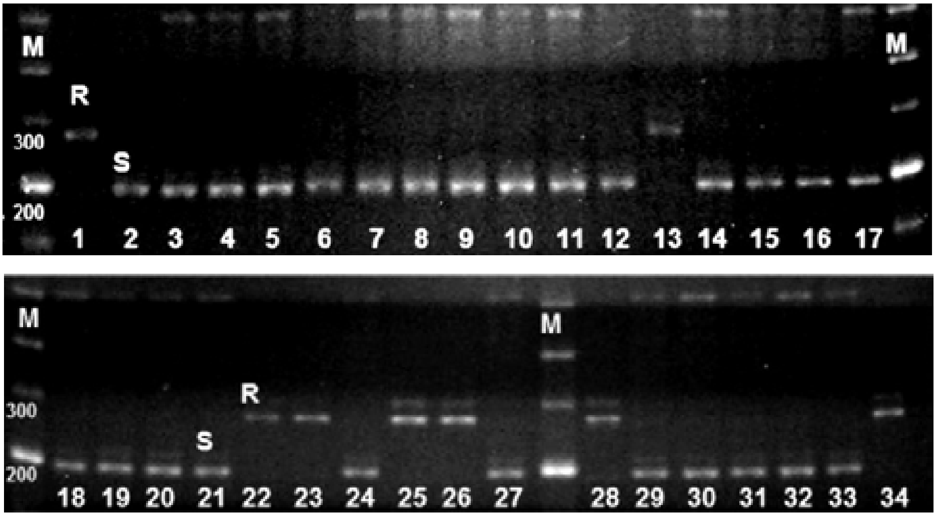


Photo 2. Satt433 amplification in 34 susceptible plants of mapping population. R, resistant allele of MJ19RR, S. susceptible allele of FT-2001. M, molecular weight marker.

Foto 2. Amplificación de Satt433 en las 34 plantas susceptibles de la población de mapeo. R, alelo resistente de MJ19RR; S, alelo susceptible de FT-2001. M, marcador de peso molecular.

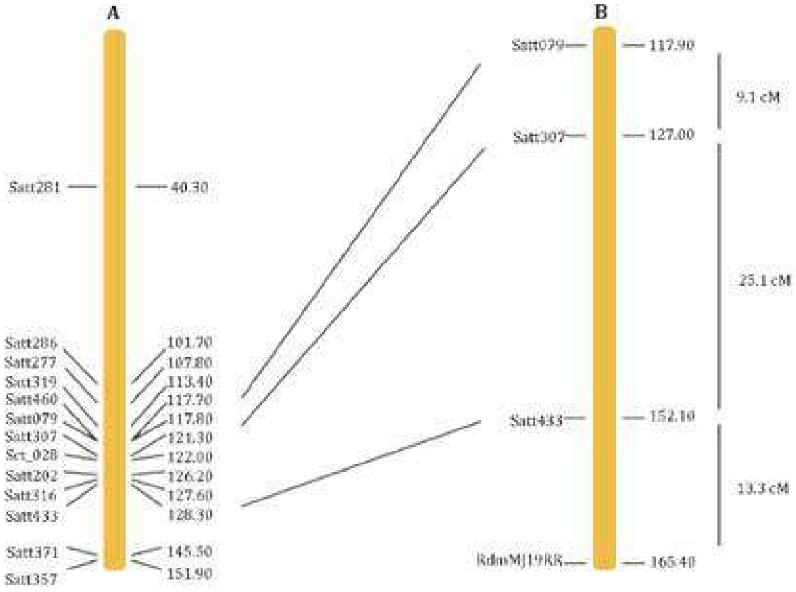


Figure 1. Section of chromosome 6 showing position of *Rdm*$_{MJ19RR}$ *locus*. A, reference map (19). B, genetic map obtained in the present study.

Figura 1. Sección del cromosoma 6 donde se localiza el *locus Rdm*$_{MJ19RR}$. A, mapa de referencia (19). B, mapa genético obtenido en el presente trabajo.

The recombination in the 34 susceptible plants was evaluated with the Satt079, Satt307 and Satt433 markers. As shown in figure 2 (page 35), recombination in all analyzed *locus,* were observed. Satt371 and Satt357, located in the distal region of Chr 6, were monomorphic for MJ19RR and FT-2001. It was not possible to analyze recombination at the distal end of this chromosome.

Resistance sources characterization

Nine soybean genotypes, among which are all known sources of SSC resistance, were analyzed with seven SSR markers of the Chr 6 region where *Rdm*$_{MJ19RR}$ was mapped (figure 3, page 36). The resistant allele from Satt433 was amplified in MJ19RR, Tracy M, Crockett and Dowling. The only resistant genotype that did not showed the resistance allele was Hutcheson. Amplifications of the *loci* Satt316 and Satt202 produced the same alleles in MJ19RR, Tracy-M, Crocket, Peking, FT-2001, Pickett71 and Hartwig, except Tracy-M and FT-2001 that showed different alleles for Satt316. With the Satt307 and Satt079 markers all genotypes amplified different alleles than MJ19RR, except Hutcheson and Dowling for the Satt079 *locus*. Analysis with Satt371 marker showed the same allele in MJ19RR, FT-2001, Pickett71 and Hartwig, while Satt357 amplified the same allele in all genotypes except in Hutcheson. Overall, these results indicate that there was no a clear relationship between the studied haplotypes.

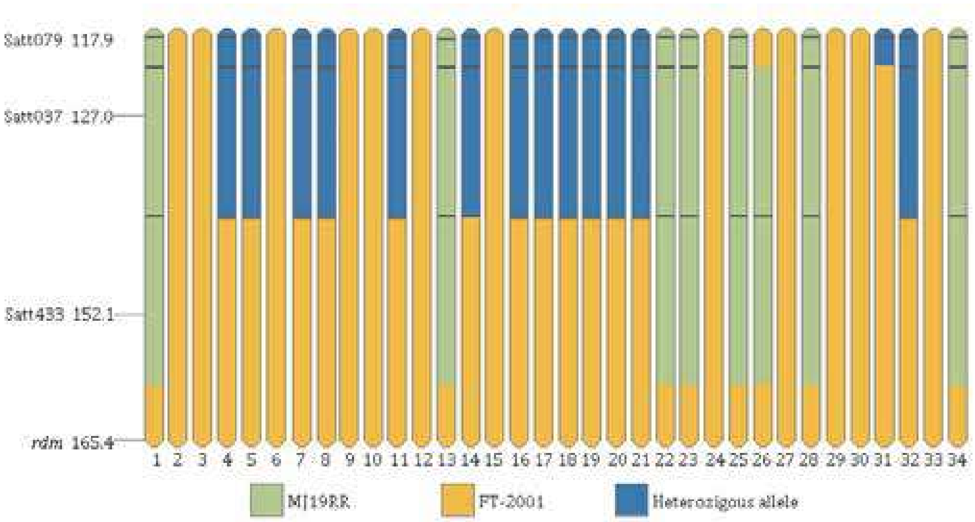


Figure 2. Recombination in distal region of chromosome 6 in 34 susceptible plants of mapping population. Orange fragments denote susceptible allele of FT-2001, green fragments denote the resistant allele of MJ19RR blue fragments, denote heterozygous regions.

Figura 2. Recombinación de la región distal del cromosoma 6 en las 34 plantas susceptibles de la población de mapeo. Los fragmentos naranjas indican el alelo susceptible de FT-2001, fragmentos verdes indican el alelo resistente de MJ19RR mientras que fragmentos azules indican regiones heterocigotas.

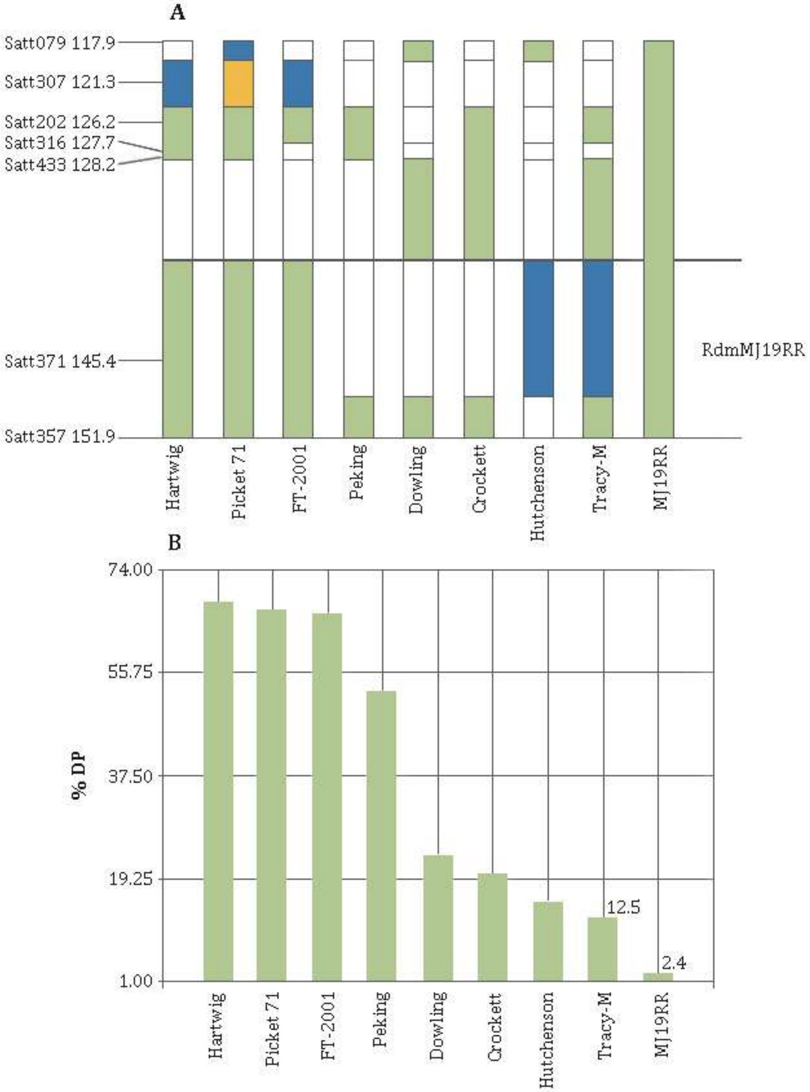


Figure 3. A: allelic combination of the distal end of chromosome 6 in all known Dpm resistance sources. Green fragments denote presence of alleles resistant of MJ19RR. **B:** percentage of dead plant index (% DP) with response to Dpm (RS12) inoculation.

Figura 3. A: combinación alélica del extremo distal del cromosoma 6 en todas las fuentes de resistencia al Dpm conocidas; fragmento verde representa los alelos del genotipo resistente MJ19RR. **B:** Porcentaje del índice de plantas muertas (% DP) como respuesta a la inoculación con Dpm (RS12).

Discussion

This research presents the first genomic approximation to stem canker resistance using bulk segregant analysis. The selection of SSR candidates according to the intensity of the resistant allele amplification in the susceptible bulk, was efficient in localizing the genomic region associated with the resistance. This strategy allowed the identification of the genomic region of interest, using few polymorphic molecular markers, a common situation when we used domestic parental for develop mapping population. When analyzing F_2 generation of the mapping population, we showed that the genetic resistance of MJ19RR was the result of a single dominant gene. In this case, the hypothesis was confirmed considering 113 F_2 plants as resistant, which did not show symptoms (immune), while the remaining 34 susceptible plants showed clearly identifiable symptoms throughout the hypocotyls (photo 1, page 29). This stark contrast between resistant and susceptible plants in the mapping population leaves no doubt of the inheritance of this gene and demonstrates the consistency of the resistance reaction of MJ19RR against an aggressive isolate of Dpm.

The genotypic analysis located the resistance at Chr 6, linked to Satt433 marker at 13.3 cM (figure 1, page 34). The recombination between Satt433 and Rdm_{MJ19RR} in eight susceptible plants suggested that resistance to SSC could be located in the distal region of Chr 6 (figure 2, page 35). The lack of polymorphism between MJ19RR and FT-2001 at Satt371 and Satt357 prevented the recombination study in the distal end of the chromosome.

This region on Chr 6 of the soybean map was previously reported as responsible for resistance to sudden-death syndrome (6, 8, 16), to *Phytophthora sojae* (12), to Asian soybean rust in cv. FT-2001 (18). Also, resistance to *Heterodera glycines* was mapped by (1, 22). These findings support the idea of a clustered location of resistance genes related to biotic stress, which is very valuable for breeding programs.

The presence of physiological breeds of Dpm that show a differential response to different *Rdm* genes was already reported (17). They observed that there are very aggressive breeds in Argentina which are controlled only by the *Rdm1* gene. However lower % DP values were observed when this gene was accompanied by *Rdm2* in the Tracy-M genotype. In our research, the Tracy-M showed % DP values corresponding to the resistance reaction (R), but Dowling, Crocket and Hutchenson showed moderately susceptible (MS) responses (table 2, page 32). This result suggests that RSF12 is a very aggressive isolate, because it is only controlled by Tracy-M (*Rdm1*/*Rdm2*).

Although we are not aware of the resistance source from where MJ19RR originated, reaction similarities between MJ19RR and Tracy-M could indicate that both genotypes share a genetic base for Dpm resistance. Using SSR markers, we aligned the haplotypes of our parental genotypes with all the possible Dpm resistance sources known and their respective reactions to RSF12 isolation (figure 3, page 36). The comparison of the genomic region from distal end of Chr 6 for genotypes MJ19RR and Tracy-M, showed similarities for Satt357 *locus* between both genotypes. In this sense, Tracy-M could be the sole potential responsible for MJ19RR resistance, considering that all the sources of resistance was tested in this experiment.

The *Rdm2* gene controls less aggressive breeds which are also controlled by other known genes (*Rdm1,3,4*) (17), while in our research the RSF12 variant was only controlled by Tracy-M genotype, a combination of *Rdm1* and *Rdm2* genes. In this way if the resistance of MJ19RR derived of Tracy-M, then it should be through the *Rdm1* gene. Nevertheless, the agronomic difference of the % DP values between MJ19RR (2.4%) and Tracy-M (12.5 %), the lack of dead plants and the sole resistance gene in MJ19RR, support the idea that these genotypes have different genetic basis for Dpm resistance.

Anyway, our experiments were unable to determine if the MJ19RR response is product of *Rdm1,* an allelic variation of this, or a new gene of other locus.

Finally, in this paper we showed that resistance of MJ19RR controls a very aggressive Dpm variant through a single gene, indicating that this genotype is a very important tool for genetics breeding programs.

Conclusions

The RSF12 was the most aggressive isolate while MJ19RR was the only genotype where no dead plants resulted from inoculations. This fact revealed MJ19RR as a promising source of resistance to SSC.

The genetic resistance of MJ19RR to SSC is determined by a single gene, located at the distal end of chromosome 6 at 13.3 cM of the Satt433 marker.

This study determined that the agronomic reaction of the soybean genotype MJ19RR against the RSF12 isolate is different from the rest of the known resistance sources, strongly indicating the presence of a new gene.

References

1. Arriagada, O.; Mora, F.; Dellarossa, J. C.; Ferreira, M. F.; Cervigni, G. D.; Schuster, I. 2012. Bayesian mapping of quantitative trait loci (QTL) controlling soybean cyst nematode resistant. Euphytica. 186: 907-917. DOI: 10.1007/s10681-012-0696-y.
2. Bowers, G. R.; Ngeleka, K.; Smith, O. D. 1993. Inheritance of stem canker resistance in soybean cultivars Crockett and Dowling. Crop Sci. 33: 67-70. DOI: 10.2135/cropsci1993.0011183X003300010010x.
3. Chiesa, M. A; Pioli, R. N.; Morandi, E. N. 2009. Specific resistance to soybean stem canker conferred by the Rdm4 *locus*. Plant Pathol. 58: 1032-1039. DOI: 10.1111/j.1365-3059.2009.02145.x.
4. Cruz, C. D.; Schuster, I. 2004. GQMOL-Aplicativo computacional para análise de dados moleculares e de suas associações com caracteres quantitativos. Universidad Federal do Viçosa. Viçosa. Brazil.
5. Fernández, A. F.; Hanlin, R. T. 1996. Morphological and RAPD analyses of *Diaporthe phaseolorum* form soybean. Micologia. 88: 425-440. DOI: 10.2307/3760884.
6. Iqbal, M. J.; Meksem, K.; Njiti, V. N.; Kassem, M. A.; Lightfoot, D. A. 2001. Microsatellite markers identify three additional quantitative trait loci for resistance to soybean sudden-death syndrome (SDS) in Essex × Forrest RILs. Theor. Appl. Genet. 102: 187-192. DOI: 10.1007/s001220051634.
7. Ivancovich, A. J.; Botta, G. L.; Annone, J. 1992. Aparición del cancro del tallo en cultivos de soja del área de la E.E.A. Pergamino. Información N°95. EEA INTA Pergamino.
8. Kazi, S.; Shultz, J.; Afzal, J.; Johnson, J.; Njiti, V. N.; Lightfoot, D. A. 2008. Separate loci underlie resistance to root infection and leaf scorch during soybean sudden death syndrome. Theor. Appl. Genet. 116: 967-977. DOI: 10.1007/s00122-008-0728-0.

9. Keeling, B. L. 1982. A seedling test for resistance to soybean stem canker caused by *Diaporthe phaseolorum* var. caulivora. Phytopathology. 72: 807-809. DOI: 10.1094/Phyto-72-807.
10. Keeling, B. L. 1988. Influence of temperature on growth and pathogenicity of geographic isolates of *Diaporthe phaseolorum* var. caulivora. Plant Dis. 72: 220-222. DOI 10.1094/PD-72-0220.
11. Kilen, T. C.; Hartwig, E. E. 1987. Identification of single genes controlling resistance to stem canker in soybean. Crop Sci. 27: 863-864. DOI: 10.2135/cropsci1987.0011183X002700050005x.
12. Li, X.; Han, Y.; Teng, W.; Zhang, S.; Yu, K.; Poysa, V.; Li, W. 2010. Pyramided QTL underlying tolerance to Phytophthora root rot in mega-environments from soybean cultivars 'Conrad'and 'Hefeng 25'. Theor. Appl. Genet. 121: 651-658. DOI: 10.1007/s00122-010-1337-2.
13. Michelmore, R. W.; Paran, I.; Kesseli, R. V. 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic region by using segregation population. P. Ntl. Acad. Sci. USA. 88: 9828-9832. DOI: 10.1073/pnas.88.21.9828.
14. Morgan-Jones, G. 1989. The *Diaporthe/Phomopsis* complex: Taxonomic considerations. Proceeding of the World Soybean Research Conference IV. Buenos Aires. Argentina. 1699-1706.
15. Murray, M. G.; Thompson, W. F. 1980. Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res. 8: 4321-4325. DOI: 10.1093/nar/8.19.4321.
16. Njiti, V. N.; Meksem, K.; Iqbal, M. J.; Johnnson, J. E.; Kassem, M. A.; Zobrist, K. F.; Kilo, V. Y.; Lightfoot, D. A. 2002. Common loci underlie field resistance to soybean sudden death syndrome in Forrest, Pyramid, Essex, and Douglas. Theor. Appl. Genet. 104: 294-300. DOI: 10.1007/s001220100682.
17. Pioli, N. R.; Morandi, E. N.; Martínez, M. C.; Lucca, F.; Tozzini, A.; Bizarro, V.; Hopp, H. E. 2003. Morphologic, molecular, and pathogenic characterization of *Diaporthe phaseolorum* variability in the core soybean-producing area of Argentina. Phytopathology. 93: 136-146. Available in: http://dx.doi.org/10.1094/PHYTO.2003.93.2.136.
18. Silva, D. G. C.; Yamanaka, N.; Polizel, A. M.; Brogin, R. L.; Pereira, S. S.; Nogueira, L. M.; Passianoto, A. L. L.; Catelli, L. L.; Arias, C. A. A.; Nepomuceno, A. L. 2006. Mapeo de genes de resistencia a roya asiática en soja. Resúmenes 3° Congreso de Soja del MERCOSUR. Rosario. Argentina.
19. Song, Q. J.; Marek, L. F.; Shoemaker, R. C.; Lark, K. G.; Concibido; V. C.; Delannay, X.; Specht, J. E.; Cregan, P. B. 2004. A new integrated genetic linkage map of the soybean. Theor. Appl. Genet. 109: 122-128. DOI: 10.1007/s00122-004-1602-3.
20. Tyler, J. M. 1996. Characterization of stem canker resistance in ¨Hutcheson¨ soybean. Crop Sci. 36: 591-593. DOI: 10.2135/cropsci1996.0011183X003600030011x.
21. Van Berloo, R. 2008. GGT2.0: versatile software for visualization and analysis of genetic data. J. Hered. 99: 232–236. DOI: 10.1093/jhered/esm109.
22. Yue, P.; Arelli, P. R.; Sleper, D. A. 2001. Molecular characterization of resistance to *Heterodera glycines* in soybean PI 438489B. Theor. Appl. Genet. 102: 921-928. DOI: 10.1007/s001220000453.

Acknowledgements

We gratefully acknowledge revision of the manuscript by Ing. Carolina Caram Di Santo (CONICET), PhD. Valeria Faggioli (INTA) and to Beatriz Masiero (INTA) for her assistance in the statistical analysis.

Molecular *versus* morphological markers to describe variability in sugar cane (*Saccharum officinarum*) for germplasm management and conservation

Marcadores moleculares y morfológicos para la descripción de variabilidad en caña de azúcar (*Saccharum officinarum*) con fines de manejo y conservación de germoplasma

Mariana I. Pocovi *, Norma G. Collavino, Ángela Gutiérrez, Gisel Taboada, Verónica Castillo, Romina Delgado, Jorge A. Mariotti

Originales: *Recepción:* 29/08/2018 - *Aceptación:* 15/10/2019

Abstract

Sugarcane is one of the most important industrial crops in tropical and subtropical regions. INTA (Argentina) administrates a Sugarcane Germplasm Bank and carries out a breeding program. The current study was designed to assess the phenotypic and genetic diversity among 65 sugarcane accessions selected from the INTA bank. Clustering and ordination methods based on quantitative and qualitative morphological traits and SSR data, were applied. Generalized Procrustes Analysis allowed evaluating the correlation between relationships established with both markers. A good fit between dendrograms and similarity matrices were revealed by high cophenetic coefficients ($r=0.82$, $p<0.0001$; $r=0.73$, $p<0.0001$; $r=0.82$, $p<0.0001$ for phenotypic quantitative, phenotypic qualitative and molecular data respectively). The presence of different reliable population structure was observed when considering different data sources. Procrustes allowed finding those accessions that should have been responsible for the low correlation found between the individual configurations (73%). Both morphologic and molecular markers resulted discriminative enough to differentiate among accessions. It was not possible, however, to correlate associations of markers with the origin of materials. Phenotypic and genetic distances based on morphology and molecular information serves to assist conservation and organization of collection of materials, and the choice of parent combinations for breeding purposes.

Keywords
multivariate analysis • morphological traits • SSR • sugarcane • genetic variability

Universidad Nacional de Salta. Facultad de Ciencias Naturales. Laboratorio de Marcadores Moleculares. Av. Bolivia 5150. C. P. 4400. Salta. Argentina.
* fcn13161@gmail.com

Resumen

La caña de azúcar es uno de los cultivos industriales más importantes de regiones tropicales y subtropicales. El INTA (Argentina) administra un Banco de Germoplasma de caña de azúcar y lleva a cabo un programa de mejora. El presente trabajo fue diseñado para estimar la variabilidad fenotípica y genética entre 65 accesiones de caña de azúcar seleccionadas del INTA. Se aplicaron métodos de clasificación y ordenamiento en el análisis de datos morfológicos y de SSR. EL Análisis de Procrustes Generalizado permitió evaluar la correlación entre las relaciones establecidas a partir de ambos tipos de marcadores. Un buen ajuste entre los dendrogramas y las matrices de similitud fue soportado por un alto coeficiente de correlación cofenética (r=0,82, p<0,0001; r=0,73, p<0,0001; r=0,82, p<0,0001 para datos cuantitativos, cualitativos y moleculares respectivamente). La presencia de una estructura poblacional fue reconocida cuando se consideraron los diferentes tipos de datos. El Procrustes permitió detectar aquellas accesiones que serían responsables de la baja correlación detectada entre configuraciones individuales (73%). Tanto los marcadores morfológicos como los moleculares resultaron lo suficientemente discriminativos para diferenciar accesiones. No obstante, no fue posible correlacionar las asociaciones establecidas por los marcadores con el origen de los materiales. Las distancias fenotípicas y genéticas basadas en información morfológica y molecular será de utilidad para asistir en la conservación y organización de los materiales de la colección y elegir combinaciones parentales con propósito de mejora.

Palabras claves

análisis multivariado • caracteres morfológicos • SSR • caña de azúcar • variabilidad genética

Introduction

Sugarcane is one of the most important industrial crops in tropical and subtropical regions. It is cultivated in more than 90 countries around the world, primarily for its ability to store high concentrations of carbohydrates to produce sugar and biofuel. INTA (Instituto Nacional de Tecnología Agropecuaria) administrates the main Sugarcane Germplasm Bank in Argentina and conducts a breeding program for this crop.

The germplasm bank fulfils aspects related to exploration, collection, evaluation, preservation and germplasm exchange. The core collection currently includes 429 sugarcane accessions and 120 clones from an annex collection with high Brix (total soluble solids) materials, an attribute related to potential sucrose yield. Some morphological traits have been measured to characterize these materials aiming at improving ttheir breeding value. However, these genetic markers have several limitations including low polymorphism, low heritability, late expression, and vulnerability to environmental influences. In addition, it is known that morphological traits do not always provide a sound measure of genetic values and may not accurately reveal the genetic variation in germplasm collections (13). Since germplasm provides the raw material for breeders to improve crop performance, knowledge on genetic variability should be an auxiliary tool for breeding and an important link between the conservation and use of sugarcane available genetic resources. Interesting genetic resources for breeders include advanced material (*e.g.* pre-bred material, breeding lines, adapted varieties, elite materials) and research material (*e.g.* advanced core collections, mapping populations). However, researchers and other users may be interested in a wider range of materials. The conservation of genetic diversity in germplasm banks broadens the spectrum of materials targeted for storage (14). The usefulness of samples held in germplasm banks is dependent on the degree and quality of information connected to the samples (14). Morphological markers reflect variation of expressed regions of genome while molecular markers indicate variation of all genome including expressed and non-expressed regions. It has been reported that the patterns of allelic variation in a species may be very different for neutral markers compared with genes under selection. Based on a meta-analysis, Latta (2008) argued that variability at neutral and selected loci are not correlated because evolutionary forces act differently on them. Reed and Frankham (2003) showed only weak correlation between neutral molecular markers and morphological quantitative measures of variation. A joint analysis of morphological and molecular variability would undoubtedly increase the resolving power of the genetic diversity analysis of the sugarcane germplasm bank. It would also allow criteria for both, the choice of progenitor combinations to maximize the genetic variability of the progeny in the breeding program and to maintain variability of the germplasm collection. For those purposes, it is necessary to deal with a large number and different types of variables. The multivariate analysis has allowed the simultaneous evaluation of many traits by summarizing information in few synthetic variables. It has also permitted a better understanding of the structure of the sugar cane germplasm collection, helping to identify which variables are more relevant in order to identify relationships among accessions (3). The current study was designed to assess the phenotypic and genetic diversity of 65 sugarcane accessions selected from INTA's Germplasm Bank (Tucumán, Argentina), determining both the discriminating power and effectiveness of different SSR primers for sugarcane genotype identification and the optimal SSR primer combination to ensure unambiguous identification of a set of sugarcane genotypes. In addition, we also evaluated the correlation between the sugarcane accessions relationships established with both morphological and molecular data in order to provide guidance for future use of sugarcane accessions in the breeding programme and germplasm bank management.

Materials and methods

Sixty five sugarcane accessions from the INTA Germplasm Bank (Tucumán, Argentina) were included in this study (table 1, page 43). Most of these genotypes are of interest for breeding purposes in Argentina due to their adaptability to subtropical growing areas (short cycle and early maturity). Some of these materials are or were used as commercial varieties in Argentina and other countries.

Three basic materials (identified as US) were also included. Sugarcane accessions were grown in the greenhouse under controlled conditions.

Table 1. Sugarcane accessions included in the genetic variability analysis and Province-Country of origin (CO).

Tabla 1. Accesiones de caña de azúcar incluídas en el análisis de variabilidad genética y sus Provincias-Países de origen (CO).

Variety	Origin	Variety	Origin	Variety	Origin
LCP85-384	Louisiana, USA	NA84-3471	Salta, Argentina	TUC72-16	Tucumán, Argentina
LCP86-454	Louisiana, USA	NA63-90	Salta, Argentina	TUC74-6	Tucumán, Argentina
LCP85-376	Louisiana, USA	NA76-128	Salta, Argentina	TUC71-7	Tucumán, Argentina
HoCP85-845	Louisiana, USA	NA73-2596	Salta, Argentina	TUC68-18	Tucumán, Argentina
HoCP92-648	Louisiana, USA	NA88-948	Salta, Argentina	TUC67-24	Tucumán, Argentina
HoCP92-645	Louisiana, USA	NA73-1454	Salta, Argentina	TUC79-9	Tucumán, Argentina
HoCP92-624	Louisiana, USA	CP48-103	Louisiana, USA	TUCCP77-42	Tucumán, Argentina
HoCP89-888	Louisiana, USA	CP68-350	Louisiana, USA	TUC77-42b	Tucumán, Argentina
HoCP91-552	Louisiana, USA	CP70-1133	Louisiana, USA	TUC78-39	Tucumán, Argentina
HoCP92-631	Louisiana, USA	CP79-1380	Louisiana, USA	TUC72-4	Tucumán, Argentina
HoCP91-555	Louisiana, USA	NA84-3471	Salta, Argentina	TUC69-2	Tucumán, Argentina
HoCP88-739	Louisiana, USA	CP79-318	Louisiana, USA	L91-281	Louisiana, USA
HoCP90-941	Louisiana, USA	CP65-350	Louisiana, USA	RA89-686	Argentina
US74-1011	USA	CP57-603	Louisiana, USA	RA87-2	Argentina
US74-1015	USA	CP57-614	Louisiana, USA	RA91-209	Argentina
US72-1289	USA	CP72-2086	Louisiana, USA	RA93-154	Argentina
L75-33	Louisiana, USA	CP66-346	Louisiana, USA	CP88-1834	Louisiana, USA
TCP81-3067	Tucumán, Argentina	CP62-258	Louisiana, USA	F98-70	Tucumán, Argentina
TCP87-388	Tucumán, Argentina	FAM81-820	Tucumán, Argentina	F97-395	Tucumán, Argentina
NA84-3013	Salta, Argentina	FAM83-11	Tucumán, Argentina	F97-786	Tucumán, Argentina
NA78-724	Salta, Argentina	TUC80-7	Tucumán, Argentina	CP65-357	Louisiana, USA
				Nco310	Sud Africa

Morphological traits

A total of 59 morphological variables from stem and leaf were evaluated. From these, 43 correspond with sugarcane UPOV (*Union for the Protection of New Varieties of Plants*) descriptors, while 16 are descriptors defined by Wagih (2004). Morphological traits comprised both qualitative (43) and quantitative (16) attributes. Most of these attributes (48) are not subjected to selection in breeding programs; 4 of them, related to stem traits, are subjected to screening as primary conditioning requisites, while other 7 are subsidiary traits related to leaves and canopy (table 2, page 44-45).

The accessions were planted in 2017-2018 in single raw evaluation plots of 1 m length (50 cm spacing) at the experimental greenhouse of Universidad Nacional de Salta (24°43'22" S and 65°24'74" W). Irrigation was provided at appropriate time according to requirements. Data on measurable morphological characters, were recorded on year after planting. Quantitative traits were measured on five random stems for each accession and data were averaged.

Table 2. Qualitative and quantitative morphological markers assessed in 65 accessions of sugarcane. Name, abbreviation and categories or units is indicated for each variable.

Tabla 2. Marcadores morfológicos cualitativos y cuantitativos estudiados en 65 accesiones de caña de azúcar. Para cada variable se indica el nombre, abreviatura y la categoría o unidades.

Plant	Abbreviation	Category and/or Units
Stool growth habit	*PC*	erect/semierect/intermediate/ semipostrate/postrate
Leaf canopy	*F*	very sparse/sparse/medium/dense
Intensity of green color of leaf canopy	*ICV*	ligth/medium/dark
Depth of growth crack	*PRC*	absent/very shallow/shalow/medium/deep
Height of stalk	*TA*	Cm
Length of cane top	*LPSC*	Cm
Width of root band	*AZR*	Mm
Bud		
Shape of bud	*FY*	triangular-pointed/oval/obovate/ pentagonal/ rhomboid/round/ovate/rectangular/beaked
Hairs of budsor		
Group 1	*P1*	
Group 2	*P2*	
Group 26	*P26*	
Group 4	*P4*	
Group 16	*P16*	
Group 8	*P8*	absent/present
Group 11	*P11*	
Group 15	*P15*	
Group 18	*P18*	
Group 19	*P19*	
Group 22	*P22*	
Group 10	*P10*	
Width of bud	*AY*	Mm
Width of bud wing	*AAY*	Mm
Bud groove	*CaY*	absent/present
Length of bud groove	*LCY*	short/medium/long
Depth of bud groove	*PCY*	very shallow/shallow/medium/deep
Position of bud tip in relation to growth ring	*PAY*	clearly below/intermediate/clearly above
Bud cushion (space between base of bud and leaf scar)	*CjY*	absent or very narrow/narrow/medium/wide
Internode		
Length of internode	*LE*	Cm
Diameter of internode	*DE*	Mm
Shape of internode	*FE*	cylindrical/tumescent/bobbin-shaped/conoidal/ obconoidal/concave-convex
Cross section of internode	*ST*	ovate/circular
Expression of zigzag alignment	*EZZ*	absent or very weak/weak/moderate/strong

Table 2 (cont.). Qualitative and quantitative morphological markers assessed in 65 accessions of sugarcane. Name, abbreviation and categories or units is indicated for each variable.

Tabla 2 (cont.). Marcadores morfológicos cualitativos y cuantitativos estudiados en 65 accesiones de caña de azúcar. Para cada variable se indica el nombre, abreviatura y la categoría o unidades.

Plant	Abbreviation	Category and/or Units
Wax ring	*AC*	Mm
Waxiness	*C*	absent or very weak/weak/moderate/strong
Leaf sheath		
Length of leaf sheath	*LV*	Cm
Distribution of hairs of leaf sheath	*DPV*	only dorsal/lateral and dorsal
Number of hairs: group 57	*P57*	absent or very few/few/medium/many/a lot
Number of hairs: group 60	*P60*	absent or very few/few/medium/many/a lot
Length of hairs: group 57	*LP57*	short/medium/long
Length of hairs: group 60	*LP60*	short/medium/long
Hairs around leaf sheath	*PAV*	absent/present
Length of hairs around leaf sheath	*LPAV*	absent/short/medium/long
Density of hairs around leaf sheath	*DPAV*	absent/scarce/medium/numerous
Adherence of leaf sheath	*AdV*	weak/medium/strong
Shape of underlapping auricle	*FASY*	transitional/deltoid/dentoid/unciform/ calcariform/ falcate/lanceolate
Shape of overlapping auricle	*FASP*	transitional/deltoid/dentoid/unciform/ calcariform/ falcate/lanceolate
Size of underlapping auricle	*TASY*	Mm
Size of overlapping auricle	*TASP*	Mm
Ligule		
Shape of ligule	*FL*	strap shaped/deltoid/crescent-shaped/bow-shaped/ asymmetrical, steeply sloping/asymmetrical horizontal
Ligule width	*Ali*	Mm
Density of ligule hairs: group 61	*DP61*	absent or very sparse/sparse/medium/dense/ very dense
Length of hairs: group 61	*LP61*	short/medium/long
Leaf blade		
Curvature	*CHL*	arched at base/curved/curved tips/arched/ straight
Width at the longitudinal mid-point	*AL*	Mm
Midrib width	*AN*	Mm
Ratio leaf blade width/midrib width	*L/N*	Mm
Length of leaf blade	*LL*	Cm
Pubescence on margin of leaf blade	*PBH*	absent or very sparse/sparse/medium/dense
Serration on margin of leaf blade	*ABH*	absent/present

All measures and observations were carried out in the greenhouse and laboratory by means of metric rule and calipter or under stereoscopic binocular loupe, by the same operators for each attribute, considered stable enough for the different genotypes.

SSR

Total genomic DNA was extracted from young leaves (+1 in Kuijper's denomination) (6) using a DNA Nucleospin II extraction kit following the manufacturer protocol. The quality and quantity of DNA was assessed using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, USA) with 1 μl sample. Based on the consistency of band patterns obtained in a previous study, twenty SSR primers were evaluated (table 3). Polymerase chain reactions (PCRs) and electrophoresis and gel staining were carried out according to Pocoví *et al.* (2013) The resulting banding pattern was scored manually. Only consistent bands with strong intensity were considered for the analysis.

Table 3. Simple Sequence Repeat (SSR) primers used for genotyping 65 sugarcane accessions from the INTA Sugarcane Germplasm Bank (Tucumán, Argentina).

Tabla 3. Cebadores de Secuencias Repetitivas Simples (SSR) usados para el genotipado de 65 accesiones de caña de azúcar del Banco de Germoplasma de INTA (Tucumán, Argentina).

SSR	Repeat motif	Size range (bp)	Annealing T	Forward primer (5'-3') Reverse primer (5'-3')
NKS26	(TG)18	194-164	54	GTT CTC GAC ATG GGC CTA CT
				CTG CAC TTT CGG TCC TTT TT
mSSCIR19	(GA)23	130-160	48	GGT TCC AAA ATA CAC AAA
				CAA TCT TAT CTA CGC ACT T
NKS38	(AG)15	92-292	55	TGA ACT CGG CAA CAG TTT TT
				CCC ACC AAG TCG TTC TGA AT
NKS 23	(GA)18	113-498	54	TAA ACC CCC GAA AAA GAA CC
				TCC GGA GGT AGA TCC ATT TG
NKS34	(GT)18 (A)31	131-214	58	CGT CTT GTG GAT TGG ATT GG
				TGG ATT GCT CAG GTG TTT CA
mSSCIR16	(GA)18	130-300	54	TGG GGA GGG CTG ACT AGA
				GGC GGT ATA TAT GCT GTG
SMC703BS	(CA)12	186-229	62	GCC TTT CTC CAAACC AAT TAG T
				GTT GTT TAT GGA ATG GTG AGG A
mSSCIR3	(GT)28	171-187	60	AAT GCT CCC ACA CCA AAT GC
				GGA CTA CTC CAC AAT GAT GC
mSSCIR18	(GA)23	170-200	52	GGG TGT TCT GTT GAG CA
				GAG GTA GGA GGG AGT GTT
SMC766BS	(CA)20(GA)16	170-270	60	TTA CTC GGC TGG GTT TTG TTC
				TAA GAA TCG TTC GCT CCA GC
SMC7CUQ	(CA)10(C)4	160-170	60	GCC AAA GCA AGG GTC ACT AGA
				AGC TCT ATC AGT TGA AAC CGA
mSSCIR78	(GTT)6	150-310	48	TGCCTTAAC CGT GACATC
				GAGGACGAGGAGCAGAA
mSSCIR34	(GA)	130-300	56	ATCGCCTCCACTAAATAAT TTGTCTTTGCTTCCTCCTC

Despite being co-dominant, SSR markers were here considered as dominant markers, because in highly polyploid genomes such as that of sugarcane, the SSR markers difficulty distinguish the alleles of homologous chromosomes, making it difficult to determine heterozygosity or homozygosity at any particular locus. From this assumption, each band was treated as a unit locus and a binary system was considered scoring each individual for presence (1) or absence (0) of a band.

Statistical multivariate analysis

Clustering methods

For quantitative variables, phenotypic relationships between pairwise of sugarcane accessions were assessed using Euclidean distance calculated with their standardized means. To measure similarities between pairwise of genotypes on the basis of multistate qualitative traits, the Simple Matching Coefficient was used (25). For molecular data, relationships between pairwise of accessions were estimated using the Jaccard Coefficient. In the three cases, the accessions were then clustered by the Unweighted Pair-Group Method with Arithmetic Averages (UPGMA). Cophenetics values matrices (25) of the UPGMA clustering were used to test goodness-of-fit of the clustering to the similarity matrix on which it was based, by means of computing the product-moment correlation (r) with 1000 permutations (Mantel, 1967). The relative support for the different groups and stability of the dendrograms were assessed by bootstrap analyses (1000 replicates). Bootstrap values exceeding a 50% cut-off are indicated above the corresponding clusters in the respective figures.

Ordination methods

A principal Component Analysis (PCA), using the canonical Euclidean distance from quantitative morphological data, was carried out. The ordination was visualized simultaneously by means of biplots where sugarcane genotypes and variables were represented in a common space. For qualitative morphological and molecular data, genetic similarities matrices were used to perform Principal Coordinate Analysis (PCoA). According to Cliff (Franco and Hidalgo, 2003), only those coordinates whose accumulated values accounted for 70% or more of the total variance were considered. To facilitate the understanding of the relationships sugarcane accessions, geometrical representations were obtained using Minimum Spanning Trees (MST).

In order to establish agreement or consensus between relationships among observations derived from morphological and molecular data, a Generalized Procrustes Analysis (GPA) was carried out.

Statical analyses were performed using Infostat v.2013 (9) and DARwin 6.0.0 software program (20).

Results and discussion

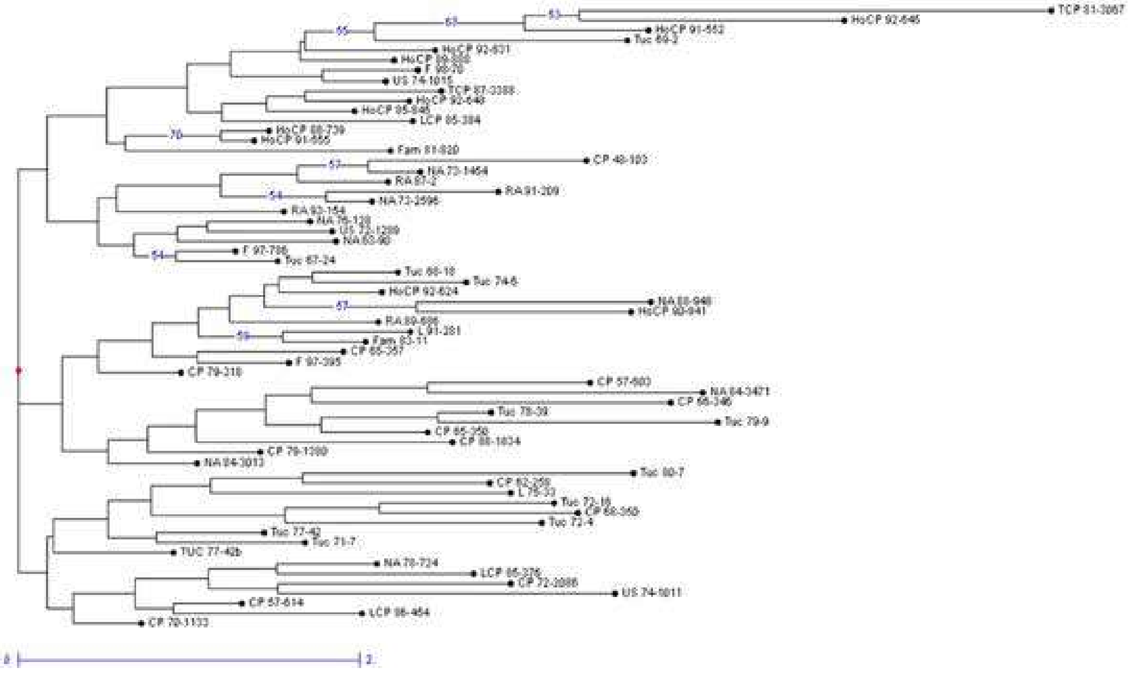
Phenotypic variability based on quantitative traits

The highest distance value was estimated between the genotypes TUC79-9 and TCP81-3067 (10.79). In opposition, HoCP88-739 and HoCP91-555 were very close to each other showing the lowest Euclidean distance value (0.48). Non-Euclidean distance between pairs of accessions was zero meaning that quantitative traits included in this study were sufficiently discriminative to differentiate unequivocally among all the accessions. The dendrogram generated with UPGMA cluster analysis of de Euclidean distance matrix, revealed nine clusters with more than 50% bootstrap values (figure 1). Probably, the small number of clusters supported by bootstrap can be explained due to many pair-wise genetic similarity coefficients with intermediate values, which allow several similar variants for dendrogram branching.

The cophenetic correlation between the dendrogram and the similarity matrix was significant (r=0.82; p<0.0001) revealing a high degree of fit.

Detail analysis of the cluster's composition does not show association patterns related to the origin or other agronomic characteristics of the materials. This result is interpreted because of the nature of the descriptors investigated, given that most of them are not associated with selection objectives of breeding. This fact can also explain the confusion of basic materials (US) with commercial ones.

The PCA analysis allowed reducing the set of correlated quantitative variables to a small number of linear combinations of these variables (principal components) such as expected (3).



Nine clusters showed in blue are those supports with more than 50% bootstrap values.

Los nueve grupos mostrados en azul son aquellos soportados por valores de bootstrap mayores a 50%.

Figure 1. Dendrogram (UPGMA) constructed with Euclidean distances based on quantitative morphological data.

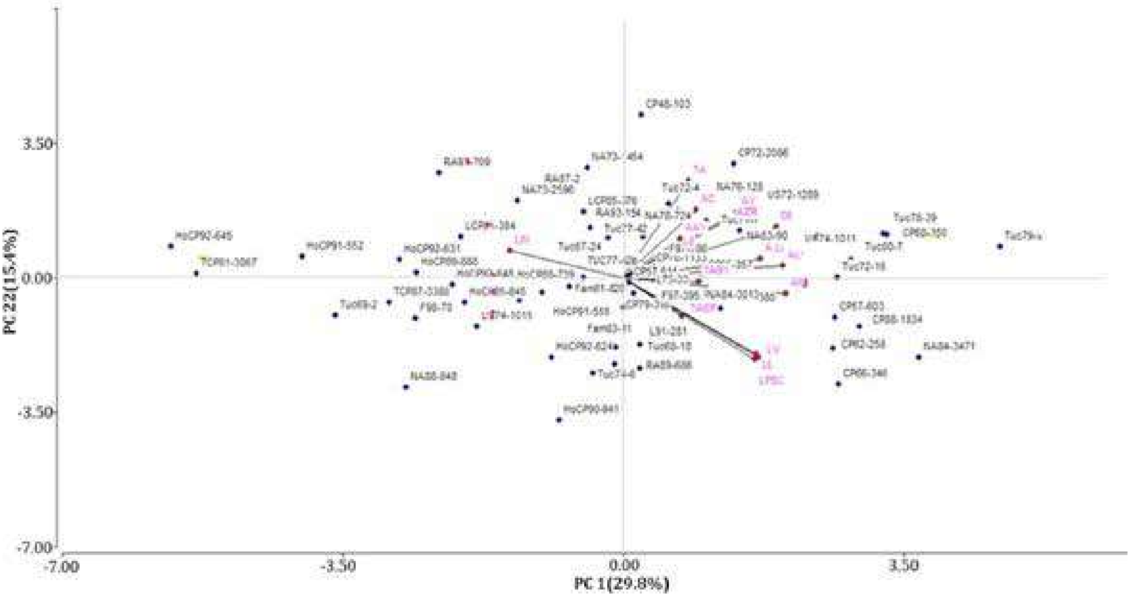
Figura 1. Dendrograma (UPGMA) basado en datos morfológicos cuantitativos construido a partir de distancias Euclídeas.

The first four principal components (PCs) had eigenvalues higher than one. The first and second synthetic variables (PC1 and PC2) explained 45% of the total variability. PC1, with an eigenvalue of 4.74, would contain equivalent information from at least four original quantitative variables. PC2, with an eigenvalue of 2.47, corresponded to two variables. According to Bhanupriya *et al.* (2014), characters with largest absolute value (eigenvectors) closer to unity within the first principal component, influence the clustering more than those with lower absolute value closer to zero. In the present study, differentiation of sugarcane accessions into different groups in PC1 can be explained because of the contribution of leaves descriptors (*Leaf sheath length, Ligule width, Midrib width, ratio Leaf blade width/Midrib width,* with eigenvalues of 0.30, 0.35, 0.30, 0.35, respectively) and cane traits (*Internode diameter, Bud width, Length of the cane top* with eigenvalues of 0.34, 0.25, 0.45, respectively) Except for diameter, the other descriptors influencing on PC1 are not primary but subsidiary traits for breeding. According to Gutiérrez-Miceli *et al.* (2002), the internode diameter is correlated with the sucrose content, so in the case of diameter it should be also considered that the range of the sample is strongly limited for being commercial type materials. These facts reinforce confounding associations discussed previously. According to Mohammadi and Prasanna (2003) when the total variation explained by the first two or three PCs is smaller than 25%, PCA provides faithful portrayal of the relationships between major groups of lines, but distances between closer genotypes are often distorted. In this study, PC1 and PC2 explained 45% of the original variation and allowed a better understanding on the structure of sugarcane genotypes. PC1 accounted for 30% of the morphological variation. Twenty of the 26 accessions (77%) classified in the first cluster (UPGMA) were grouped to the left of PC1, these genotypes would have greater *ratio Leaf blade width/Midrib width* than those on the right of CP1 (figure 2, page 50). According to Di Rienzo *et al.* (2013), the orthogonality of the principal components ensures that CP2 provides new information on variability compared to that provided by CP1. In this study, genotypes that could not be differentiated by leaf traits on PC1 could be identified by PC2, being stem height the main attribute associated to this component. Accession CP48-103 is the genotype with greater stem height.

Again, in this study, PCA analysis could not clearly differentiate materials according to their origin or nature (US) based on the morphological descriptors investigated.

Phenotypic variability based on qualitative traits

Morphological qualitative traits were also discriminative. Although some pairs of sugarcane accessions were phenotypically very close, with dissimilarities coefficients near zero (0.102), none of them showed a zero value. The histogram of pairwise dissimilarity from the qualitative data indicates a normal distribution. The dissimilarity coefficients ranged from 0.102 to 0.731. The fact that most of the dissimilarity coefficients ranged between 0.35 and 0.50 can probably explain that few internal branches (3) in the dendrogram (UPGMA) supported by bootstrapping (figure 3, page 51).



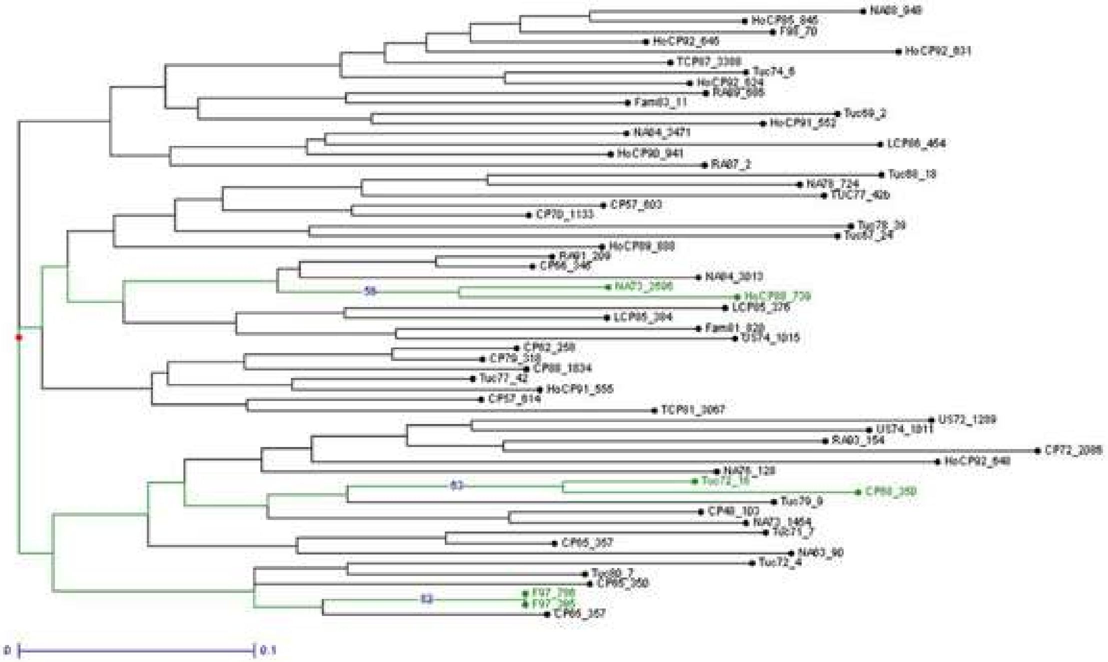
TA: Height of stalks; LPSC: Length of cane top; AZR: Width of root band; AY: Width of bud; AAY: Width of bud wing; LE: Length of internode; DE: Diameter of internode; AC: Wax ring; LV: Length of leaf sheath; TABY: Size of underlapping auricle; Ali: Ligule width; AN: Midrib width; L/N: Ratio leaf blade width/midrib width; LL: Length of leaf blade.

TA: Altura de tallo; LPSC: Longitud parte superior de la caña; AZR: Anchura de zona radicular; AY: Anchura de yema; AAY: Anchura del ala de la yema; LE: Longitud del entrenudo; DE: Diámetro del entrenudo; AC: Anillo ceroso; LV: Longitud de la vaina; TASY: Tamaño aurícula subyacente; Ali: Anchura de la lígula; AN: Anchura de la nervadura principal; L/N: Anchura del limbo/Anchura de la nervadura principal; LL: Longtud del limbo.

Figure 2. PCA biplot of 16 quantitative morphological traits with 65 sugarcane accessions and eigenvalue vectors for the traits.

Figura 2. Biplot PCA de 16 caracteres morfológicos cuantitativos con 65 accesiones y vectores de autovalores para los caracteres.

The cophenetic correlation between the dendrogram and the similarity matrix was significant ($r=0.73$; $p<0.0001$) indicating a good agreement between the graphical display of distances and the original matrix, supporting the visual inferences suggested in figure 3 (page 51). None of the sugarcane accession pairs with minimum and maximum distance values coincided with genotype pairs that appeared closer and more distant on the bases of quantitative data.



Numbers shown in clusters indicate those supported with more than 50% bootstrap values (clusters shown in green).

Los números mostrados en los grupos indican aquellos agrupamientos soportados por más de 50% de valores de bootstrap.

Figure 3. Dendrogram (UPGMA) constructed with Simple Matching Coefficients based on qualitative morphological data.

Figura 3. Dendrograma (UPGMA) basado en datos morfológicos cualitativos utilizando el Coeficiente de Simple Matching.

These differences could be explained because both types of quantitative and qualitative descriptors have different genetic bases and imply different genomic regions. It is expected that qualitative traits are mainly under monogenic or oligogenic control, conversely, quantitative traits have more complex genetic base as they are usually governed by multiple genes and their interactions (7). A much wider genomic area is expected to be considered when phenotypic relationships are estimated from quantitative data.

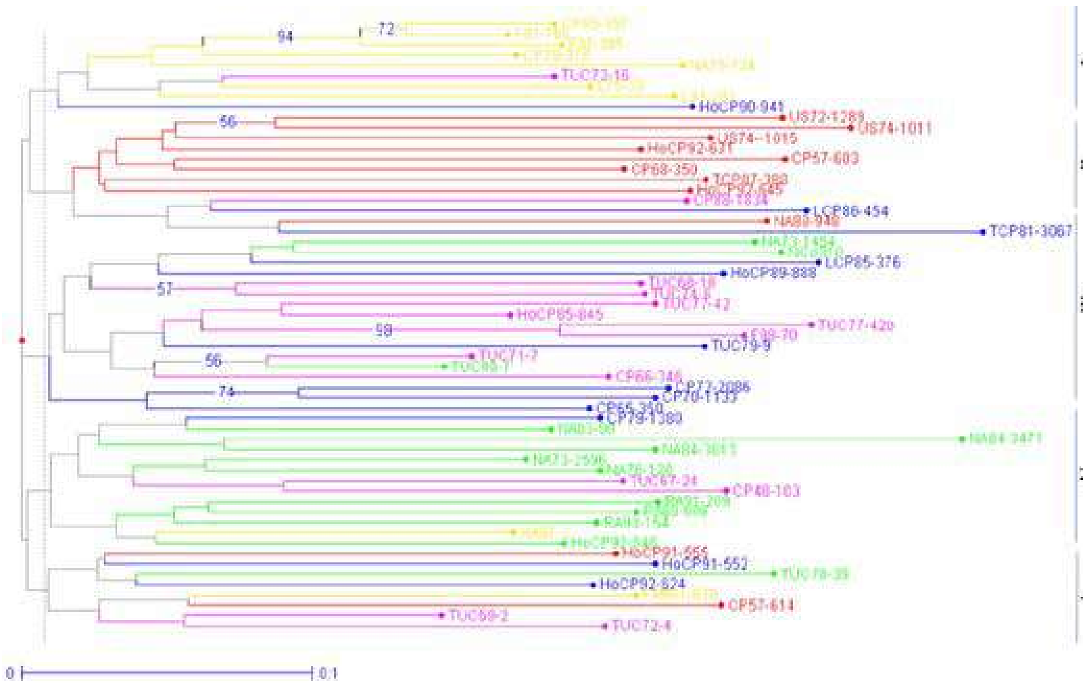
As in the case of the analysis based on quantitative traits, it was not possible to distinguish associations between qualitative based arrangements with the origin of materials. US 74-1011 and US72-1289 appear closely related and separated of commercial type accessions, while US74-1015 appears confounded with commercial types in a separate group.

PCA results based on qualitative data were not considered due to eigenvalues lower than 1, meaning that no PC explained even an original variable and small proportion of variance accounted for by the first two components (21%). ACP based on these qualitative data seemed to be inefficient to conglomerate defined sugarcane accessions groups.

Genotypic variability based on SSR

With 13 SSR primers, a total of 107 bands were detected of which, 94% were polymorphic among the studied sugarcane accessions.

The dissimilarity matrix, calculated from binary data, expressed the similarity pair to pair between sugarcane genotypes. The histogram of frequency distribution of the pairwise genetic distances fitted a normal distribution. Distance coefficients values among a total of 1711 pairs of genotypes showed an overall mean of 0.43. Of note, no dissimilarity value was zero, indicating that SSR included in this study were sufficiently discriminative for the sugarcane accessions. Most of the pairs of genotypes showed distances between 0.4 and 0.5, which allowed several similar variants for dendrogram branching and probably could explain the small number of clusters supported by bootstrap. Clustering percentage values above 50% for 1000 bootstrap cycles occurred in only seven groups (figure 4).



Numbers shown in clusters indicate those supported with more than 50% bootstrap values.

Los números de los clusters indican aquellos grupos con valores de bootstrap mayores a 50%.

Figure 4. Consensus dendrogram (UPGMA) constructed with dissimilarity genetic distances based on SSR data.

Figura 4. Dendrograma consenso (UPGMA) construido sobre la base de datos SSR utilizando medidas de disimilitud genética.

These dissimilarities values are like those reported by other authors in this species (18). According to the information indicated in table 4, we suggest that thirteen pairs of sugarcane accessions, with dissimilarities values higher than 0.65, might be considered as parental combinations accessions in the Breeding Programme, and thus, it could to some degree, benefit the broadening of the genetic basis in sugarcane hybridization. According to You *et al.* (2013), the innovation of parents with higher genetic diversity showed a positive role in sugarcane breeding programs in China. They suggested that more attention should be paid in the future to the selection of new parents in sugarcane hybrid breeding.

Differences were clear within clusters derived from quantitative morphological and molecular data. In both cases, cophenetic correlation coefficients were 0.82 indicating a high correlation between cophenetic distances and input distance matrices obtained from the data. Since cophenetic distance between two accessions is the distance at which two genotypes are first clustered together in a dendrogram from the bottom to the top (19), the cophenetic correlation coefficient, therefore, measures the relationships between the original pair wise distances between accessions (true distance) and pair wise distances predicted using dendrogram. In both cases, dendrograms corresponded graphically to 82% of the dissimilarity matrices. According to Odong *et al.* (2011) cophenetic correlation coefficient ≤ 0.8 is an indicator for strength of subgroup differentiation. Our results showed the presence of different reliable population structure in the studied sugarcane accessions when morphological and molecular data were considered. The phenotypic variation does not always follow the genetic pattern of variation and diversity of plant populations. The lack of congruence between morphological and genetic diversity has been reported in different plant species (1, 24).The different clustering can be explained due to a partial and insufficient genome representation when morphological data are used. Semang (2000) explained the lack of correspondence between molecular and morphological results, when stated that molecular markers cover a larger proportion of the genome, (including coding and noncoding regions), than the morphology ones. In addition, a large portion of the genetic variation detected by molecular markers is non-adaptive and, therefore, they are not subjected to either natural or artificial selection as many morphological traits.

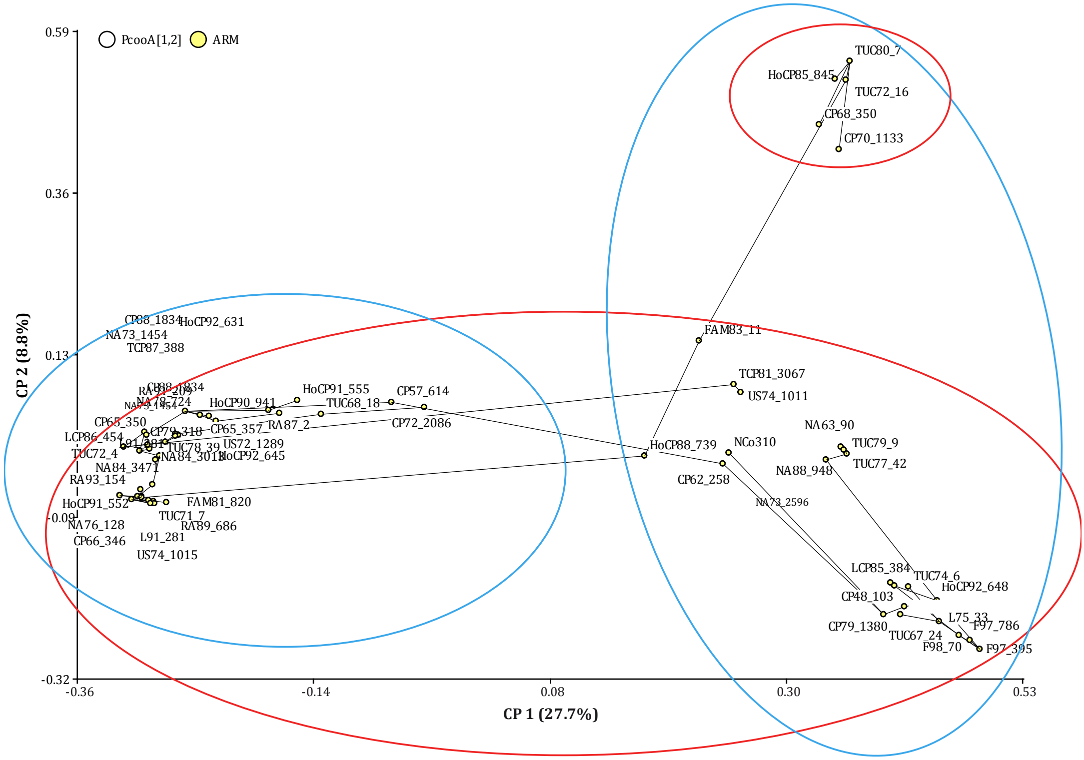
Table 4. Pairs of sugarcane accessions, with dissimilarities values higher than or equal to 0.60.

Tabla 4. Pares de accesiones de caña de azúcar con valores de disimilitud mayores o iguales a 0,60.

Pairs of sugarcane accessions		D=1-S_{ij}
F97-395	NA78-724	0.65
F97-395	L75-33	0.64
RA91-209	NA78-724	0.64
CP57-603	NCo310	0.63
RA91-209	L75-33	0.63
L75-33	HoCP91-555	0.62
TCP81-3067	LCP85-376	0.62
CP68-350	NA78-724	0.61
NA73-1454	NA78-724	0.61
TUC72-16	NA78-724	0.60
FAM83-11	L75-33	0.60
CP79-1380	L75-33	0.60
NA78-724	US74-1011	0.60

Differences in clustering can also be explained due to an absence of linkage between the loci that control the studied morphological characters and the evaluated SSR markers.

The first PCo summarized most of the variability present in the original data (28%) relative to all remaining PCos. The second PCo explained 9% of the variability and because PCos are orthogonal and independent to each other, they reveal different properties of the original data. According to Cliff criterion (10), the first 10 PCos explained 70% of cumulative variance, but only the first five showed eigen-values equal to or greater than one. The minimum spanning tree (MST) imposed on the PCoA improved the representation of sugarcane relationships (figure 5).



The numbers in parenthesis refer to the proportion of the variance explained by the main coordinates. The blue and red circles indicate the different groups of PC1 and PC2, respectively. The colour of the accessions is related to their origin (blue: Louisian, USA; green: Salta, Argentina; Fuchsia: Tucumán, Argentina; red: USA).

Los números entre paréntesis indican la proporción de la varianza explicada por la coordenada principal. Los círculos azul y rojo muestran los diferentes grupos en PC1 y PC2 respectivamente. El color de las accesiones está relacionada con su origen (azul: Luisiana, USA; verde: Salta, Argentina; Fuccia: Tucumán, Argentina; rojo: USA).

Figure 5. Arrangement by Minimum Spanning Tree (MST) in the plane of the coordinates PC1 and PC2 of the 67 sugarcane accessions based on SSR data.

Figura 5. Árbol de recorrido mínimo (ARM) con proyección en el plano de las coordenadas PC1 y PC2 de las 67 accesiones de caña de azúcar basada en datos de SSR.

Although it was computed on the full dimension of data, the MST provided information about the quality of the projection on the low dimensional space, showing relationships that may have not been seen by inspection on the reduced space.

According to Balzarini *et al.* (2011) if many branches and segments cross each other, it suggests distortion problems in the projection which could bias regular interpretations. Even PCo2 explained only 9% of the variability; there is a group of accessions projected onto PCo2 that is clearly differentiated from the rest of the accessions (TUC 80-7; TUC72-16; CP68-350; CP70-1133 and HoCP85-845).

Qualitative morphological traits vs molecular markers

The distribution of values for morphological and genetic dissimilarity (calculated with qualitative traits and SSRs data) did not differ substantially. The distribution based on morphological data was slightly biased toward small values of distance (figure 6). Differences in the frequency distributions indicate that both types of markers detected a distinct pattern of association between sugarcane accessions. Consequently, complementary studies based on morphological and SSR will provide relevant information for establishing relationships among plant materials and a better description and interpretation of the available variability in germplasm banks and breeding programmes, as well as a foundation for promoting breeding and for germplasm conservation.

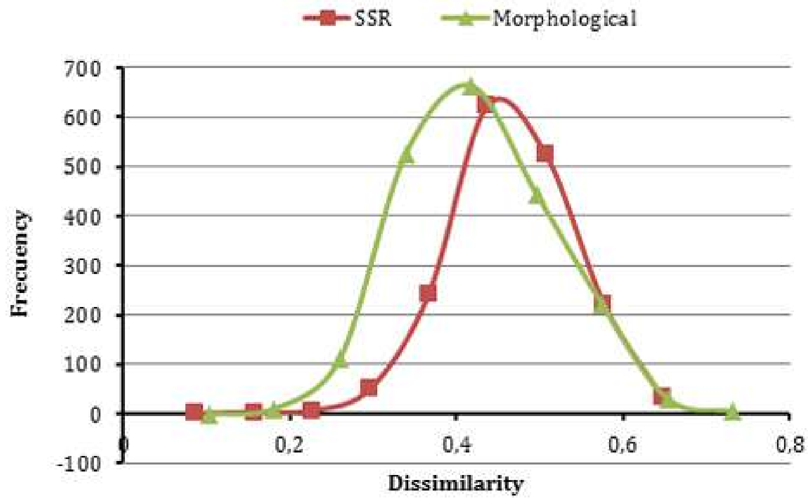


Figure 6. Frequency distribution of genetic dissimilarity among pairwise combinations of 65 sugarcane accessions based on morphological and SSR.

Figura 6. Distribución de frecuencias de disimilitudes genéticas entre pares de combinaciones de 65 accesiones de caña de azúcar basada en datos morfológicos y SSR.

Generalized Procrustes Analysis (GPA): Consensus between morphological and molecular data

GPA allowed a deeper study of the relationships among relative ordinations of the same sugarcane accessions under morphological and SSR data. Gower's (1975) recommended calculating an ANOVA to comparatively break down the total sums of squares into the between and within configurations.

Table 5. ANOVA Consensus between molecular and morphological ordinations. Accessions in bold showed the greatest discrepancy between the morphological and SSR data due to their higher relative values of Residual Sum of Squares (RSS).

Tabla 5. ANOVA Consenso entre ordenamientos basados en marcadores moleculares y morfológicos. Las accesiones en negrita mostraron las mayores discrepancias entre datos morfológicos y de SSR debido a sus valores de Sumas de Cuadrados Residuales (SCR) más altos.

	Consensus	**Residue**	**Total**	**Consensus proportion**
LCP85-384	0.014	0.007	0.021	0.673
LCP86-454	0.018	**0.012**	0.030	0.598
LCP85-376	0.031	**0.013**	0.044	0.706
HoCP85-845	0.014	0.007	0.021	0.673
HoCP92-648	0.022	**0.010**	0.031	0.684
HoCP92-645	0.022	0.009	0.031	0.701
HoCP92-624	0.011	0.007	0.018	0.628
HoCP89-888	0.016	**0.012**	0.027	0.576
HoCP91-552	0.018	0.008	0.026	0.675
HoCP92-631	0.024	0.004	0.028	0.852
HoCP91-555	0.012	**0.018**	0.030	0.406
HoCP88-739	0.014	0.009	0.023	0.604
HoCP90-941	0.040	0.009	0.050	0.816
US74-1011	0.050	0.006	0.056	0.890
US74--1015	0.030	0.007	0.037	0.800
US72-1289	0.030	0.008	0.038	0.784
L75-33	0.021	0.008	0.029	0.733
TCP81-3067	0.036	**0.013**	0.049	0.729
TCP87-388	0.023	0.006	0.029	0.781
NA84-3013	0.015	**0.010**	0.025	0.608
NA78-724	0.014	**0.016**	0.030	0.460
NA84-3471	0.026	0.008	0.034	0.763
NA63-90	0.028	0.008	0.036	0.778
NA76-128	0.028	0.006	0.034	0.816
NA73-2596	0.012	0.008	0.020	0.581
NA88-948	0.024	0.005	0.029	0.835
NA73-1454	0.032	**0.015**	0.047	0.680
CP48-103	0.037	0.009	0.046	0.814
CP68-350	0.023	0.007	0.030	0.755
CP70-1133	0.016	0.005	0.021	0.763

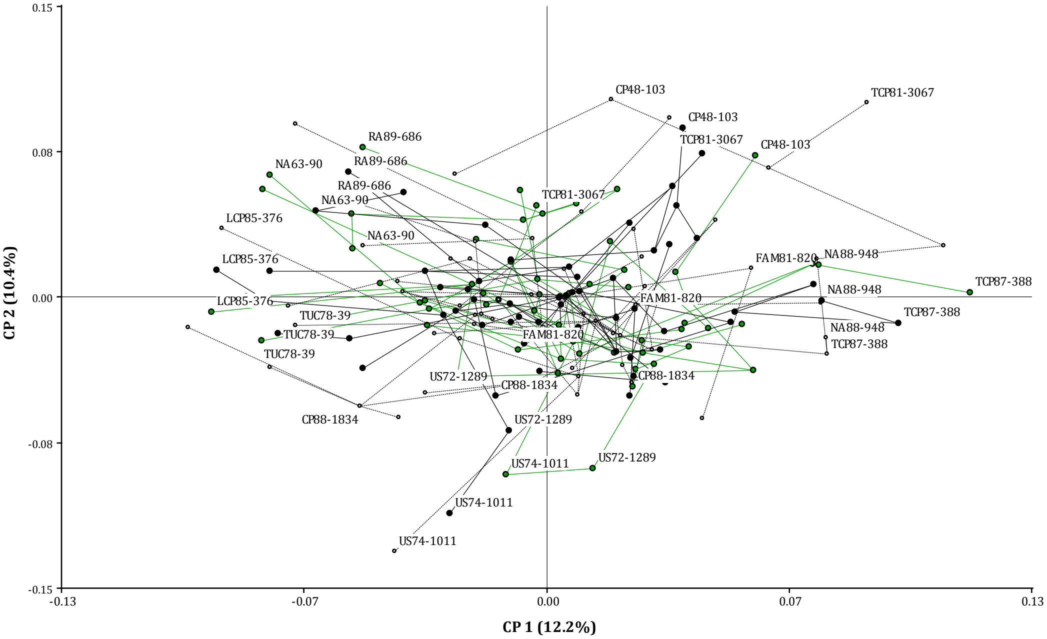
Table 5 (cont.). ANOVA Consensus between molecular and morphological ordinations. Accessions in bold showed the greatest discrepancy between the morphological and SSR data due to their higher relative values of Residual Sum of Squares (RSS).

Tabla 5 (cont.). ANOVA Consenso entre ordenamientos basados en marcadores moleculares y morfológicos. Las accesiones en negrita mostraron las mayores discrepancias entre datos morfológicos y de SSR debido a sus valores de Sumas de Cuadrados Residuales (SCR) más altos.

	Consensus	**Residue**	**Total**	**Consensus proportion**
CP79-1380	0.019	0.008	0.027	0.690
CP79-318	0.016	0.003	0.019	0.828
CP65-350	0.018	0.007	0.024	0.726
CP57-603	0.043	0.007	0.051	0.858
CP57-614	0.021	**0.011**	0.032	0.651
CP72-2086	0.034	0.007	0.040	0.834
CP66-346	0.026	**0.011**	0.037	0.706
CP62-258	0.034	**0.010**	0.044	0.769
FAM81-820	0.018	**0.012**	0.030	0.594
FAM83-11	0.049	0.005	0.055	0.900
TUC80-7	0.019	0.011	0.030	0.635
TUC72-16	0.033	0.010	0.043	0.761
TUC74-6	0.016	**0.018**	0.034	0.476
TUC71-7	0.018	**0.010**	0.027	0.649
TUC68-18	0.028	0.008	0.037	0.772
TUC67-24	0.024	**0.012**	0.036	0.661
TUC79-9	..0.020	0.006	0.026	0.769
TUC77-42	0.016	0.005	0.021	0.770
TUC78-39	0.026	0.008	0.034	0.775
TUC72-4	0.024	0.006	0.030	0.791
TUC69-2	0.014	0.008	0.023	0.629
L91-281	0.020	0.006	0.026	0.765
RA89-686	0.031	0.009	0.040	0.778
RA87-2	0.033	0.009	0.041	0.788
RA91-209	0.011	0.020	0.030	0.356
RA93-154	0.025	**0.010**	0.035	0.702
CP88-1834	0.0.18	0.006	0.024	0.757
F98-70	0.025	0.005	0.029	0.841
F97-395	0.014	0.003	0.017	0.798
F97-786	0.014	0.004	0.017	0.798
CP65-357	0.014	0.005	0.020	0.733
Total	1.459	0.541	2.000	0.730

According to Bramardi *et al.* (2005), the latter is broken into the consensus and the residual sum of squares. This residual sum of squares measures the divergence between the two points corresponding to the morphological and molecular characterization to the consensus one, respectively (table 5, page 56-57). The ratio between the consensus value (1.459) and the total sum of squares revealed a consensus of 73% between molecular and agronomic ordinations (2).

This percentage of consensus is an univariate measure of association between both groups of markers. According to table 5, accessions in bold letter are those that have shown a high discrepancy between morphological and molecular data, because they have grater residual sum of square values, therefore they should have been responsible for the low correlation found between the individual configurations.



The continuous green line indicates the MST based on morphological data and the black dotted line; the MST based on molecular data.

La línea verde indica el ARM obtenido con datos morfológicos y la línea discontinua negra, el ARM basado en datos moleculares.

Figure 7. Configuration of consensus matrix of GPA between morphological and molecular data with Minimum Spanning Tree (continuous black line).

Figura 7. Configuración consenso GPA con datos morfológicos y moleculares que incluye el Árbol de Recorrido Mínimo (ARM) en línea negra.

The consensus configuration of GPA with Minimum Spanning Tree (MST) is presented in figure 7 (page 58). The large number of accessions included in this study and the close genetic relationship among materials, hinders the identification of individuals in the consensus configuration.

In most of the references found for sugarcane, the assessment of the genetic variability is based, independently, on the analyses of morphological or molecular markers data.

Some papers estimate a correlation coefficient between distance matrices. According to Demey (2008), conclusions based only on correlation coefficient values can be inaccurate since the correlation is not only affected by the size of the compared samples but also because the configurations belong to the same reference system.

Conclusions

Based on results formerly presented and discussed, we propose the following general conclusions:

Both morphologic (quantitative and qualitative) and molecular markers included in this research resulted discriminative enough to differentiate among the studied accessions. It was not possible, however, to correlate associations of markers with the origin of materials.

The large number of pair-wise similarity coefficients with intermediate values determined a rather small number of nodes in clustering, which, on time, reflects the near genetic origin of most of the studied materials.

Diversity detected for morphological descriptors contributing to explain PC1 and PC2 (except for diameter), are expected since they are not usually subjected to selection in breeding.

Phenotypic and genetic distances based on morphology and molecular information serves to assist conservation and organization of collection of materials, and the choice of parent combinations for breeding purposes.

References

1. Ayele, T. B.; Gailing, O.; Finkeldey, R. 2011. Assessment and integration of genetic, morphological and demographic variation in *Hagenia abyssinica* (Bruce) J. F. Gmel to guide its conservation. Journal for Nature Conservation. 19: 8-17.
2. Balzarini, M.; Bruno, C.; Peña, A.; Teich, I.; Di Rienzo, J. 2010. Estadística en Biotecnología. Aplicaciones en InfoGen. Encuentro Grupo Editor. Córdoba. Argentina.
3. Balzarini, M.; Teich, I.; Bruno, C.; Peña, A. 2011. Making genetic biodiversity measurable: a review of statistical multivariate methods to study variability at gene level. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 43(1): 261-275.
4. Bhanupriya, B.; Satyanarayana, N.; Mukherjee, S.; Sarkar, K. 2014. Genetic diversity of wheat genotypes based on principal component analysis in Gangetic alluvial soil of West Bengal. J. Crop Weed. 10: 104-107.
5. Bramardi, S. J.; Bernet, G. P.; Asíns, M. J.; Carbonell, E. A. 2005. Simultaneous agronomic and molecular characterization of geno types via the generalised procrustes analysis. An Application to Cucumber. Crop Science. 45: 1603-1609.
6. Casagrande, A. A. 1991. Tópicos de morfologia e fisiología da cana-de-açúcar. Jaboticabal: Funep. 157 p.

7. Corrado, G.; La Mura, M.; Ambrosino, O.; Pugliano, G.; Varricchio, P.; Rao, R. 2009. Relationships of Campanian olive cultivars: comparative analysis of molecular and phenotypic data. Genome. 52: 693-700.
8. Demey, J. F. 2008. Diversidad genética en bancos de germoplasma: un enfoque biplot. Universidad de Salamanca. Departamento de estadística. PHD thesis Salamanca. España.
9. Di Rienzo, J. A.; Casanoves, F.; Balzarini, M. G.; Gonzalez, L.; Tablada, M.; Robledo, C. W. 2013. InfoStat versión 2013. Grupo InfoStat. FCA. Universidad Nacional de Córdoba. Argentina. URL http://www.infostat.com.ar
10. Franco, F. L.; Hidalgo, R. 2003. Análisis estadístico de datos de caracteres morfológicos de recursos fitogenéticos. Boletín Técnico N°8. IPGRI. Cali. Colombia.
11. Gower, J. 1975. Generalized Procrustes Analysis. Psychometrika. 40: 33-51.
12. Gutiérrez Miceli, F.; Arias Castro, C.; Dendooven, L.; Méndez Salas, R.; Rodriguez Mendiola, M.; Nefaltí Ochoa, A.; Bello Peréz, L. 2002. Regulación enzimática de la acumulación de sacarosa en plantas de caña de aúcar (Saccharum ssp). Agrociencia 36 (4):149.156.
13. Jansky S.; Dawson, J.; Spooner, D. M. 2015. How do we address the disconnect between genetic and morphological diversity in germplasm collections? American Journal of Botany. 102(8): 1213-1215.
14. Khoury, C.; Achicanoy, H. A.; Bjorkman, A. D.; Navarro-Racines, C.; Guarino, L.; Flores-Palacios, X.; Engels, J. M. M.; Wiersema, J. H.; Dempewol, H.; Sotelo, S.; Ramírez-Villegas, J.; Castañeda-Alvarez, N. P.; Fowler, C.; Jarvis, A.; Rieseberg, L. H.; Struik, P. C. 2017. Origins of food crops connect countries worldwide. Proceedings of the Royal Society B. 283(1832): 20160792. https://doi.org/10.1098/rspb.2016.0792
15. Latta, R. 2008.Conservation genetics as applied evolution: from genetic pattern to evolutionary process. Evolutionary applications. doi: 10.1111/j.1752-4571.2007.00008
16. Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27(2): 209-20.
17. Mohammadi, S. A. 2003. Analysis of genetic diversity in crop plants. Salient statistical tools and considerations. Crop Science. 43: 1235-1248.
18. Nair, N. V.; Selvi, A.; Srinivasan, T. V.; Pushpalatha, K. N. 2002. Molecular diversity in Indian sugarcane cultivars as reveled by Randomly Amplified DNA polymorphisms. Euphytica. 127: 219-225.
19. Odong, T. L.; Van Heerwaarden, J.; Janse, J.; Van Hintum, T. J. L.; Vaneeuwijk, F. A. 2011. Determination of genetic structure of germplasm collections: are traditional hierarchical clustering methods appropriate for molecular marker data? Theoretical and Applied Genetics. 123: 195-205.
20. Perrier, X.; Jacquemoud-Collet, J. P. 2006. DARwin software http://darwin.cirad.fr/darwin.
21. Pocovi, M.; Taboada, G.; Collavino, G.; Gutiérrez, A.; Mariotti, J. 2013. Optimal use of SSRs for establishing genetic relationships and variety identification in a collection of Sugarcane hybrids. Indian J. Plant Genet. Resour. 26(2): 103-112.
22. Reed, D. H.; Frankham, R. 2003. Correlation between fitness and genetic diversity. Conservation Biology. 17(1): 230-237.
23. Semagn, K. 2002. Genetic relationships among ten endod types as revealed by a combination of morphological, RAPD and AFLP markers. Hereditas. 37: 149-156.
24. Smissen, R. D.; Heenan, P. B. 2010. A taxonomic appraisal of the Chatham Islands flax (*Phormium tenax*) using morphological and DNA fingerprint data. Australian Systematic Botany. 23: 371-380.
25. Sneath, P. H.; Sokal, R. R. 1973. Numerical Taxonomy: the principles and practice of numerical classification. W. H. Freeman. San Francisco.
26. Wagih M. E.; Musa, Y.; Ala, A. 2004. Fundamental botanical and agronomical characterization of sugar cultivars for clonal identification and monitoring genetic variations. Sugar Tech. 6(3): 127-140.
27. You, Q.; Xu, L.; Zheng, Y.; Que, Y. 2013. Genetic diversity analysis of sugarcane parents in chinese breeding programmes using ssr markers. The Scientific World Journal doi:10.1155/2013/613062.

Water and radiation productivity in different cropping sequences in the north center of Santa Fe

Productividad del agua y la radiación en diferentes secuencias de cultivos en el centro norte de Santa Fe

Horacio Omar Imvinkelried, Marianela Pietrobón, Ignacio Miguel Dellaferrera

Originales: *Recepción:* 27/03/2018 - *Aceptación:* 11/08/2018

Abstract

Dry matter and grain productions depend on the ability of crops to capture resources. Productivity of resourses is defined based on the amount of grain or dry matter produced per unit of available resource (solar radiation or rainfall) during the year. Our main objective was to evaluate the effect of different crop sequences on the productivity of water (WP) and radiation (RP) resources for grain production and total dry matter (DM). The trial was carried out during 2014/15 and 2015/16. Nine sequences were established, including different cultures and fertilization doses with a 25, 50 or 75 % -variable-participation of grasses.

Increases of the order of 125 and 125 % were determined in WP, and of 141 and 142 % for RP for grain and DM respectively, in the sequence b/fc-w/s respect v/s-w/s (b: barley; w: wheat; s: soybean; v: vicia; fc: fertilized corn). The results showed that the sequences of crops that included higher percentage of grasses and the adequate fertilization, increased water and radiation efficiency and productivity, achieving a greater contribution of carbon from crop residues.

Keywords

water use efficiency • radiation use efficiency • soybean • wheat • vicia • corn • barley

Universidad Nacional del Litoral. Facultad de Ciencias Agrarias. Departamento de Producción Vegetal. Cátedra de Cultivos Extensivos. R. P. Kreder Nº 2805. Esperanza, Santa Fe. Argentina. horaim@fca.unl.edu.ar

Resumen

La producción de MS y grano dependen de la capacidad de los cultivos para capturar recursos. Para estudiar la eficiencia a nivel de la secuencia de cultivos se define la productividad de los recursos basada en la cantidad de granos o materia seca producidos por unidad de recurso (radiación solar o lluvias) disponible durante el año. El objetivo principal del trabajo fue evaluar el efecto de diferentes secuencias de cultivos sobre la productividad de los recursos agua (PA) y radiación (PR) para la producción de grano y materia seca total (MS). El ensayo se realizó durante las campañas 2014-15 y 2015-16, estableciendo 9 secuencias que incluyeron diferentes cultivos y niveles de fertilización, teniendo las gramíneas un 25, 50 o 75 % de participación en las mismas.

Incrementos en el orden del 125 y 125 % fueron determinados en la PA y del 141 y 142 % en la PR para la producción de grano y MS respectivamente, en la secuencia c/mf-t/s respecto a v/s-t/s. (c: cebada; t: trigo; s: soja; v: vicia; mf: maíz fertilizado). Los resultados mostraron que las secuencias de cultivos que incluyeron mayor porcentaje de gramíneas y la adecuada fertilización, aumentaron la eficiencia y productividad en el uso del agua y radiación, logrando un mayor aporte de carbono proveniente de los rastrojos al suelo.

Palabras clave

eficiencia uso del agua • eficiencia uso radiación • soja • trigo • vicia • maíz • cebada

Introduction

During the last 200 years, population growth has considerably increased the pressure on productive lands. Worldwide food demand is expected to increase by 60-100 % by 2050 (25). Plowing of agricultural soils and the low return of harvest residues to them has caused the reduction of carbon (C) in these soils (17). The absence of winter crops also reduce the capture of resources (water and radiation) which are not used to produce grains or dry matter (3). In this sense, in regions where growing season is broad and the supply of resources is favorable, a huge amount of these resources is wasted instead of being exploited by intensifying the sequences in a sustainable way.

The sustainable intensification of agriculture aims to maintain or increase the current production levels with a more intense and rational use of the resources of the environment and of lands with greater aptitude. Improvements based on sustainable intensification must be economically viable, socially acceptable and environmentally sustainable (4).

Intensive farming involving multiple crops per year could improve resource capture and productivity. Resource productivity is defined as the ratio between output (dry matter or grain yield) and annual input of Photosynthetically Active Radiation (PAR) or rainfall.

Dry Matter (DM) and grain production depend on the ability of the crop to capture resources. The efficiency of a crop sequence is defined according to the amount of grain or DM produced by resource unit, available during the year (3). This concept integrates capture and use efficiencies.

Increased radiation capture could improve the cycling of nutrients and the return of crop residues to the agricultural systems (22). This is associated with improved C balance (24) and soil aggregation (20). In similar way, the application of fertilizers increases the productivity of crops, achieving in the medium and long term a positive effect on the soil by increasing the production of crop residues (16).

The Intensification Sequence Index (ISI) depends, basically on the period of the year occupied by crops in each sequence. Besides, there is exist a negative correlation between ISI and runoff or erosion, and a positive correlation soil C content (9, 19, 23). The wheat/soybean double cropping is the more widespread sequence. This sequence occupies a great proportion of the growing season, obtaining a high and efficient capture of resources, with a value of ISI=2. On the other hand, soybean monoculture has a lower ISI value (ISI=1).

We hypothesize that the productivity of water and radiation resources can be modified according to the different participation of grasses in the sequence crop. The main objective of this work was to evaluate the effect of different crop sequences of two years on the productivity of water and radiation resources for the production of grain and dry matter.

The inclusion of winter crops for grains, allows implementing the double sequential crop, sowing a summer crop after harvesting a winter crop. Enough information for the Argentine Pampas area states that soybean monoculture causes highly negative C balances.

Other crop rotations are necessary, including such as corn with other winter alternatives other than wheat, such as vicia or barley.

Materials and Methods

The trial was carried out in the Experimental Unit of Extensive Crops, in the city of Esperanza, Santa Fe, Argentina (31º 24' 54.14''S 60º 54' 28.64''O), the soil is typic Argiudoll, Esperanza series, moderately deep and drained, with agricultural history of 8-year direct seeding and soybean predecessor.

Organic matter was determined in 2.2 % [considered medium-low (6)] and phosphorus Bray levels at 10 ppm (below the critical response levels for soybeans (10) and wheat (12)). Meteorological data (rain, radiation and temperature) were taken from the weather station located in the Facultad de Ciencias Agrarias of Esperanza.

Three criteria are proposed for the selection of agricultural sequences: 1) maintain an ISI=2 with four crops in two years; 2) soybean present in all the sequences; and 3) include alternative winter crops for wheat, both for harvest and as cover crop. The design was in randomized complete blocks with arrangement in divided plots and three repetitions.

The main plot corresponded to the crops of first occupation (winter) and the sub-plot to the rest of the crops in the sequence.

The crops were: vicia (*Vicia villosa*) -as a cover crop-; wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), soybean (*Glycine max*) and corn (*Zea mays*). Second corn had two levels of fertilization: fertilized corn (fc) [150 kg_N ha^{-1} according to the replacement dose of the N extracted by the crop to maximum yields as defined in Maddonni *et al*. (2003)] balance method, and without fertilization (c).

The grasses represented 25, 50 or 75 % of the sequence crops. In total 9 sequences were used 1) vicia/soybean-wheat/soybean (v/s-w/s); 2) vicia/soybean-wheat/soybean (v/s-w/s); 3) wheat/soybean-wheat/soybean (w/s-w/s); 4) barely/soybean-wheat/soybean (b/s-w/s); 5) wheat/corn-wheat/soybean (w/c-w/s); 6) vicia / fertilized corn-wheat/soybean (v/fc-w/s); 7) barely corn-wheat/soybean (b/c-w/s); 8) wheat /fertilized corn-wheat/soybean (w/fc-w/s); 9) barely/fertilized corn–wheat/soybean (b/fc-w/s).

A basic fertilization according to the requirements of each crop, was provided. Fertilization was performed according to diagnosis at the beginning and end of each crop of the rotation. Management practices were implemented to maximize their production in terms of nutrition, weed control, pests and diseases.

In the culture of vicia, the production of DM at the time of drying was determined. This was done with 2 l ha^{-1} of glyphosate at the beginning of flowering (20 % flowering -a flowered knot within the top five knots-).

Grain yield was determined by harvesting plants of 8 linear meters of each experimental unit. The samples were dried in an oven with forced air circulation at 65 °C until constant weight. Then, they were re-weighted to obtain total DM and threshed by hand to determine grain yield. Final weight was corrected to commercial humidity. The contribution of C in crop residues was estimated by subtracting the total aerial DM from the DM in grain, and knowing that 40 % of the DM is C (1).

The Photosynthetically Active Radiation intercepted (PARi) by crop was obtained using a radiometer LI-COR (LI-250) in each plot, PAR above (I_0) and below (I’) the canopy was measured around noon (*i.e.* 1200–1400 h) on sunny days, according to methodology proposed by Gallo & Daughtry (1986). The percentage of intercepted PAR (% iP) was calculated as $100*[1-(I'/I_0)]$. The cumulative PAR intercepted by the crop (PARi) was obtained by multiplying the incident PAR by % iP during crop ontogeny.

Initial useful water stored in the profile up to 1.2 m depth, and the residual at the time of physiological maturity or at the time of drying with herbicide in the case of cover crops, was calculated by gravimetry. With the variation of soil water content (Δ h), the water balance method was used to obtain crop evapotranspiration (Etc), clearing the formula:

$$\Delta h = Etc - Pe - Per$$

where:

Pe = effective precipitation, considered as 0.8 of the total precipitation when it exceeds 20 mm (7)

Per = deep percolation (zero was assumed).

Water Productivity (WP) in different crop sequences was estimated as:

$$WP\ (g\ m^{-2}\ mm^{-1}) = WUE*Wc$$

where:

WUE = Water Use Efficiency

Wc = Water capture efficiency.

The WUE was calculated as the quotient between the sum of the yields or total DM of summer and winter crops and, the sum of the ETc of crops.

The Wc resulted from the quotient between the sum of the ETc of the crops and the rainfall from June 1, 2014 to April 20, 2016.

Radiation productivity (RP) was calculated as:

$$RP\ (g\ m^{-2}\ MJ^{-1}) = RUE*Rc$$

where:
RUE = radiation use efficiency
Rc = radiation capture efficiency.

The RUE was calculated as the quotient between the sum of the yields or total DM of the crops in the different sequences and the PARi. The Rc was the quotient between crops PARi and the photosynthetically active radiation, incident from June 1, 2014 to April 20, 2016.

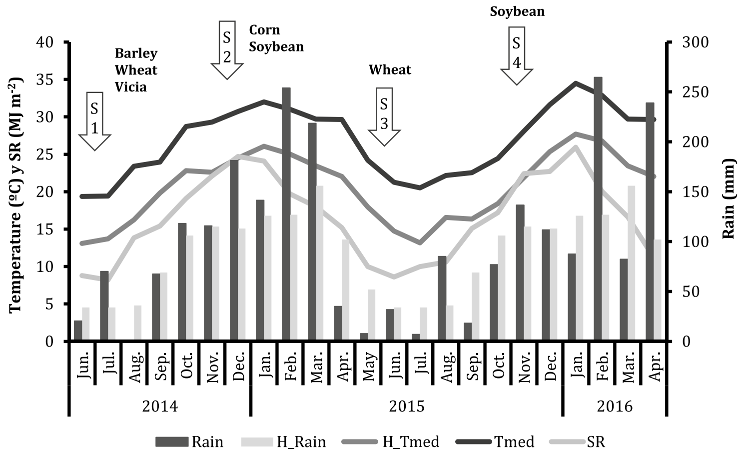
The data were evaluated by means of ANOVA for repeated measurements by sequence culture and following the structure of plots and treatments defined in the design.

When the differences between treatments for one variable were significant, the means were compared according to the LSD test (α= 0.05). InfoStat Professional version software was used (11).

Results and discussion

Meteorological conditions

During the 2014 campaign, photothermal conditions were sub optimal for the flowering period of wheat and barley crops (September 21-October 12) with temperatures higher than the historical record (figure 1). Plant growing period occurred with scarce offer of rains. The period of stem elongation was favored by the precipitations of September, followed by filling of grains with scarce precipitations and high temperatures (figure 1).



Arrow indicates date of sowing [S1: sowing winter crops –wheat, barley and vicia (cover crop)-; S2: sowing summer crops -soybean and corn-; S3: sowing winter crop –wheat-; S4: sowing summer crop–soybean-].

La flecha indica las fechas de siembra [S1: siembra cultivos de invierno -trigo, cebada y vicia (cultivo de cobertura)-; S2: siembra cultivos de verano -soja y maíz-; S3: siembra cultivo invierno –trigo-; S4: siembra cultivo verano –soja-].

Figure 1. Average temperatures (Tmed) and Historical (H_Tmed), solar radiation (SR) and monthly rains from June 2014 to April 2016 (Rain), Historical rains (H_Rain).

Figura 1. Temperaturas medias (Tmed) e históricas (H_Tmed), radiación solar (SR) y lluvias mensuales desde junio 2014 a abril 2016 (Rain) e históricas (H_Rain).

For summer crops, rainfall totaled 830 mm in the cycle (December-April), 33 % above the historical average. It should be noted that February excess rainfall had no negative effects on crops.

The 2015 wheat campaign had average temperatures around 19 °C, recommended to ensure optimum grain filling (figure 1, page 65). In the initial stages of the crop, rainfall was very scarce.

However, the period of stem elongation (September) was favored by timely and above normal rainfall during August (figure 1, page 65). For soybean cultivation, of soybean the rains in November, February and April (2015-2016) were above normal, with excesses in the month of April affecting quality grain and harvest.

Total Dry Matter and carbon contribution to the soil

The highest total DM productions were achieved with the sequences b/fc-w/s and w/fc-w/s (table 1). On average and, with respect to the lower production sequence (v/s-w/s), 4971 g m^{-2} were obtained, 93 % superior to the sequence v/s-w/s.

The rotations with 75 % of grasses and high fertilization achieved the highest productions of dry matter and contributions of crop residues C (without roots) to the soil (table 1). It is known that nitrogen fertilization, mainly in grasses, increases biomass production of crops, causing a greater accumulation of C in plant tissues and a high return to the soil (13, 14).

Table 1. Total Dry Matter (Total DM) and contribution of harvest residue carbon -without roots- (Contribution of C) in different sequences of 2 years duration crops (v= vicia, w= wheat, b= barley, s= soybean, c= corn without fertilization, fc= fertilized corn).

Tabla 1. Materia seca total (Total DM) y aporte de carbono del rastrojo -sin raíces- (Aporte de C) en diferentes secuencias de cultivos de 2 años de duración (v= vicia, t= trigo, c= cebada, s= soja, m= maíz sin fertilización, mf= maíz fertilizado).

Crop sequence	Total DM (g m^{-2})	Contribution of C (T ha^{-1}year^{-1})
v/s-w/s (25)	2396 a	2.7 a
v/c-w/s (50)	2933 b	3.5 ab
w/s-w/s (50)	3139 bc	3.8 b
b/s-w/s (50)	3362 bc	4.1 b
w/c-w/s (75)	3727 cd	4.1 bc
v/fc-w/s (50)	4218 de	5.1 d
b/c-w/s (75)	4429 ef	5.4 d
w/fc-w/s (75)	4871 fg	5.7 d
b/fc-w/s (75)	5071 g	5.9 d

The percentage of grasses in the sequence is expressed in parentheses. Different letters, within the same column, indicate differences according to LSD test ($p \leq 0.05$).

El porcentaje de gramíneas en la secuencia está expresado entre paréntesis. Letras diferentes, dentro de la misma columna, indican diferencias según el test de LSD ($p \leq 0,05$).

The contributions of C to the soil were lower in the sequence v/s-w/s and v/c-w/s. However, the proper fertilization of corn in the same sequence (v/fc-w/s) allowed a 46 % increase in this variable, marking the positive and significant effect to the addition of higher doses of nitrogen. The contribution values of C ha^{-1} $year^{-1}$ of crop residues in the double crop wheat/soybean in this study, are slightly lower than those reported by Álvarez (2005) for soils of the South-eastern Pampa.

Productivity, use efficiency and capture of water

The sequences that included the highest percentage of grasses, increased the efficiency and productivity in the use of water for grain production and total DM (table 2). Increases of the order of 102, 103, 125 and 125 % were determined in WUE and WP for grain and DM in the sequence b/fc-w/s respect v/s-w/s and 64, 57, 80 and 63 % compared with the w/s-w/s, the most widespread in the region.

In double cropping w/s, the WP to produce grains (0.50 g m^{-2} mm^{-1}) was within the range reported by Caviglia *et al*. (2013) for the southeast of Buenos Aires (0.47 a 0.75 g m^{-2} mm^{-1}). However, it was lower than those of Paraná (Entre Ríos) which were in 0.84 g m^{-2} mm^{-1}. On the other hand, the WP to produce DM (1.27 g m^{-2} mm^{-1}) was lower than what was reported for two different campaigns (2.22 y 1.83 g m^{-2} mm^{-1}) in Balcarce (Buenos Aires) (5).

Table 2. Efficiency Use Water in grain and Dry Matter (WUE_g and WUE_DM), Productivity of Water in grain and in Dry Matter (WP_g and WP_DM) and efficiency of Water capture (Wc) in different sequences of crops of 2 years duration (v= vicia, w= wheat, b= barley, s= soybean, c= corn without fertilization, fc= fertilized corn).

Tabla 2. Eficiencia uso agua en grano y en materia seca (WUE_g and WUE_DM), productividad del agua en grano y en materia seca (WP_g and WP_DM) y eficiencia de captura del agua (ECA) en diferentes secuencias de cultivos de 2 años de duración (v= vicia, t= trigo, c= cebada, s= soja, m= maíz sin fertilización, mf= maíz fertilizado).

Crop sequence	WUE_g (g m^{-2} mm^{-1})	WUE_DM (g m^{-2} mm^{-1})	WP_g (g m^{-2} mm^{-1})	WP_DM (g m^{-2} mm^{-1})	Wc
v/s-w/s (25)	0.65 a	1.53 a	0.40 a	0.92 a	0.60 a
v/c-w/s (50)	0.78 ab	1.93 ab	0.47 ab	1.23 b	0.62 ab
w/s-w/s (50)	0.80 abc	1.97 ab	0.50 ab	1.27 b	0.62 abc
b/s-w/s (50)	0.84 abc	2.17 bc	0.53 b	1.30 bc	0.63 abc
v/fc-w/s (50)	1.05 abc	2.60 cd	0.67 c	1.53 cd	0.63 abc
w/c-w/s (75)	1.10 abc	2.63 cd	0.67 c	1.70 de	0.61 abc
b/c-w/s (75)	1.14 abc	2.87 d	0.70 c	1.83 ef	0.61 abc
w/fc-w/s (75)	1.20 bc	2.97 d	0.83 d	1.97 f	0.69 bc
b/fc-w/s (75)	1.31 c	3.10 d	0.90 d	2.07 f	0.69 c

The percentage of grasses in the sequence is expressed in parentheses. Different letters, within the same column, indicate differences according to LSD test ($p \leq 0.05$).

El porcentaje de gramíneas en la secuencia está expresado entre paréntesis. Letras diferentes, dentro de la misma columna, indican diferencias según el test de LSD ($p \leq 0,05$).

The values obtained from WUE_MS and the WUE_g in the sequence w/s-w/s (1.97 and 0.80 g m^{-2} mm^{-1}, respectively) were lower than those reported in Balcarce (Buenos Aires) with WUE_DM of 3.12 to 3.41 g m^{-2} $mm^{-1}$and WUE_g between 0.88 and 1.02 g m^{-2} mm^{-1}. These differences were probably given by the lower vapor pressure deficit (VPD) of the environment explored by the crops in Balcarce.

Regarding the WUE_G, it was similar to that reported by Daniels & Scott (1991), with 0.79 g m^{-2} mm^{-1}, value obtained as a general average of several sources of variation including year, irrigation and stubble management.

With respect to Wc, significant differences were achieved between the sequence v/s-w/s y v/c-w/s and b/fc-w/s, with 13.1 % in favor of the sequence with the highest % of grasses. This coincides with what was reported by Ojeda *et al.* (2018) for forage crop sequences, where the highest seasonal WP was obtained with the highest proportion of maize in the sequences, being corn a C4 species with high-WUE (26).

The values reached in the different sequences (between 0.60 and 0.69) were much lower than what was reported for the Paraná area (0.99) and, closer to the data from southeastern Buenos Aires (0.54 and 0.70) (2, 4).

The lower values of Wc and WP_g could be associated to the abundant rains registered in February 2015 and 2016 (figure 1, page 65); where rainfall exceeded the water needs of corn and soybean crops. This situation has been evaluated, explaining that 66 % of the variability of the WP_G may be due to water excess during the campaign (5).

Productivity, use efficiency and capture of radiation

Productivity and efficiency of radiation use for the production of total DM and grain, showed significant differences in favor of rotations with higher percentage of grasses[v/s-w/s (25), w/s-w/s (50), c/s-w/s (50) y v/c-w/s (50) vs. w/c-w/s (75), v/fc-w/s (50), b/c-w/s (75), w/fc-w/s (75) y b/fc-w/s (75)] (table 3, page 69).

Among the sequences with the extreme values of RUE RP and Rc (v/s-w/s vs. b/fc-w/s), increases of the order of 78.3, 80.7, 141, 142 y 34.5 % were determined for RUE_g, RUE_DM, RP_g, RP_DM and Rc, respectively, in favor of the sequence with the highest % of grasses. When comparing the most used sequence in the region (w/s-w/s) with b/cf-w/s, the achieved values were 69.8, 61.1, 105, 98.0 and 21.9 % for RUE_g, RUE_DM, RP_g, RP_DM and Rc, respectively.

In the sequence w/s-w/s, the RP_g and RUE_g was 0.20 and 0.63 g m^{-2} $MJ^{-1}$and the RP_DM and RUE_DM 0.50 and 1.57 g m^{-2} MJ^{-1} respectively; being RP_g similar to what was reported for Paraná (0.21 g m^{-2} MJ^{-1}) by Caviglia *et al*. (2004), and lower than those measured in Balcarce (0.34 g m^{-2} MJ^{-1}) as well as RUE_g and RUE_DM (0.71 and 2.07 g m^{-2} MJ^{-1} respectively) (5).

The similar response in Wc and Rc, added to the fact that water is a cumulative resource and radiation is not, reinforces the concept that increasing productivity requires radiation capture. Water can be stored in the soil, thus attenuating the imbalances between the availability of the resource and the demand. Radiation capture, however, depends on the size and structure of the canopy at a given moment, so there are no compensatory mechanisms for the recovery of radiation not intercepted by the crop.

Table 3. Radiation use efficiency in grain and dry matter (RUE_G and RUE_DM), radiation productivity in grain and dry matter (RP_G and RP_MS) and radiation capture efficiency (Rc) in different sequences of crops of 2 years of duration (v= vicia, w= wheat, b= barley, s= soybean, c= corn without fertilization, cf= corn fertilized).

Tabla 3. Eficiencia uso radiación en grano y en materia seca (RUE_G and RUE_DM), productividad de la radiación en grano y en materia seca (RP_G and RP_MS) y eficiencia de captura de la radiación (Rc) en diferentes secuencias de cultivos de 2 años de duración (v= vicia, t= trigo, c= cebada, s= soja, m= maíz sin fertilización, mf= maíz fertilizado).

Crop sequence	RUE_G ($g\ m^{-2}\ MJ^{-1}$)	RUE_DM ($g\ m^{-2}\ MJ^{-1}$)	RP_G ($g\ m^{-2}\ MJ^{-1}$)	RP_DM ($g\ m^{-2}\ MJ^{-1}$)	Rc
v/s-w/s (25)	0.60 a	1.40 a	0.17 a	0.41 a	0.29 a
w/s-w/s (50)	0.63 a	1.57 ab	0.20 a	0.50 b	0.32 ab
b/s-w/s (50)	0.70 ab	1.60 ab	0.23 a	0.53 b	0.33 abc
v/c-w/s (50)	0.77 bc	2.07 c	0.26 a	0.70 b	0.34 bcd
w/c-w/s (75)	0.90 bc	2.17 c	0.31 b	0.76 c	0.35 bcde
v/cf-w/s (50)	0.93 bc	2.27 c	0.32 b	0.79 cd	0.35 bcde
b/c-w/s (75)	0.97 c	2.37 c	0.35 b	0.87 d	0.37 cde
w/cf-w/s (75)	0.97 c	2.50 c	0.36 c	0.92 e	0.37 de
b/cf-w/s (75)	1.07 c	2.53 c	0.41 c	0.99 e	0.39 e

The percentage of grasses in the sequence is expressed in parentheses. Different letters, within the same column, indicate differences according to the LSD test ($p \leq 0.05$).

El porcentaje de gramíneas en la secuencia está expresado entre paréntesis. Letras diferentes, dentro de la misma columna, indican diferencias según el test de LSD ($p \leq 0,05$).

Conclusion

The sequences of crops with the same rate of intensification but with a greater percentage of grasses, increased the efficiency and productivity in the use of water and radiation for the production of grain and total DM, achieving a greater contribution of C from the crop residues to the soil. The differential response for water and radiation offer ideas for the development of strategies based on the improvement of radiation uptake to raise annual water productivity, considering different crop sequences and their strategic fertilization.

References

1. Álvarez, R. 2005. Carbon stocks in pampean soils: a simple regression model for estimation of carbon storage under non degraded scenarios. Comm. Soil Sci. Plant Anal. 36: 1583-1589.
2. Caviglia, O. P.; Sadras, V. O.; Andrade, F. H. 2004. Intensification of agriculture in the south-eastern pampas. I. Capture and efficiency in the use of water and radiation in double cropped wheat-soybean. Field Crops Res. 87: 117-129.

3. Caviglia O. P.; Van Opstal, N. V.; Gregorutti, V. C.; Melchiori, R. J. M.; Blanzaco, E. 2008. El invierno: estación clave para la intensificación sustentable de la agricultura. En: INTA Paraná (eds.). Agricultura Sustentable, actualización técnica. EEA Paraná. Serie Extensión Nº 51. 7-13.
4. Caviglia, O. P.; Andrade, F. H. 2010. Sustainable intensification of agriculture in the Argentinean Pampas: capture and use efficiency of environmental resources. Am. J. Plant Sci. Biotechnol. 3: 1-8.
5. Caviglia, O. P.; Sadras, V. O.; Andrade, F. H. 2013. Modelling long-term of cropping intensification reveals increased water and radiation productivity in the South-eastern Pampas. Field Crops Res. 149: 300-311.
6. Conti, M. E. 2000. Materia orgánica del suelo. En: Conti, M. E. (ed.). Principios de edafología, con énfasis en suelos argentinos. FAUBA. Buenos Aires. Argentina. 67-86.
7. Critchley, W.; Siegert, K.; Chapman, C.; Finkel, M. 1991. Water harvesting. A manual for the design and construction of water harvesting schemes for plant production. Ed. FAO. Roma. Italia. 133 p.
8. Daniels, M. B.; Scott, H. D. 1991. Water use efficiency of double-cropped wheat and soybean. Agron. J. 83: 564-570.
9. Darder, M. L.; Sasal, M. C.; Andriulo, A. E.; Wilson, M. G.; Chagas, C. I. 2010. Coeficientes de enriquecimientos de fósforo, nitrógeno y carbono de sedimentos erosionados en diferentes secuencias de cultivos bajo siembra directa. Actas del XXII Congreso Argentino de la Ciencia del Suelo. Rosario, Sante Fe. Argentina.
10. Díaz Zorita, M.; García, F.; Melgar, R. 2002. Fertilización en soja y trigo-soja: respuesta de la fertilización en la región pampeana. Boletín Proyecto Fertilizar. EEA Pergamino. INTA. 44 p.
11. Di Rienzo, J. A.; Casanoves, F.; Balzarini, M. G.; González, L.; Tablada, M.; Robledo, C. W. 2014. InfoStat versión 2014. Grupo InfoStat. FCA. Universidad Nacional de Córdoba. Argentina.
12. Echeverría, H.; Barbieri, P.; Sainz Rosas, H.; Covacevich, F. 2005. Fertilización nitrogenada y métodos de diagnóstico de requerimientos de nitrógeno en trigo. Informaciones Agronómicas. 26: 8-15.
13. Eiza, M. J. 2005. Dinámica de la materia orgánica particulada bajo distintas rotaciones y sistemas de labranza. Tesis de Magister Scientiae en Producción Vegetal. FCA. UNMdP. Balcarce, Buenos Aires. Argentina. 69 p.
14. Fabrizzi, K. P.; García, F. O.; Costa, J. L.; Picone, L. I. 2005. Soil water dynamics, physical properties and corn and wheat responses to minimum and no-tillage systems in the southern Pampas of Argentina. Soil Till. Res. 81: 57-69.
15. Gallo, W. P.; Daughtry, C. S. T. 1986. Techniques for measuring intercepted and absorbed photosynthetically active radiation in crop canopies. Agron. J. 78: 752-756.
16. Haynes, R. J.; Naidu, R. 1998. Influence of lime, fertilizer and manure applications on soil organic matter content and soil physical condition. Nut. Cyc. Agroecosys. 51: 123-137.
17. Lal, R. 2004. Soil Carbon sequestration impacts on global climate change and food security. Science. 5677 (304): 1623-1627.
18. Maddoni, G.; Villariño, P.; Garça De Salamone, I. 2003. Dinámica de los nutrientes en el sistema suelo planta. En: Satorre E. H.; Benech Arnold, R. L.; Slafer, G. A.; de la Fuente, E. B.; Miralles, D. J.; Otegui, M. E.; Savin, R. (eds.). Producción de granos: bases funcionales para su manejo. FAUBA. Buenos Aires. Argentina. 443-474.
19. Novelli, L.; Melchiori, R.; Caviglia, O. 2009. Efecto de la fertilización nitrogenada sobre el contenido de C y N en diferentes tamaños de agregados. Revista Científica Agropecuaria. 13: 25-32.
20. Novelli, L. E.; Caviglia, O. P.; Melchiori, R. J. 2011. Impact of soybean cropping frequency on soil carbon storage in Mollisols and Vertisols. Geoderma. 167: 254-260.
21. Ojeda, J. J.; Pembleton, K. G.; Caviglia, O. P.; Islam, M. R.; Agnusgei, M. G.; García, S. C. 2018. Modelling forage yield and water productivity of continuous crop sequences in the Argentinian Pampas. European Journal of Agronomy. 92: 84-96. doi: 10.1016/j. eja.2017.10.004.

22. Reeves, D. W. 1997. The role of soil organic matter in maintaining soil quality in continuous cropping systems. Soil Till. Res. 43: 131-167.
23. Sasal, M. C.; Castiglioni, M. G.; Wilson, M. G. 2010. Effect of crop sequences on soil properties and runoff on natural rainfall erosion plots under no tillage. Soil Till. Res. 108: 24-29.
24. Studdert, G. A.; Echeverría, H. E. 2000. Crop rotations and nitrogen fertilization to manage soil organic carbon dynamics. Soil Sci. Soc. Am. J. 64: 1496-1503.
25. Valin, H.; Sands, R. D.; van der Mensbrugghe, D.; Nelson, G. C.; Ahammad, H.; Blanc, E.; Bodirsky, B.; Fujimori, S.; Hasegawa, T.; Havlik, P.; Heyhoe, E.; Kyle, P.; Mason-D'Croz, D.; Paltsev, S.; Rolinski, S.; Tabeau, A.; van Meijl, H.; von Lampe, M.; Willenbockel, D. 2014. The future of food demand: understanding differences in global economic models. Agricultural Economics. 45: 51-67. doi: 10.1111/agec.12089.
26. Zhang, Z.; Whish, J. P.; Bell, L. W.; Nan, Z. 2017. Forage production, quality and water use-efficiency of four warm-season annual crops at three sowing times in the Loess Plateau region of China. Eur. J. Agron. 84: 84-94. doi: 10.1016/j.eja.2016.12.008.

Acknowledgement

To the financial support of the Universidad Nacional del Litoral (UNL) through the project CAI+D PI 2011 (50120110100112LI) *"Efecto de diferentes secuencias de cultivos sobre la productividad de los recursos y la sustentabilidad del sistema agrícola"*.

Rev. FCA UNCUYO. 2020. 52(1): 72-86. ISSN (en línea) 1853-8665.

Effects of hydropriming on maize seeds (*Zea mays* L) on growth, development, and yield of crops

Efecto del hidroacondicionamiento de semillas de maíz (*Zea mays* L) en el crecimiento, desarrollo y rendimiento del cultivo

Francisco Marcelo Lara-Viveros [1], Nadia Landero-Valenzuela [1], Graciano Javier Aguado-Rodríguez [1], Edna Irene Bautista-Rodríguez [2], Eduardo Martínez-Acosta [2], Judith Callejas-Hernandez [2]

Originales: *Recepción:* 17/02/2017 - *Aceptación:* 17/10/2018

Abstract

Seed germination is a process that involves several phases, beginning with the uptake of water by dry seeds and ending with emergence. Based on current knowledge, several methodologies have been developed to manipulate this process in order to produce beneficial effects on crops. The hydropriming of maize seeds is one technique that has been used to lower the in-field germination time. The objective of the present study was to measure the effect of different hydropriming times on maize seeds and the subsequent growth, development, and yield of plants. The results demonstrated that hydroprimed seeds for 12 and 18 hours, germinated more rapidly in comparison with the control and 36-hour treatment. Yield was also affected as a function of the imbibition time. The generated data allowed for an optimal soaking time of 22.12 hours to be determined, resulting in an estimated yield of 16.6 t per hectare.

Keywords
hydropriming • Seed corn • germination of seed corn

1 CONACYT . Programa de Maestría en Ciencias en Desarrollo Agrotecnológico Sustentable. Carretera Tepatepec. San Juan Tepa. Km. 2. C.P. 46660. Francisco I. Madero Hidalgo México. gjaguadoro@conacyt.mx
2 Universidad Politécnica de Francisco I. Madero. Ingeniería en Agrotecnología. Carretera Tepatepec. San Juan Tepa. Km. 2. C. P. 46660. Francisco I. Madero Hidalgo México.

Resumen

La germinación de las semillas es un proceso que inicia con la toma de agua por la semilla seca y concluye con la emergencia. Con base en los conocimientos actuales se han desarrollado metodologías para manipular este proceso y obtener efectos benéficos en los cultivos. El hidroacondicionamiento de las semillas de maíz ha sido utilizado para obtener menores tiempos de germinación en campo. El objetivo del presente trabajo fue medir el efecto de diferentes tiempos de hidroacondicionamiento en agua de semillas de maíz sobre su crecimiento, desarrollo y rendimiento. Los resultados mostraron que las semillas hidroacondicionadas por 12 y 18 horas germinaron más rápidamente en comparación con el tratamiento testigo y 36 horas. El rendimiento fue afectado en función de los tiempos de imbibición. Los datos permitieron estimar un tiempo óptimo de imbibición que fue de 22,12 horas con lo que se obtiene un rendimiento estimado de 16,6 toneladas por hectárea.

Palabras clave

Hidroacondicionamiento • semillas de maíz • germinación de semillas de maíz

Introduction

Germination begins with the uptake of water by a dry seed (imbibition) and ends when a portion of the seed (the embryonic axis in dicotyledons or the radicle in monocotyledons or gymnosperms) emerges from the surrounding structure, known as the emergence phase (15). The uptake of water by seeds is triphasic and begins with the rapid initial absorption of water (phase I), followed by a plateau phase (phase II). Finally, a subsequent increase in water absorption (phase III) corresponds with the elongation of the embryonic axis and the aperture of the surrounding sheath (17). During these phases, important physiological changes occur assuring the survival of the seedling. These events have been widely studied by different authors. For example, during phase I, the structures damaged during the previous dehydration phase are repaired, and during phase II, protein synthesis resumes (4). The duration of each phase is variable and depends on seed-specific characteristics, including size, content of hydratable substrates, permeability of seed covering, and available O_2 and CO_2, in addition to other external conditions during imbibition, such as temperature, substrate composition, and moisture content (19).

For sowed crops, large volumes of water are commonly used to provide optimal germination conditions. However, during this period, water losses may also be significant due to a lack of vegetation cover, leading to greater water evaporation. Consequently, recently emerged seedlings experience a greater level of stress. In this context, Mullan and Reynolds (2010) list several genotypes that are capable of rapidly developing their leaf area, increasing the surface of shaded soil and decreasing water evaporation. This results in a more efficient use of water. In this sense, it is preferable that seeds initiate the imbibition-germination process as quickly as possible (7), as this favours a more efficient usage of water and minimizes the time of exposure to pathogens and other adverse environmental factors present in agricultural systems. In addition, this technique can improve other corn production systems such as corn silage, which represents an important alternative in several countries of America (24).

Based on current knowledge, various methods have been attempted for manipulating imbibition and germination to obtain beneficial effects on crops. One of the most studied techniques for achieving such benefits is hydropriming. In this method, seeds are placed in contact with water or an osmotic solution to initiate the imbibition process, but without arriving at the germination stage (12).

Several studies have shown this method to be effective in increasing the germination percentage and vigour of seedlings. A study spanning back several decades, as well as a more recent investigation (26) that tested four methods of hydroconditioning, found a resultant improvement in the germination and vigour of okra seedlings (*Abelmoschus esculentus* L. Moench). Previous research highlights that physiological changes may be initiated during imbibition, and these remain even after seeds are dehydrated (3). For this reason, osmoconditioned seeds rapidly re-initiate their metabolism, improving the percent and uniformity of germination (8).

Because water is a limiting factor in agricultural systems, the use of the aforementioned technologies may shorten the imbibition-germination time and improve the initial vigour of seedlings. Thus, the objective of the present study was to evaluate the effect of different periods of soaking in water on maize seeds and to examine their effects on growth, development, and yield of crops.

Materials and methods

First phase: Determination of the imbibition-germination times

For this phase, hybrid maize seeds (1503 by Aspros®) were placed in a humidity chamber with 90% relative humidity at a temperature of 27±3°C. The humidity chamber consisted of a plastic container. Paper saturated with sterile water was placed in the bottom of the container. Subsequently, the seeds were placed, the container was sealed and kept at constant temperature in complete darkness. Twenty seeds were placed in 50 humidity chambers (1000 seeds in total). Initial weight along with increases in weight every six hours, were recorded. The number of germinated seeds was recorded over the period of evaluation. A seed was considerd as germinated when it showed a radicle measuring at least, 2 mm. The recorded data were tested for normality and homoscedasticity. When assumptions of normality were not met, the data were transformed using the following formula:

$$Y=\sqrt{X} + 10$$

where:
Y= transformed data
X= data

The previous procedure facilitated the determination of the germination times and weight gain due to water absorption. Based on the generated data, three periods of hydroconditioning and control were then selected (0, 12, 18, and 36 hours). For each time period, 20 seeds were placed in humidity chambers for the corresponding treatment time. As mentioned, their initial weights, as well as weight gains assessed every 6 h, were recorded.

Temperature and RH were kept constant (27±3 °C and 90% HR). Once the imbibition time was finalised, the seeds were removed from the humidity chamber and replaced on absorbent paper at room temperature until they returned to their original weight prior to imbibition (approximately 120 hours). Afterwards, the seeds were placed in humidity chambers to determine the number of germinated seeds over time; measurement were recorded every six hours. The recorded data were subjected to a regression analysis in Microsoft Excel®.

Second phase: Field experiments

First, 200 g of maize seed were placed in a humidity chamber at 90% humidity and 25±2°C for each one of the previously established time periods (12, 18, and 36 hours and control). After the treatment periods, the seeds were removed from the humidity chamber and placed on absorbent paper at room temperature until they recovered their initial weight (approximately 120 hours). Afterwards, the seeds were sown in an open-field plot located in the experimental field of the Polytechnic University of Francisco I. Madero on Hidalgo estate México (14Q 490716.79 m E y 2236223.96 m N). The study was carried during 2014-2015. The experimental desing was a completely randomised block. For each treatment, a surface area of 50 m^2 was sown with seeds, with a distance of 0.75 m between furrows and 0.13 m between plants. Each treatment was replicated four time. Drip irrigation system was used, and sowing was carried out at the same time for all treatments under consideration. Weed control was mechanical in all treatments and no fertilizer was applied.

The mean of temperature in the experimental field was 26.2 °C and the HR was 22%.

Variables analysed during the fieldwork

For each treatment (period of hydro-conditioning), the number of emerged seeds was counted six days after sowing (DAS). A plant was considered to have emerged if the coleoptile had a minimum height of 4 cm. The number of plants was also determined at the harvest time.

The variables plant height, foliar temperature, and soil temperature were also recorded. Plant and soil temperatures were measured with a Benetech® infrared thermometer at an approximate distance of 20 cm from the foliage or ground surface. This variables have been previously used for estimation of the water use status on plant (10) in addition to the percentage of ground area covered by foliage every 7-9 days.

The percentage of ground area covered by foliage was determined by digital imaging, according to the method proposed by Mullan and Barcelo-García (2012). The digital images were then processed with Adobe Photoshop CS5 Extended® software, adjusting the parameters of saturation and luminosity to constant values (+60 and -20, respectively) aiming to contrast and compare the colours corresponding to leaves and ground surface. With this method, the area of the image corresponding to the green colours of leaves was substituted by absolute white (R=255, G=255, and B=255) in an RGB colour system, and the area corresponding to soil and the related colour gamma was substituted by absolute black (R=0, G=0, and B=0) in the same colour system.

The last step involved determining the ratio of white to black pixels in the RGB colour system, using the measuring tools provided by the software. With this information, the percentage of soil cover with foliage was calculated according to the following formula:

%SCPAT=(AWP/255)X100;

where:

%SCPAT=percentage of soil covered with photosynthetically active tissue

AWP=average number of white pixels

Once a female maize flower appeared, the length and diameter of each cob was measured. During harvest, the average percentage humidity of the seeds for each treatment was determined, and the grains of each cob per treatment were separately weighed in order to calculate yield per hectare.

Data analysis

The generated data were submitted to analysis of variance, and a means comparison test was performed if the results were significant (Tukey, P=0.05). For the data on plant height and length, diameter of the cob, and percent of ground cover, the area under the curve was calculated following the polygon method. The analysis of the area under the curve is a method used to estimate the total growth through time and the result is a dimensionless value. This method was described by Liengme, 2002. Yield data were analysed by Regression analysis.

Results

Determination of the imbibition-germination times

Time of imbibition was correlated with seed weight. Before the seeds were placed in humidity chambers, average weight of 20 seeds was 8.3 g. Thirty six hours after the start of imbibition, average weight was 11.8 g (figure 1b, page 77). The greatest number of germinated seeds after reaching an average imbibed weight of 12.4 g, occurred after 70 hours in the humidity chamber. Seed weight had a correlation coefficient of 0.53 with germination percentage. However, time of imbibition was statistically more significant in explaining germination (data not shown). According to the regression model (figure 1a, page 77), 50% germination (G_{50}) was achieved after 54.8 hours of imbibition.

During the initial hours (0-20 hours), a rapid increase in weight was observed, corresponding to the first phase of imbibition, characterised by a rapid increase in water absorption. Afterwards, increases in weight were stable, followed by another increase in weight corresponding to the appearance of the radicle.

Germination of hydroprimed seeds

The seeds showed a distinct behaviour as a function of imbibition time. The most rapid germination time was found for the 36-hour imbibition treatment, resulting in 50% germination of seeds (G_{50}) after 16.7 hours in the humidity chamber. The 12-hour (T12) and 18-hour (T18) imbibition treatments took more time to germinate (G_{50}) at 33.4 and 30.27 hours, respectively. The control treatment (unimbibed seeds) had the highest germination time (G_{50}) at 48.2 hours. Seeds that were treated for 12 hours had an overall rate of germination of 94.1% after 48 hours in a humidity chamber (figure 2, page 78).

Field experiments

Emergence of seeds

Hydropriming time affected seed emergence emergence in the field. The emergence of seedlings at six DAS, increased in direct proportion with the imbibition time of seeds up to 18 hours, when the number of emerged seeds began to diminish. The 36-hours treatment had the lowest number of emerged seedlings (figure 3, page 78). A similar behaviour has been observed in

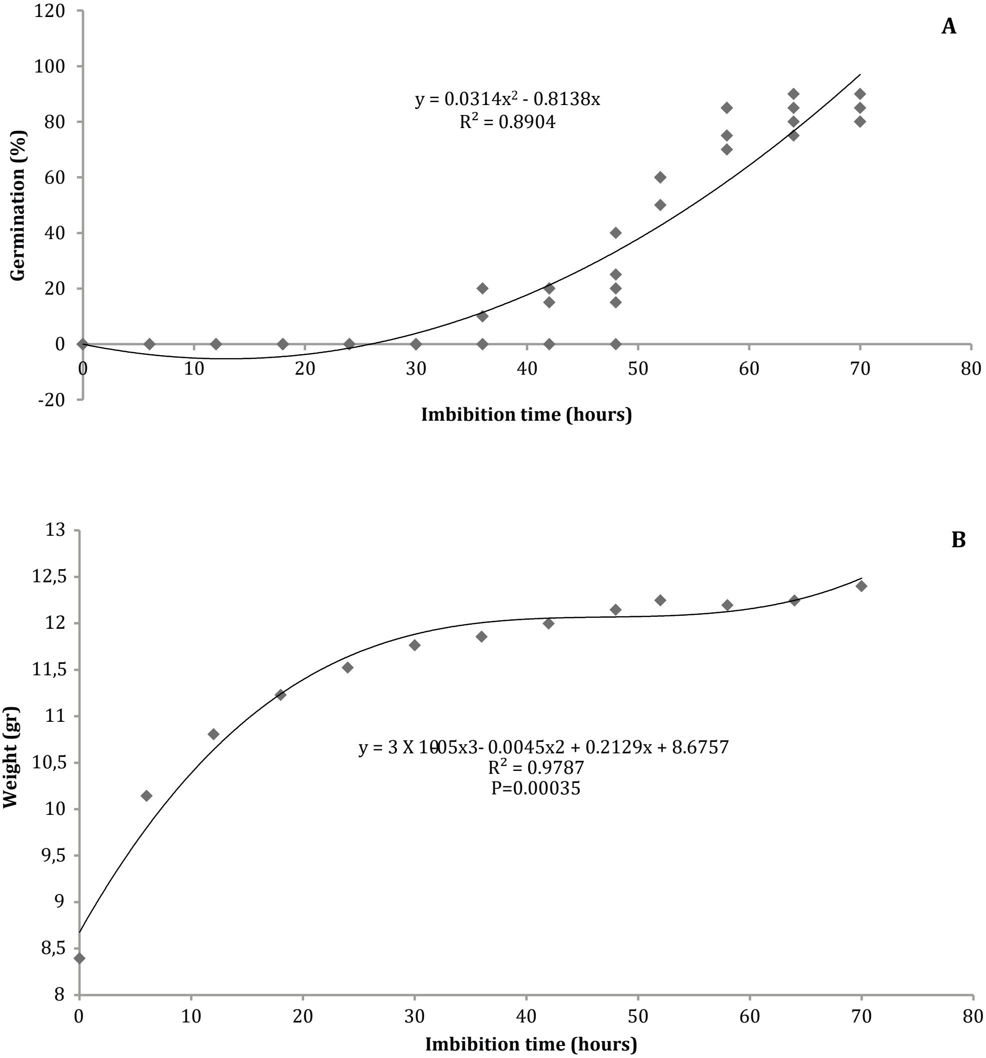
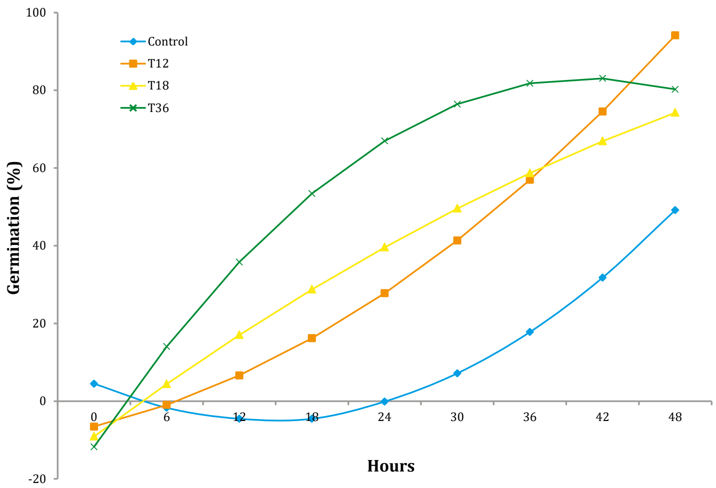


Figure 1. Effect of imbibition time on A) germination percentage of maize seed and B) seed weight.

Figura 1. Efecto del tiempo de imbibición sobre A) porcentaje de germinación de semillas de maíz y B) peso de semillas de maíz.

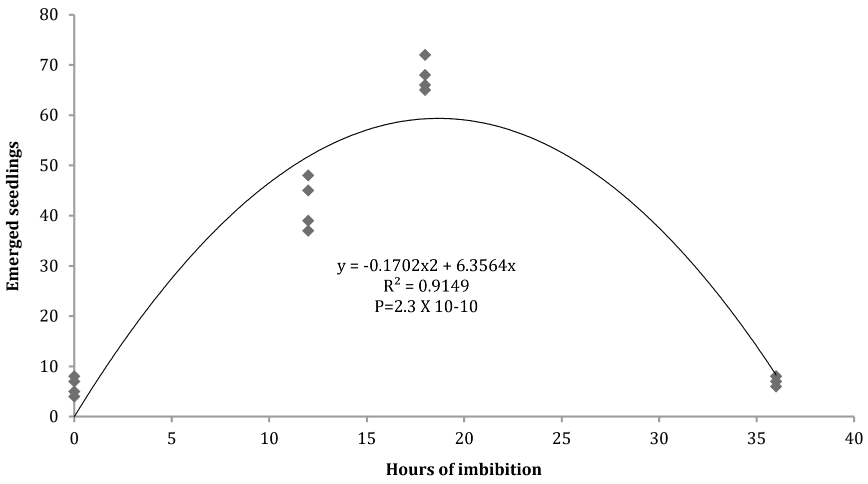


G_{50}= time (hours) to reach 50% germination; G_{48h}= percentage of germinated seeds at 48 hours. All equations are significant with alpha≤0.05.

G_{50}= tiempo (horas) al 50% de germinación; G_{48h} = porcentaje de germinación a las 48 horas. Todas las ecuaciones son significativas con un alpha≤0,05.

Figure 2. Effect of different hydropriming times on the time to germination of maize seeds.

Figura 2. Efecto de diferentes tiempos de hidroacondicionamiento sobre el tiempo de germinación en semillas de maíz.



Gmax = Maximum number of emerged seeds. / Gmax = Máximo número de semillas emergidas.

Figure 3. Average number of emerged seeds at different imbibition time, six DAS.

Figura 3. Promedio de número de semillas emergidas a diferentes tiempos de imbibición, seis DAS.

other species; for example, Marín Sanchez *et al.* (2007) found an increase in the percentage of abnormal onion seedlings after 72 hours of hydroconditioning, and the percentage of abnormalities further increased after 96 hours. In another study, Sadeguhi *et al.* (2011) found an average dry weight of 1.55 g for soy seeds hydroconditioned for 12 hours. However, when the time of osmoconditioning increased to 18 and 24 hours, the average dry weight decreased to 1.3 and 1.2 g, respectively.

Plant height

Plant height demonstrated significant differences at 37 and up to 43 DAS. During this period, seeds hydroconditioned for 36 hours showed an average height equal to the control treatment, while the 12- and 18-hour treatments had a greater height. For the rest of the sampled dates, plants did not show significant differences. Overall, and in terms of accumulated growth, seeds imbibed for 12 or 18 hours resulted in plants of greater height in comparison with the control treatment (table 1).

Plant and soil temperature

For the 0-, 12-, and 18-hour treatments, plant and soil temperatures significantly differed differed significantly at 16, 22, and 30 DAS. A lower difference in temperature after 16 days, was found with the 36-hour treatment, while the 12-hour treatment presented the greatest temperature difference. At 22 DAS, an increase in solar energy resulted in a higher soil temperature; causing that plants from imbibed seeds of all treatments showed a difference in relation to non-treated seed. At 30 DAS, only the 12-hour treatment demonstrated a statistically significant difference in comparison with the other treatments. For the rest of the sampling dates, no significant differences were found (table 2, page 80).

Percent of soil cover

Although soil cover percentage largely depends on leaf lamina growth, it is also determined by a number of other factors, such as luminosity, nutrient availability in water and soil, leaf insertion angle to the

Table 1. Plant height expressed in centimeters from seeds with different imbibition times.

Tabla 1. Alturas de plantas expresadas en centímetros provenientes de semillas con diferentes tiempos de imbibición.

	Days after sowing									
Imbibition time	**16**	**22**	**30**	**37**	**43**	**51**	**65**	**71**	**79**	**AUC**
0	19.5 a	32.1 a	52.1 a	68.1 ab	80.6 b	103.14 a	179.4 a	201 a	232.4 a	5781.2 b
12	18.2 a	37.5 a	51.7 a	76.5 a	89.5 ab	112.2 a	178.2 a	225.57 a	251 a	6155.9 a
18	18 a	33.7 a	49.9 a	73.5 a	94.3 a	109.2 a	194.3 a	224.7 a	243 a	6225.4 a
36	17.7 a	32.2 a	48.1 a	63.8 b	85.8 ab	101.5 a	188.2 a	192.2 a	235.4 a	5923.9 ab
P	0.59	0.04	0.3	0.005	0.059	0.004	0.11	0.06	0.22	0.04
MSD	**3.94**	**11.13**	**6.93**	**9.38**	**13.4**	**11.3**	**22.27**	**39.4**	**25.8**	**367**

†ABC = Area under the curve for plant height (Dimensionless number). MSD = Minimum significant difference. Different letters indicate statistically significant differences (Tukey, P=0.05).

†ABC = Área bajo la curva del peso de la planta. MSD = Diferencia mínima significativa. Letras diferentes indican diferencias significativas (Tukey, P=0,05).

Table 2. Soil and maize plant temperatures on different days after planting for a given time of imbibition.

Tabla 2. Temperaturas de suelo y planta en diferentes días después de la siembra para diferentes tiempos de imbibición.

	Days after planting											
TI (Hours)	**16**			**22**			**30**			**37**		
	TS	TP	D	TS	TP	D	TS	TP	D	TS	TP	D
0	33.7	29.7	4.7 bc	64.8	38.4	30.4 b	62.7	31.4	31.3 b	35.5	24.7	10.8 a
12	39.2	31.1	8.0 a	54.8	23.7	35.1 a	68.2	31	37.2 a	34.9	24.9	10.0 a
18	34.6	29.4	5.2 ab	54.3	20.3	34.0 a	64.3	29.2	34.1 b	35.3	25.4	9.9 a
36	35.3	33.2	2.1 c	59.7	24.5	35.2 a	64	31.2	31.8 b	35	24.7	10.3 a
DMS			3.6			3.8			3.0			4.1
P			0.04			0.03			0.02			0.90

	Days after planting								
TI (Hours)	**51**			**71**			**79**		
	TS	TP	D	TS	TP	D	TS	TP	D
0	26.8	22.2	4.6 a	29.4	22.5	6.9 a	38.01	31.5	6.5 a
12	32.6	31.1	1.5 a	29.3	22.9	6.4 a	33.7	30.3	3.4 a
18	26.5	23.5	3.0 a	26.1	22.1	3.9 a	33.1	28.4	4.7 a
36	29.9	23.9	6.0 a	29.8	23.5	6.3 a	28.3	28.6	0.7 a
DMS			4.6			4.7			7.5
P			0.08			0.35			0.56

†TI = Time of imbibition; TS=Soil temperature; TP = Plant temperature; D = TS-TP; DMS = Minimum significant temperature (Tukey, P = 0.05). The value of temperatures and difference are expressed in °C.

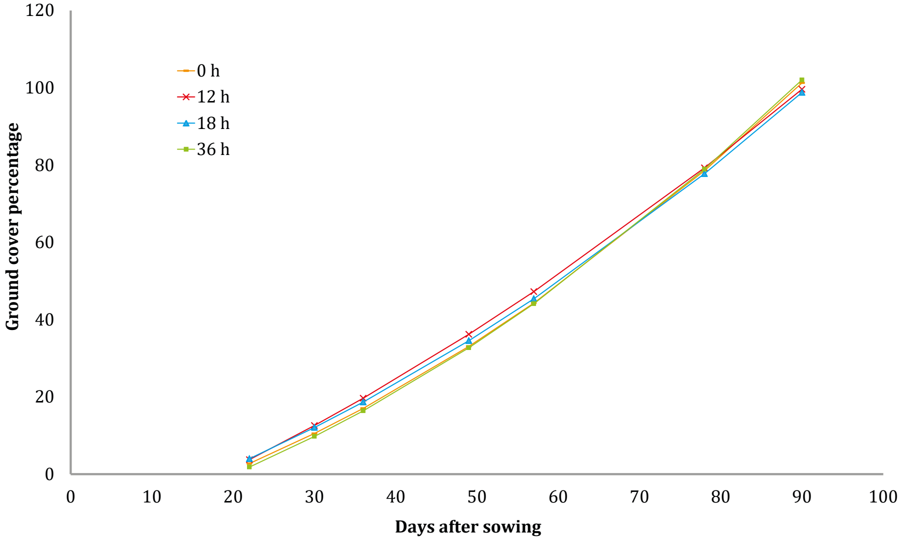
†TI = Tiempo de imbibición; TS = Temperatura del suelo; TP = Temperatura de la planta; D = TS-TP; DMS = Diferencia mínima significativa (Tukey, P = 0,05). El valor de las temperaturas y la diferencia se expresan en °C.

main stem, planting density and spatial distribution. In addition, any incidence of plague or disease may also have an effect. In the current study, all treatments showed a similar percentage of soil cover, indicating that the hydropriming treatments did not affect this variable (figure 4, page 81).

Although from 16 to 30 DAS the incidence of solar radiation caused a difference in temperature between soil and plants (table 2), all of the treatments maintained a similar foliage area. Even so, the 12- and 18-hour treatments maintained a greater difference in temperature, which implies that even though none of the plants were affected in terms of growth, only the 12- and 18-hour treatments were capable of transpiring under these conditions. This finding is possibly due to the plants' ability to maintain open stomata, allowing transpiration and gas exchange and thereby favouring the process of photosynthesis.

Yield

The imbibition treatments showed an effect on yield. The generated data were used as inputs for a mathematical model to estimate maximum yield, resulting in 16.6 t per hectare and corresponding with 22.12 hours of imbibition. Upon surpassing this timeframe, plant yield decreased to 15.6 t per hectare (figure 5, page 82).



For the 12-, 18-, and 36-hour treatments, regression models were adjusted to the following form: $y=ax^2+bx+c$. For the control treatment (0 hours), the model was adjusted to the following form: $y=ax+b$, where y=ground cover percentage and x=number of days after sowing. In all cases, the models were highly significant and had a coefficient of determination (R^2) of 0.93 or higher.

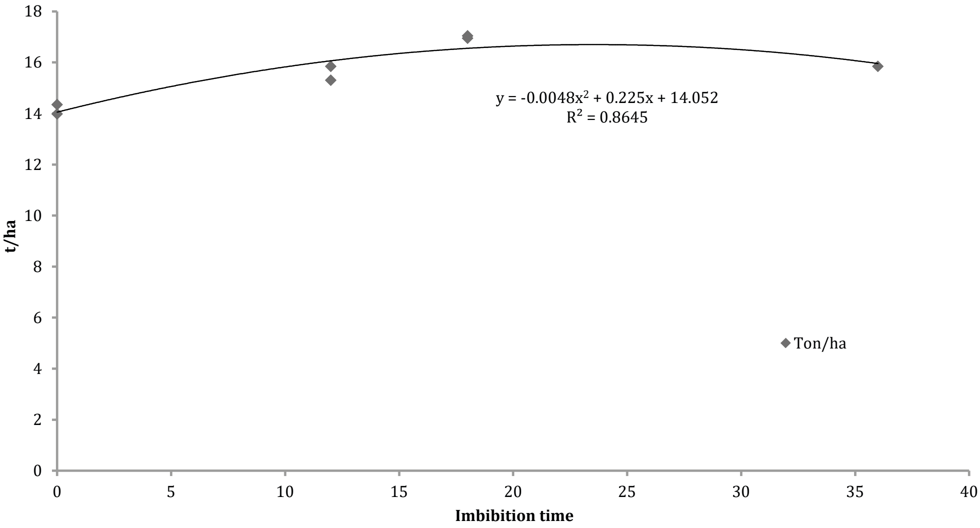
Para los tratamientos de 12, 18 y 36 horas, los modelos de regresión fueron ajustados a la forma $y=ax^2+bx+c$. Para el tratamiento control (0 horas) el modelo ajustado fue de la forma $y=ax+b$, donde y=Porcentaje de cobertura de suelo y x=Número de días después de la siembra. En todos los casos los modelos fueron altamente significativos y el coeficiente de determinación (R^2) fue de 0,93 o superior.

Figure 4. Effect of different imbibition times of maize seeds on ground cover percentage over time.

Figura 4. Efectos de diferentes tiempos de imbibición de semillas de maíz en el porcentaje de cobertura de suelo a través del tiempo.

Discussion

The germination value is greater than that reported, who found a G50 value of 46 hours. After 36 hours of imbibition, an 11% increase in seed germination was observed. In the present study, this time period was considered to be the maximum imbibition period before the seeds began to germinate. The germination time behaviour is typical of the majority of seeds (19). For hybrid corn, other authors have reported similar results. For example, by Yu-quin and Song-quan (2008) tested different imbibition times and evaluated the water content of seeds under different treatments. Phase 1 was observed between 0 and 12 hours, followed by a decrease in water content from 12 to 36 hours and, finally, a subsequent increase in water uptake. The hydropriming of seed resulted in earlier germination for maize seeds. However, this also affected the final number of germinated seeds. This behaviour has also been reported for other species. For example, in soybean



Max yield = maximum yield estimated by the following model: $y = ax^2+bx+c$, where y = output in kg per hectare and x = time of imbibition.

Max yiel = rendimiento máximo estimamdo por el modelo $y = ax^2+bx+c$, donde y = rendimiento en kg por hectárea y x = tiempo de imbibición.

Figure 5. Effect of imbibition time of maize seeds on yield per hectare.

Figura 5. Efecto del tiempo de imbibición de semillas de maíz sobre el rendimiento.

crops (25) 50% germination of seeds was found to increase at imbibition periods of 18 and 24 hours, even though the final germination percentage decreased in comparison with seeds hydroprimed for 12 hours. Hydropriming of seeds induces a series of biochemical changes (11) that are necessary for the germination process to occur, Heydecker and Coolbear (1977) stimulating, for example, the activation of enzymes or the metabolism of germination inhibitors (1). However, during the hydration process, seeds may also suffer temporary changes in the permeability of their cellular membranes, loosing solutes and metabolites of low molecular weight to the surrounding environment (19). These compounds are necessary for development during the first stages of germination (4), and their excessive loss could represent a disadvantage for the seed (5). This described phenomenon may explain what occured in this study, when seeds were hydroconditioned for 36 hours and germinated more rapidly even when the final number of germinated seeds resulted lower. This finding is possibly due to irreparable damage from excessive loss of nutritional compounds during imbibition phase II. In the field experiments, results demonstrated that hydroconditioning treatments are only beneficial, when practiced for an adequate period of time. When the optimal hydroconditioning time is surpassed, physiological damage may occur leading to a lower percentage of emergence. The results of plant height were similar to those reported by Sharma *et al.* (2014), who found an average height for okra plants of 27.1 cm when the seeds were imbibed for 12 hours. By contrast, with the control treatment, an average height of 14.8 cm was found, but when the imbibition time increased to 18 hours, the seedlings displayed a height of 22 cm. Plant surface temperature is related to transpiration rate; increased transpiration lowers leaf temperature by dissipation of sun heat (20). This has demonstrate that plant temperature is the result of several physiological processes, which, in addition to transpiration, involve stomatal conductance, hydric status of the plant, water use efficiency, leaf area index, and yield (23). In the present study, plants from hydroconditioned seeds showed a greater difference in plant and soil temperatures, compared with non-imbibed seeds. It may be inferred that imbibition causes a physiological effect that enables a greater capacity for transpiration in conditions of high irradiance, which may lead to an elevated rate of photosynthesis as stomata remain open for longer periods of time. Some studies have revealed that the hydroconditioning of seeds results in significant effects during plant development. In the case of wheat, it has been shown that hydroconditioning leads to a decrease in the concentration of sodium ions in plants growing in saline soils, favouring plant growth (22).

Further research has highlighted how hydroconditioned plants behave in comparison with control treatment plants as a function of environmental conditions. For example, Chen and Arora (2011) found that spinach seedlings from hydroconditioned seeds showed an increase in the expression of the *CAP85* gene when plants were submitted to stress by desiccation. However, no similar expression of the gene occured under optimal conditions. This gene encodes for LEA (late embryogenesis abundant) proteins that play an important role in water stress resistance (22). In the specific case of maize (6), an increase in the expression of LEA genes and their respective proteins was found for osmoconditioned seeds compared with normal seeds. Although the function of these proteins is not precisely known, evidence suggests that these proteins protect cellular structures from water stress or cold (27). In the present study, it is likely that 12- and 18-hour treatments would have affected the expression of the genes that encode LEA proteins, enabling these plants to exhibit a greater resistance to stress between 22 and 30 DAS, which corresponds to the period of high irradiance (table 2, page 80).

Dry matter and grain production depend on the ability of crops to capture resources, and radiation is an important factor for yield (14). However, a high solar radiation causes an increase in leaf and soil temperatures. When sun incidence was not sufficient to elevate soil temperature above 50°C, plants did not show significant differences in leaf temperature. This finding suggests that during the hydroconditioning of maize seeds, gene expression may be affected, leading to an improved stress response.

Other studies have demonstrated that seed imbibition may lead to an agronomic advantage. For example, Ghiyasi *et al.* (2008) found an increase in maize plant yield when seeds were hydroconditioned with water at a potential of -0.5 MPa for 24 hours. A similar behaviour has been observed for other crop species. For example, Arif *et al.* (2014) found that soy plants from seeds hydroconditioned with polyethylene glycol (PEG) for six hours at -1.1 MPa, had a greater yield, compared with plants from non-treated seeds. Plants from treated seeds also flowered and presented mature seeds before the control. In line with these findings, recent evidence has shown that hydroconditioning treatments enable moderate resistance to drought because the physiological mechanisms behind this tolerance are activated as a consequence of these treatments (9).

Intermembrane proteins called aquaporins, transport water from cell to cell and therefore, play an important role during germination. Another key process might involve guard cells and their changes in volume with the addition of water. Finally, phloem loading and unloading, as well as stomatal movements (17), may partly explain drought resistance as previously reported. In the current study, plants underwent high temperatures from 16 to 30 DAS (table 2, page 80).

Under these conditions, certain treatments (12 and 18 hours) showed a greater difference between soil and foliage temperature, indicating that these plants had greater resistance. This resistance also led to greater growth at 37 to 43 DAS (table 1, page 79), which may be an indicator of the capacity of the 12- and 18-hour treatments to continue development in conditions under which other treatments (control and 36 hours) were interrupted. As a consequence of these effects, seeds that are hydroconditioned for 12 and 18 hours develop into plants with greater stress tolerance, representing a significant advantage in comparison to non-treated plants.

Conclusions

Hydroconditioned maize seeds for 12 and 18 hours presents an agronomic advantage for adult plants. Several of these advantages include more uniform germination, earlier germination, greater growth during periods of thermal stress, and greater grain yields.

References

1. Ajouri, A.; Haben, A.; Becker, M. 2004. Seed priming enhances germination and seedling growth of barley under conditions of P and Zn deficiency. Journal of Plant Nutrition and Soil Science. 167: 630-636.
2. Arif, M.; Jan, M.; Mian, I.; Khan, S.; Hollington, P.; Harris, D. 2014. Evaluating the impact of osmopriming varying with polyethylene glycol concentrations and durations on soybean. International Journal of agricultural and biology. 16 (2): 359-364.
3. Asgedom, H.; Becker, M. 2001. Effects of seed priming with nutrient solutions on germination, seedling growth and weed competitiveness of cereals in Eritrea. In H. Asgedom, & M. Becker, Procedures Deutscher Tropentag. 282 p. Bonn, Alemania: Magrraf Publishers Press.
4. Bewley, J. D. 1997. Seed germination and dormancy. The Plant Cell. 9: 1055-1066.
5. Bewley, J. D.; Blck M. 2012. Physiology and biochemistry of seeds in relation to germination: Volume 2: Viability, dormancy, and environmental control. Springer Science & Business Media. 375 p.
6. Campos-Álvarez, F.; Cruz-García, F.; Torres-Espinosa, A.; Sánchez-Jiménez, M.; Colmenero-Flores, J. M.; Smith-Espinoza, C.; Vázquez-Ramos, J. M .2002. Expression of late embryogenesis abundant (lea) protein codifying genesduring osmopriming of maize and bean seeds. Agrociencia. 36: 461-470.
7. Caseiro, R.; Bennet, M, A.; Marcos-Filho, J. 2004. Comparison of three priming techniques for onion see lots differing in initial quality. Seed Science and Technology. 32: 365-375.

8. Chen, K.; Arora, R. 2011. Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in Spinach (*Spinacia oleracea*). Plant Science. 180: 212-220.
9. Chen, K.; Fessehaie, A.; Arora, R. 2012. Dehydrin metabolism is altered during seed osmopriming and subsequent germination under chilling and desiccation in *Spinacia oleracea* L. *cv.* Bloomsdale: possible role in stress tolerance. Plant Science. 183: 27-36.
10. Ghiyasi, M.; Pouryoursef, M.; Tajbakhsh, M.; Zalehzade, H.; Meshkat, M. 2008. Influence of differents osmopriming treatments on emergency and yield of maize (*Zea mays* L). Research Journal of biological sciences. 12(3): 1452-1455.
11. Giri, G. S.; Schillinger, W. F. 2003. Seed priming winter wheat for germination, emergence, and yield seed priming winter wheat for germination, emergence, and yield. Crop Science. 43(6): 2135-2141.
12. Heydecker, W.; Higgins, J.; Guliver L. 1973. Accelerated germination by osmotic seed treatment. Nature. 246: 42-44.
13. Heydecker, W.; Coolbear, P. 1977. Seed treatment for improved performance survey and attempted prognosis. Seed Science and Technology. 5: 353-42.
14. Imvinkelried, H. O.; Pietrobón, M.; Dellaferrera, I. M. 2020. Water and radiation productivity in different cropping sequences in the north center of Santa Fe. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 52(1): 61-71.
15 Iqbal, M.; Ashraf, M. 2007. Seed preconditioning modulates growth, ionic relations, and photosynthetic capacity in adult plants of hexaploid wheat under salt stress. Journal of Plant Nutrition. 30 (3): 381-396. doi:10.1080/01904160601171330.
16. Liengme, B. V. 2002. A Guide to Microsoft Excel 2002 for Scientists and Engineers. Academic Press.
17. Manz, B.; Müller, K.; Kucera, B.; Volke, F.; Leubner-Metzger, G. 2005. Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. Plant Physiology. 138: 1538-1551.
18. Marín-Sanchez, J.; Mejia-Contreras, J. A.; Hernández-Livera, A.; Carballo-Carballo, A.; Peña-Lomeli, A .2007. Acondicionamiento osmótico de semillas de cebolla (*Allium cepa* L.). [Osmotic conditioning of onion seeds (*Allium cepa* L.)] Agricultura Técnica en México. 33(1): 63-71.
19. Matilla, A. J. 2008. Desarrollo y germinación de semillas. [Development and seed germination]. In J. Azcón-Bieto, & M. Talón (Eds.). Fundamentos de Fisiología Vegetal. Madrid. España: McGraw-Hill.
20. Mullan, D. J.; Reynolds, M. P. 2010. Quantifying genetics effects of ground cover and soil water evaporation using digital imaging. Funtional plant biology. 37: 703-712.
21. Mullan, D.; Barcelo-García, M. 2012. Crop ground cover. Chapter 10. In A. Pask, J. Pietragalla, D. Mullan, & M. Reynolds, Physiological Breeding II: A Field Guide To Wheat Phenotyping. México. D. F.: CIMMYT. 132 p.
22. Neven, L. G.; Haskell, D. W.; Hofig, A.; Li, Q.-B.; Guy, C. L .1993. Characterization of a spinach gene responsive to low temperature and water stress . Plant Molecular Biology, 21: 291-305.
23. Pietragalla, J. 2012. Canopy temperature. In A. Pask, J. Pietragalla, D. Mullan, & M. Reynolds, Physiological Breeding II: A field guide to wheat phenotyping. México D. F.: CIMMYT. 10-14 p.
24. Rebora, C.; Ibarguren, L.; Barros, A.; Bertona, A.; Antonini, C.; Arenas, F.; Calderón, M.; Guerrero, D. 2018. Corn silage production in the northern oasis of Mendoza, Argentina. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 369-375.
25. Sadeghi, H.; Khazaei, F.; Yari, L.; Sheidaei, S .2011. Effec of seed osmopriming on seed germination behavior and vigor of soybean. ARPN Journal of Agricultural and Biological Science, 6(1): 39-43.
26. Sharma, A. D.; Rathore, S. V.; Srinivasan, K.; Tyagi, R. K .2014. Comparison of various seed priming methods for seed germination, seedling vigour and fruit yield in okra (*Abelmoschus esculentus* L. Moench). Scientia Horticulturae, 165: 75-81.

27. Swire-Clark, G. A., Marcotte, R. W. 1999. The wheat LEA protein Em functions as an osmoprotective molecule in *Sacharomyces cerevisiae*. Plant Molecular Biology, 39: 117-128.
28 . Yu-qin, M.; Song-quan, S .2008. Early morphological and physiological events occurring during germination of maize seeds. Agricultural Sciences in China. 7(8): 950-957.

Acknowledgements

The authors are grateful for the support provided by the Francisco I. Madero Polytechnic University (Universidad Politécnica de Francisco I. Madero) to perform the present study. Special thanks are extended to the personnel that assisted with the experimental fieldwork and that offered logistical support over the course of the study.

Assessing growth and antioxidant properties of greenhouse-grown lettuces (*Lactuca sativa* L.) under different irrigation and carbon fertilization management

Evaluación de la producción y propiedades antioxidantes de lechuga (*Lactuca sativa* L.) bajo invernadero en función del manejo del riego y la fertilización carbónica

Idoia Garmendia [1*], Marcelle M. Bettoni [2], Nieves Goicoechea [3]

Originales: *Recepción:* 05/06/2018 - *Aceptación:* 17/12/2018

Abstract

Previous studies have demonstrated that moderate water restrictions and enhanced CO_2 concentration can maintain or improve yield and accumulation of secondary compounds in lettuce under greenhouse conditions. Therefore, the aim of this study was to evaluate the combination of reduced soil moisture and carbon fertilization in shoot fresh weight (FW) and antioxidant capacity of two Batavia lettuce cultivars (Batabia Rubia Munguia; BRM and Maravilla de Verano; MV). Moderate water restriction treatment was equivalent to 2/3 of the field capacity and elevated CO_2 concentration (ECO_2) was fixed at ~700 µmol mol^{-1}. While CO_2 enrichment exerted a positive effect on shoot FW of MV, especially in combination with water restrictions, the yield of the cultivar BRM was not affected by CO_2 concentration, nor by irrigation regime. However, antioxidant capacity of BRM plants was increased under ECO_2 conditions. These results demonstrate that carbon fertilization and/or moderate water limitations can be strategically used to enhance nutritional value and growth of greenhouse lettuce.

Keywords
antioxidant activity • carbon dioxide • *Lactuca sativa* • yield • water restriction • fertilization

1 Universidad de Alicante. Facultad de Ciencias. Departamento Ciencias de la Tierra y del Medio Ambiente. Ctra. San Vicente del Raspeig, s/n. Apdo. Correos 99. E-03080 Alicante. Spain. * idoia.garmendia@ua.es
2 Universidade Federal do Paraná. Setor de Ciências Agrárias Departamento de Fitotecnia e Fitossanitarismo. Rua dos Funcionários. 1540. Juvevê. Curitiba. Brasil.
3 Universidad de Navarra. Facultades de Ciencias y de Farmacia y Nutrición. Departamento de Biología Ambiental. Grupo de Fisiología del Estrés en Plantas (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño). Irunlarrea 1. 31008. Pamplona. Spain.

Resumen

Estudios previos han demostrado que la moderada reducción del riego y el aumento de la concentración de CO_2 pueden mantener o incrementar la producción vegetal e inducir la acumulación de compuestos secundarios en lechuga bajo condiciones de invernadero. Por ello, el objetivo del presente estudio fue evaluar la combinación de la reducción del contenido de humedad del suelo y la fertilización carbónica sobre la materia fresca (MF) de la parte aérea y capacidad antioxidante de dos cultivares de lechuga Batavia (Batavia Rubia Munguia; BRM y Maravilla de Verano; MV). El tratamiento de déficit hídrico moderado fue equivalente a 2/3 de la capacidad de campo y la elevada concentración de CO_2 (ECO_2) se fijó en ~700 µmol mol^{-1}. Mientras que el enriquecimiento de CO_2 ejerció un efecto positivo sobre la MF de la parte área de MV, especialmente en combinación con la restricción del riego, la producción del cultivar BRM no se vio afectado ni por la concentración de CO_2 ni por el régimen de riego aplicado. Sin embargo, la capacidad antioxidante de las plantas BRM se incrementó bajo condiciones de ECO_2. Los resultados demuestran que la fertilización carbónica y/o una moderada limitación hídrica pueden estratégicamente ser utilizados para mejorar el valor nutricional y rendimiento de la lechuga bajo invernadero.

Palabras clave

actividad antioxidante • dióxido de carbono • *Lactuca sativa* • rendimiento • restricción del riego • fertilización

Introduction

Lettuce (*Lactuca sativa* L.) is considered a major food crop within the European Union. It is one of the most popular vegetables given its healthy properties, attributable to the presence of fiber, antioxidant compounds and minerals (12, 16, 18). Batavia (*Lactuca sativa* L. var. *capitata*) is extensively cultivated in greenhouses and highly commercialized in the North of Spain. Batavia Rubia Munguia (BRM) and Maravilla de Verano (MV) are two cultivars of Batavia. BRM develops a round, dense head and has yellow-green leaves, with very ruffled borders and a consistent, crisp texture. MV has rounded and very broad leaves with crunchy texture and red pigmentation it develops a firm head and can be grown all year long, especially in summer due to its resistance to high temperatures.

Cultivation of lettuce requires frequent irrigation for better growth and development because this plant lacks of a deep root system. However, soil moisture ranging from 50 to 75% of field capacity (FC) allow lettuce plants to produce similar biomass than those fully irrigated (20). Moreover, Baslam and Goicoechea (2012) observed that the nutritional quality of BRM and MV had even increased when plants had received 2/3 of FC. When grown with limited irrigation the levels of anthocyanins in the leaves of BRM were higher than in well-watered plants and so were the concentrations of total carotenoids and chlorophylls in leaves of MV.

Regarding the increase of atmospheric CO_2 as a consequence of global change and/or horticultural practices, it is well known that it affects plant growth and development. The enhanced CO_2 concentration increases the potential net photosynthesis in C3 plants, such as lettuce, and therefore can improve yield over short-term exposures (14). These responses may occur in natural ecosystems, but also can be used to increase the production, the nutritional quality and the accumulation of secondary compounds. Under greenhouse conditions, CO_2 fertilization facilitated rapid nursery production of olive trees during the winter season under Mediterranean conditions (5), induced the synthesis of secondary compounds with pharmacological interest in several plant species (17) and induced the accumulation of phenolic compounds in lettuces cv. BRM and MV when plants were not associated with mycorrhizal fungi (2). In fact, Becker and Kläring (2016) observed that CO_2 enrichment can result in high yields of red lettuce rich in phenolic compounds.

All these previous findings lead us to hypothesize that the increased levels of some secondary compounds in leaves of BRM and MV cultivated with restricted irrigation or carbon fertilization may enhance their antioxidant properties.

Objective

The objective was to study whether water deficit and/or carbon fertilization could improve plant growth together with leaf antioxidants in greenhouse grown lettuce.

Materials and methods

Plant material and experimental design

Seeds of BRM and MV were surface sterilized by 10% bleach for 10 min and then germinated in a mixture of light peat and sand (1:1, v:v). When seedlings had 2-3 fully developed leaves (three weeks after sowing) 24 plants of each cultivar (BRM and MV) were transplanted to 13 L pots filled with a mixture of vermiculite-siliceous sand-light peat (2.5:2.5:1, v:v:v).

The peat had a pH of 5.2-6.0, 70-150 mg L^{-1} of nitrogen, 80-180 mg L^{-1} of total P_2O_5 and 140-220 mg L^{-1} K_2O and it was previously sterilized at 100°C for 1 h on three consecutive days.

During transplant, the plants were transferred to four $[CO_2]$ controlled greenhouses located on the Universidad de Navarra campus (42.80 N, 1.66 W; Pamplona, Spain) (two ambient CO_2 (ACO_2) and two elevated CO_2 (ECO_2) greenhouses).

The design of the greenhouses was similar to that described by Morales *et al.* (2014). Twelve BRM plants and twelve MV plants were placed in ACO_2 greenhouses (six plants from every lettuce cultivar in each ACO_2 greenhouse). Twelve BRM plants and twelve MV plants were placed in ECO_2 greenhouses (six plants from every lettuce cultivar in each ECO_2 greenhouse). In the two ACO_2 greenhouses, no CO_2 was added and the $[CO_2]$ in the atmosphere was approximately 392 µmol mol^{-1}. In the other two greenhouses (ECO_2), $[CO_2]$ was fixed at ~700 µmol mol^{-1} by injecting pure CO_2 (purity up to 99.99%) from cylinder-gases (34 L of CO_2 per cylinder) through the two inlet fans during the light hours.

The CO_2 was provided by Air Liquide (Bilbao, Spain). The $[CO_2]$ was continuously monitored using a Guardian Plus gas monitor (Edinburgh Instruments Ltd, Livingston, UK).

Different irrigation regimes were also imposed at transplanting. Six BRM and six MV lettuces cultivated at ACO_2 concentration in the air (three plants from each cultivar and ACO_2 greenhouse) were always watered at FC and kept as well-watered (WW) treatments. Field capacity was calculated as the maximum water retained by a pot after complete drainage of water excess (900 mL per pot as an average value for all pots). Previous experience (2) demonstrated that WW lettuce plants performed better by dividing this amount of total water into three irrigations per week as follows: 300 mL of Hewitt's nutrient solution with some modifications (1) once a week and 300 mL of distilled water twice a week.

Plants subjected to 2/3 FC received 300 mL of modified Hewitt's solution once a week and 150 mL of distilled water twice a week. In all cases, nutrient solution was alternated with distilled water in order to avoid salt deposition. As results obtained by Baslam and Goicoechea (2012) showed that the leaf water content in WW lettuce plants was at least 90% after receiving the abovementioned nutrient solution and water supplies for 7 weeks, the irrigation regimes in the present study were kept through the whole experiment.

Total antioxidant capacity of leaves

Leaf extracts were obtained as described by Chapuis-Lardy *et al.* (2002) with some modifications. Samples (0.5 g of FW) were pulverized in liquid nitrogen, mixed with 20 mL of 80% methanol, and homogenized at room temperature for 1 min. After filtration, 0.5 mL of each sample was mixed with 10 mL of distilled water.

The total antioxidant capacity was evaluated by applying the free α, α-Diphenil-β-picrylhydrazyl radical scavenging activity ($DPPH^{\bullet}$ assay).

The free radical scavenging activity using the free radical $DPPH^{\bullet}$ (6) was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction in parafilm-sealed glass cuvettes (to avoid methanol evaporation) at 25°C (9).

The reaction was started by adding 20 μL of the corresponding sample to the cuvette containing 80 μM (methanol solution) (980 μL) of the free radical ($DPPH^{\bullet}$) (11).

The final volume of the assay was 1 mL. Reaction was followed with a spectrophotometer (Jasco V-630, Analytical Instruments, Easton, MD, USA). The trapping potential for DPPH radicals scavenging activity in the leaf extracts was calculated as the percentage of inhibition (*I* %) against blank:

$$I\ \% = (A\ \text{blank} - A\ \text{sample}) * 100 / A\ \text{blank}$$

where:

A blank = the absorbance of the control reaction (containing all reagents except the test compound)

A sample = the absorbance of the test compound.

Statistical analysis

Within each lettuce cultivar, data were subjected to a two-factor ANOVA (factorial 2 × 2) (IBM SPSS v. 24).

The variance was related to the main treatments (atmospheric CO_2 concentration, CO_2 and water regime, W) and to the interaction between both parameters (CO_2 × W). Means ± standard errors (SE) were calculated and, when the F ratio was significant ($p \leq 0.05$), a Duncan Multiple Range Test was applied. Tests results were always considered significant at $p \leq 0.05$.

Results

Although the shoot FW of the cultivar BRM tended to be higher when plants were cultivated under ECO_2 (table 1), the final size achieved by the lettuces was not significantly affected by either the concentration of CO_2 or the irrigation regime (table 1). In contrast, the antioxidant capacity measured in leaves of BRM was significantly enhanced when plants were exposed to ECO_2 (CO_2, $p \leq 0.01$). The interaction between CO_2 and water supply was not significant for either growth or antioxidant properties in this cultivar.

Contrary to BRM, growth of MV was clearly increased under ECO_2 (table 2, page 92). Moreover, such enhancement was reinforced when ECO_2 interacted with restricted irrigation ($CO_2 \times W$, $p \leq 0.05$), so that the highest shoot FW (67.33 g plant^{-1}) was achieved by plants cultivated under ECO_2 with a water supply of 2/3 FC. In this red-leaf cultivar, the antioxidant capacity measured in leaves was not affected by the irrigation regime, the concentration of CO_2 in the air or the interaction between both environmental factors (table 2, page 92).

Table 1 Shoot fresh weight (FW) (g plant^{-1}) and trapping potential for DPPH radicals scavenging activity (*I* %) in Batavia Rubia Munguia (BRM) cultivated at either ambient (~370 µmol mol^{-1}) (ACO_2) or elevated (ECO_2) (~700 µmol mol^{-1}) CO_2 concentration in the air, and grown with optimal (FC, field capacity) or restricted (2/3 FC) water supply.

Tabla 1 Materia fresca (FW) de la parte aérea (g planta^{-1}) y capacidad para eliminar radicales DPPH (*I* %) en Batavia Rubia Munguia (BRM) cultivada tanto en concentraciones de CO_2 ambiente (~370 µmol mol^{-1}) (ACO_2) como en elevadas (ECO_2) (~700 µmol mol^{-1}), y crecidas bajo riego óptimo (FC, capacidad de campo) o bajo restricción (2/3 FC).

Treatments		Shoot FW (g plant^{-1})	*I* %
ACO_2	FC	47.12 ± 3.95	68.12 ± 1.59
	2/3 FC	45.96 ± 4.96	74.49 ± 1.92
ECO_2	FC	50.48 ± 2.49	78.17 ± 2.66
	2/3 FC	56.92 ± 3.61	77.31 ± 1.55
Main effects			
CO_2	ACO_2	46.75 ± 2.93	71.02 ± 1.54 b
	ECO_2	53.41 ± 2.25	77.74 ± 1.47 a
Water supply	FC	48.95 ± 2.27	73.15 ± 2.12
	2/3 FC	51.44 ± 3.39	76.03 ± 1.23
CO_2		NS	**
Water supply (W)		NS	NS
$CO_2 \times W$		NS	NS

Values are means (n = 6) ± S.E. Within each column, data followed by the same letter indicate that values did not differ significantly ($p \leq 0.05$). ANOVA: NS, not significant; **, significant at $p \leq 0.01$.

Los valores corresponden a medias muestrales (n = 6) ± S.E. Dentro de cada columna, el valor acompañado por la misma letra indica que las medias no difieren significativamente ($p \leq 0,05$). ANOVA: NS, no significativo; **, significativo a $p \leq 0,01$.

Table 2 Shoot fresh weight (FW) (g plant^{-1}) and trapping potential for DPPH radicals scavenging activity (*I* %) in Maravilla de Verano (MV) cultivated at either ambient (~370 µmol mol^{-1}) (ACO_2) or elevated (ECO_2) (~700 µmol mol^{-1}) CO_2 concentration in the air, and grown with optimal (FC, field capacity) or restricted (2/3 FC) water supply.

Table 2 Materia fresca (FW) de la parte aérea (g planta^{-1}) y capacidad para eliminar radicales DPPH (*I* %) en Maravilla de Verano (MV) cultivada tanto en concentraciones de CO_2 ambiente (~370 µmol mol^{-1}) (ACO_2) como en elevadas (ECO_2) (~700 µmol mol^{-1}), y crecidas bajo riego óptimo (FC, capacidad de campo) o bajo restricción (2/3 FC).

Treatments		Shoot FW (g plant^{-1})	*I* %
ACO_2	FC	55.90 ± 3.66 bc	70.27 ± 4.19
	2/3 FC	47.28 ± 2.67 c	75.32 ± 3.74
ECO_2	FC	60.27 ± 2.34 ab	73.07 ± 3.78
	2/3 FC	67.33 ± 4.84 a	74.66 ± 2.23
Main effects			
CO_2	ACO_2	51.59 ± 2.52 b	72.79 ± 2.79
	ECO_2	64.00 ± 2.80 a	73.86 ± 2.11
Water supply	FC	58.08 ± 2.17	71.67 ± 2.72
	2/3 FC	57.51 ± 4.06	74.99 ± 2.08
CO_2		**	NS
Water supply (W)		NS	NS
CO_2 × W		*	NS

Values are means (n = 6) ± S.E. Within each column, data followed by the same letter indicate that values did not differ significantly ($p \leq 0.05$). ANOVA: NS, not significant; *, significant at $p \leq 0.05$; **, significant at $p \leq 0.01$.

Los valores corresponden a medias muestrales (n = 6) ± S.E. Dentro de cada columna, el valor acompañado por la misma letra indica que las medias no difieren significativamente ($p \leq 0,05$). ANOVA: NS, no significativo; *, significativo a $p \leq 0,05$; **, significativo a $p \leq 0,01$.

Discussion

The statistical study of the main effects of CO_2 and water supply demonstrated that moderate water restriction (2/3 FC) did not reduce yield in any of the two cultivars of lettuce (table 1, page 91 and table 2), despite the high sensitiveness of plant growth to water deficit.

Enhanced CO_2 concentration in the air increases the potential net photosynthesis in C3 plants (8), which results in improved plant growth. However, when the synthesis of carbohydrates in plants exposed to ECO_2 exceeds the capacity to produce new sinks, their photosynthetic rate declines as a consequence of a product feedback inhibition (19).

In this study, BRM and MV plants were harvested when their vegetative development had not still finished, which means that the youngest leaves could act as sink organs. This fact may explain why CO_2 enrichment exerted a positive effect on shoot growth, being this effect more evident in MV (table 2) than in BRM (table 1, page 91).

The antioxidant capacity measured in leaf extracts from the green-leaf BRM was similar to that found in the red-leaf MV (table 1, page 91 and table 2), which contrasts with the significantly higher DPPH scavenging activity found by Llorach *et al.* (2008) in red lettuces in comparison with that of green varieties. According to Baslam *et al.* (2012), leaves of MV have more anthocyanins than those of BRM when plants are fully irrigated and cultivated at ACO_2, but the amount of total phenolic compounds is higher in leaves of BRM than in leaves of MV. This suggests that the antioxidant capacity in the red-leaf MV may be mainly due to the anthocyanins (15) and the antioxidant properties of the green-leaf BRM may be related to total phenolics (10).

Baslam *et al.* (2012) also observed that the concentrations of anthocyanins significantly increased in leaves of BRM exposed to ECO_2, which could explain the enhancement of the antioxidant capacity observed in this cultivar fertilized with CO_2 (table 1, page 91). At ACO_2, moderate water restriction (2/3 FC) also induced the accumulation of anthocyanins in leaves of BRM (2), which may explain the higher trapping potential for DPPH radicals scavenging activity in BRM receiving irrigation equivalent to 2/3 FC (74.49%) than in plants grown with full irrigation (68.12 %) (table 1, page 91).

However, no additive effect between restricted irrigation (2/3 FC) and ECO_2 was observed for improving the antioxidant activity in neither of the two cultivars (table 1, page 91 and table 2, page 92). This lack of interaction between CO_2 and water restriction for increasing the antioxidant properties may be due to the preferential use of the photoassimilates to improve growth in detriment to the synthesis and accumulation of secondary compounds in plants cultivated with restricted water supply (2/3 FC) under ECO_2.

In BRM (table 1, page 91), while atmospheric CO_2 fertilization increased shoot FW by 7% in fully irrigated plants (from 47.12 to 50.48 g plant^{-1}), the increase in plants grown with water restriction (2/3 FC) was 24% (from 45.96 to 56.92 g plant^{-1}).

In MV (table 2, page 92), ECO_2 enhanced shoot FW by 8% in plants with full irrigation (from 55.90 to 60.27 g plant^{-1}) and by 42% (from 47.28 to 67.33 g plant^{-1}) in plants receiving 2/3 FC.

Conclusions

Moderate limitation of water supply may allow the obtention of greenhouse-grown lettuces without significant decreases in the final plant size. Carbon fertilization can enhance yield and/or the antioxidant properties of greenhouse-grown lettuces, but results are highly dependent on the variety or cultivar evaluated.

Only in the red-leaf cultivar of lettuce, MV, plant growth (but not the antioxidant activity) was improved by the simultaneous application of restricted irrigation and CO_2 enrichment.

References

1. Baslam, M.; Garmendia, I.; Goicoechea, N. 2011. Arbuscular mycorrhizal fungi (AMF) improved growth and nutritional quality of greenhouse-grown lettuce. Journal of Agricultural and Food Chemistry. 59: 5504-5515.
2. Baslam, M.; Goicoechea, N. 2012. Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. Mycorrhiza. 22: 347-359.
3. Baslam, M.; Garmendia, I.; Goicoechea, N. 2012. Elevated CO_2 may impair the beneficial effect of arbuscular mycorrhizal fungi (AMF) on the mineral and phytochemical quality of lettuce. Annals of Applied Biology. 161: 180-191.
4. Becker, C.; Kläring, H. P. 2016. CO_2 enrichment can produce high red lettuce yield while increasing most flavonoid glycoside and some caffeic acid derivate concentrations. Food Chemistry. 199: 736-745.

5. Biel, C.; de Herralde, F.; Savé, R.; Evans, R. Y. 2008. Effects of CO_2 atmospheric fertilization on greenhouse production of olive trees (*Olea europaea* L. 'Arbequina'). European Journal of Horticultural Science. 73: 227-230.
6. Brand-Williams, W.; Cuvelier, M. E.; Berset, C. 1995. Use of free radical method to evaluate antioxidant activity. Food Science and Technology. 28: 25-30.
7. Chapuis-Lardy, L.; Contour-Ansel, D.; Bernhard-Reversat, F. 2002. High performance liquid chromatography of water-soluble phenolics in leaf litter of three *Eucalyptus* hybrids (Congo). Plant Science. 163: 217-222.
8. Drake, B. G.; González-Meler, M. A.; Long, S. P. 1997. More efficient plants: a consequence of rising atmospheric CO_2? Annual Review of Plant Physiology. 48: 609-39.
9. Espín, J. C.; Soler-Rivas, C.; Wichers, H. J. 2000. Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2,2-diphenyl-1-picrylhydrazyl radical. Journal of Food Biochemistry. 24: 225-250.
10. Gangwar, M.; Gautam, M. K.; Sharma, A. K.; Tripathi, Y. B.; Goel, R. K.; Nath, G. 2014. Antioxidant capacity and radical scavenging effect of polyphenol rich *Mallotus philippenensis* fruit extract on human erythrocytes: an *in vitro* study. The Scientific World Journal Article ID 279451. 12 p.
11. Llorach, R.; Tomás-Barberán, F. A.; Ferreres, F. 2004. Lettuce and chicory by products as a source of antioxidant phenolic extracts. Journal of Agricultural and Food Chemistry. 52: 5109-5116.
12. Llorach, R.; Martínez-Sánchez, A.; Tomás-Barberán, F. A.; Gil, M. I.; Ferreres, F. 2008. Characterisation of polyphenols and antioxidant properties of five lettuce varieties and escarole. Food Chemistry. 108: 1028-1038.
13. Morales, F.; Pascual, I.; Sánchez-Díaz, M.; Aguirreolea, J.; Irigoyen, J. J.; Goicoechea, N.; Antolín, M. C.; Oyarzun, M.; Urdiain, A. 2014. Methodological advances: Using greenhouses to simulate climate change scenarios. Plant Science. 226: 30-40.
14. Oliveira, V. F.; Zaindan, L. B. P.; Braga, M. R.; Aidar, M. P. M.; Carvalho, M. A. M. 2010. Elevated CO_2 atmosphere promotes plants growth and inulin production in the cerrado species *Vernonia herbacea*. Functional Plant Biology. 37: 223-231.
15. Rice-Evans, C. A.; Miller, N. J.; Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science. 2: 152-159.
16. Romani, A.; Pinelli, P.; Galardi, C.; Sani, G.; Cimato, A.; Heimler, D. 2002. Polyphenols in greenhouse and open-air-grown lettuce. Food Chemistry. 79: 337-342.
17. Savé, R.; de Herralde, F.; Codina, C.; Sánchez, X.; Biel, C. 2007. Effects of atmospheric carbon dioxide fertilization on biomass and secondary metabolites of some plant species with pharmacological interest under greenhouse conditions. Afinidad. 64: 237-241.
18. Serafini, M.; Bugianesi, R.; Salucci, M.; Azzini, E.; Raguzzini, A.; Maiani, G. 2002. Effect of acute ingestion of fresh and stored lettuce (*Lactuca sativa*) on plasma total antioxidant capacity and antioxidant levels in human subjects. British Journal of Nutrition. 88: 615-623.
19. Thomas, R. B.; Strain, B. R. 1991. Root restriction as a factor in photosynthetic acclimation of cotton seedlings grown in elevated carbon dioxide. Plant Physiology. 96: 627-34.
20. Tsabedze, M. W.; Wahome, P. K. 2010. Influence of different irrigation regimes on production of lettuce (*Lactuca sativa* L.). American-Eurasian Journal of Agricultural and Environmental Sciences. 8: 233-238.

Acknowledgments

The authors wish to thank Amadeo Urdiáin for technical support.
This study has been supported by the Ministerio de Economía y Competitividad (MINECO, Spain) (AGL2011-30386-C02-02, BFU 2011-26989).
Marcelle M. Bettoni received a grant from' Los CAPES y Coordenação do Programa de Pós-graduação em Agronomia - Produção Vegetal' from the Brazilian Government.

Rev. FCA UNCUYO. 2020. 52(1): 95-105. ISSN (en línea) 1853-8665.

Effect of two sources of zinc on the physiological quality of seed and nutrition of rice (*Oriza sativa*) seedlings

Efecto de dos fuentes de zinc sobre la calidad fisiológica de semilla y nutrición de plántulas de arroz (*Oriza sativa*)

Flávia Mendes dos Santos Lourenço [1], Mariely de Abreu dos Santos [1],
Charline Zaratin Alves [1], Cid Naudi Silva Campos [1], Ana Carina da Silva Cândido [1],
Renato de Mello Prado [2], Gabriel Barbosa da Silva Júnior [3*]

Originales: *Recepción:* 11/04/2018 - *Aceptación:* 18/05/2019

Abstract

Rice seeds coating with microparticulate zinc oxide can maximize the absorption of this micronutrient by the seedling and favor seeds vigor when compared with zinc sulfate. This work aimed to evaluate the sources and doses of zinc in seed quality and in the nutrition of rice seedlings. The experiment consisted of a completely randomized design with four replications, five doses of zinc in the form of oxide (0; 20; 40, 80 and 160 g kg^{-1}), and a control treatment of zinc in the form of sulfate (40 g kg^{-1}). The following parameters were evaluated: germination, accelerated aging, seedlings emergence, emergence speed index, cold test, dry matter, and zinc concentration in the root and shoot of the seedlings. Seed coating with zinc, regardless of the source, improved seed physiological quality and seedling nutrition. Zinc sources did not affect germination; however, the oxide form induced better seed vigor, except for the highest dose (160 g kg-1). Increasing doses of zinc oxide augmented the concentration of this nutrient in rice seedlings dry matter. Zinc coating of rice seeds in the form of microparticulate, depending on the dose, can maximize seed vigor and increase the concentration of this micronutrient in the seedlings when compared with zinc sulfate, the traditional source.

Keywords
micronutrient • seed coating • microparticulate zinc oxide

1 Universidad Federal de Mato Grosso do Sul. Campus Chapadão do Sul. Carretera MS-306. Zona Rural. C. P. 79560-000. Chapadão do Sul. Estado de Mato Grosso do Sul. Brasil.
2 Universidade Estadual Paulista "Julio de Mesquita Filho". Facultad de Agricultura y Ciencias Veterinarias. Departamento de Suelos y Fertilizantes. Vía de acceso Prof. Paulo Donato Castellane. CP: 14884-900. Jaboticabal. Estado de São Paulo. Brasil.
3 Universidade Federal de Piauí. Departamento de Ciencia de las Plantas. Centro de Investigación de Ciencias Agrícolas. Campus Ministro Petrônio Portella. C. P. 64049-550. Teresina. Estado de Piauí. Brasil. * gabrielbarbosa@ufpi.edu.br

Resumen

El revestimiento de semillas de arroz con óxido de zinc microparticulado puede maximizar la absorción de este micronutriente por las plántulas y favorecer el vigor de las semillas en comparación con el sulfato de zinc. Este trabajo tuvo como objetivo evaluar las fuentes y dosis de zinc en la calidad de semillas y en la nutrición de plántulas de arroz. El experimento fue desarrollado en delineamiento completamente al azar con cuatro repeticiones, con un testigo sin aplicación de zinc, cuatro dosis de óxido de zinc (20, 40, 80 y 160 g kg^{-1} de Zn), y un tratamiento en la forma de sulfato de zinc, 40 g kg^{-1} de Zn. Se evaluaron los siguientes parámetros: germinación, velocidad de envejecimiento, emergencia de plántulas, índice de velocidad de emergencia, prueba fría de semillas, materia seca y acumulación de zinc en la raíz y parte aérea de las plántulas . La cobertura de semillas con zinc, independientemente de la fuente, mejoró la calidad fisiológica de las semillas y la nutrición de las plántulas de arroz. Las fuentes de zinc no alteraron la germinación; sin embargo, el óxido de zinc proporcionó un mejor vigor de las semillas, con la excepción de la mayor dosis, 160 g kg^{-1}. El aumento en la dosis de óxido de zinc aumentó la acumulación de ese nutriente en la materia seca de las plántulas de arroz. El recubrimiento con zinc en semillas de arroz en forma de micropartículas, dependiendo de la dosis, puede maximizar el vigor de las semillas y aumentar la concentración de este micronutriente en las plántulas en comparación con el sulfato de zinc, que es la fuente tradicional utilizada por los productores.

Palabras clave

micronutriente • revestimiento de semillas • óxido de zinc microparticulado

Introduction

Seed Zinc (Zn) application is an effective method to guarantee the greater availability and absorption of this element at the beginning of the crop's development. At this stage, plants do not absorb large amounts of nutrients from soil or leaf applications due to the little-developed root system and leaf area (21). Thus, Zn soil fertilization does not increase the concentration of this element in rice plants (*Oryza sativa L.)* (25).

Seed coating enhances nutrient distribution uniformity, reduces losses, lowers application costs, and allows the rational use of non-renewable natural reserves due to the small amounts used (3, 4). Moreover, Zn application may increase germination percentage, physiological response, and crop's growth, especially in grasses (2), and depending on the source used.

Some studies have demonstrated divergent results for the different Zn sources applied to grasses. Tunes *et al.* (2012) reported that zinc sulfate ($ZnSO_4$) did not increase the germination potential of wheat seeds, whereas Santos *et al.* (2017) verified that zinc sulphate application in maize seeds decreased germination and whole plant dry matter (DM). However, the application of zinc oxides (ZnO) forms to the seeds, increased growth and DM of sorghum (14) and maize (15) seedlings, as well as root and shoot Zn in rice seedlings (3). These results confirm the higher efficiency of ZnO when compared with $ZnSO_4$. To the moment, studies have reported the use of non-microparticulate oxides; therefore, the use of a ZnO source with microparticles in the range of 1.90 to 18.00 μm could be tested in order to find if it may increase the benefits in germination, growth, and nutrition of rice seedlings.

Considering the aforementioned, the work hypothesis is that plants absorb more ZnO in the form of microparticulate than conventional $ZnSO_4$ due to the particles size. In addition, the use of ZnO promotes the slower zinc availability in relation to the soluble source, $ZnSO_4$. This fact favors a high absorption of the element without inducing toxicity, which normally occurs with $ZnSO_4$ seed application, as observed in maize seedlings (19).

Thus, rice seeds coating with microparticulate ZnO can maximize the absorption of the micronutrient in the seedling and favor seed vigor and seedling development when compared with zinc sulfate. This work aimed to evaluate the sources and doses of zinc in seed quality and in the nutrition of rice seedlings, cultivar ANA 5015.

Material and methods

The study was carried out at the Seed Technology Laboratory of the Federal University of Mato Grosso do Sul, Campus of Chapadão do Sul - CPCS/UFMS, using seeds of the rice cultivar ANA 5015.

The experiment consisted of a randomized complete design with four replications. Treatments consisted of a control without application of Zn (0 g kg $^{-1}$ of Zn), four doses of microparticulate zinc oxide (20, 40, 80, and 160 g kg^{-1} of Zn in seeds), corresponding to the amount of Zn to coat 1 kg of seed and a treatment containing a dose of 40 g kg^{-1} of Zn in seeds, with zinc sulfate (35% Zn). The zinc oxide source contains 50, 90, and 100% of the particles with a diameter below 1.90; 8.96 and 18.00 μm, respectively. In addition, 85% of Zn is associated with lignin at the concentration of 1,0 g L^{-1} in order to keep the zinc in suspension. Zinc doses were chosen according to recommendations for application of Zn in seed treatment (15).

Seed coating was carried out by hand in a plastic bag, by mixing the respective dose of ZnO with 200 g of seeds moistened with 50 mL of water and shaking for three minutes until complete homogenization. The effects of the treatments were evaluated by germination and vigor tests.

Germination test was carried out with four replications of 50 seeds distributed on germitest paper, moistened with water equivalent to 2.5 times the mass of the non-hydrated substrate, in a germinator at 25°C. The evaluation was carried out 14 days after installation (11).

The evaluation of the accelerated aging used four replications and 50 seeds per treatment and was carried out in gerbox-type boxes (11.5 x 11.5 x 3.5 cm) covered by a metal screen. Seeds were distributed in a single layer, containing 40 mL of sodium chloride solution (40 g NaCl/100 mL H_2O), with 76% RH (6). The boxes were covered and maintained in a Biochemical Oxigen Demand (B.O.D) for 24 hours at 41°C (21). After the aging process, seeds were subjected to the germination test, as previously described, and evaluated five days after sowing.

Seedlings emergence was evaluated by placing the seeds in expanded polystyrene box containing Plantmax® substrate with two daily irrigations. Seedlings were evaluated at 15 days after sowing, by counting the emerged seedlings (12), using four replications of 50 seeds. The emergence speed index was calculated by the Maguire formula (8), with daily evaluations from the beginning of seedlings emergence, by counting the number of seedlings emerged for 15 days after sowing.

For the cold test, four replications of 50 seeds were used for each treatment. Seeds were distributed in germitest paper rolls and moistened with water equivalent to 2.5 times the mass of the non-hydrated substrate. The rolls were stored in plastic bags and maintained in a B.O.D. chamber at 10°C for seven days. Afterwards, they were transferred to the germinator at 25°C, where they remained for another 7 days, followed by counting normal seedlings.

For seedlings DM (dry matter), 20 seeds were sown in germitest paper previously moistened with water equivalent to 2.5 times the mass of the non-hydrated substrate, remaining in the germinator at 25°C for 19 days. Shoots and roots of normal seedlings of each replication were separated using a blade and placed in kraft paper bags and dried in a forced-air-circulation-oven at 65°C until constant weight. Afterwards, shoot and root DM (mg per seedling) were obtained. The Zn concentration was determined according to the methodology proposed by Bataglia *et al.* (1983).

Data were subject to ANOVA, and the doses of ZnO were analyzed by polynomial regression. Three statistical contrasts were established and analyzed by the t-test at 5% probability: ZnO (40 g kg^{-1}) x $ZnSO_4$ (40 g kg^{-1}) (C1), absence of Zn x $ZnSO_4$ (40 g kg^{-1}) (C2), and absence of Zn x ZnO (40 g kg^{-1}) (C3).

Results and discussion

For germination, no significant difference was observed between treatments (table 1, page 99). However, this experiment does not consider the growth rate, which is fundamental for a successful seedling establishment. Therefore, if the seedlings development is relatively slow, but is complete at the expected period of time for the laboratory test, the results may not be the same under less favorable environmental conditions (10).

Although zinc coating did not affect the germination, it influenced seeds vigor. In the cold test, when comparing the oxide form of Zinc with respect to the sulfate form, contrast 1 (C1), and the absence of zinc, contrast 2 (C2), the oxide presented higher averages of seedlings (table 1, page 99). Seeds under adverse conditions of temperature and humidity presented a decreased germination. In these circumstances, zinc oxide was beneficial in relation to the absence of zinc, causing Zn absorption even under low seed metabolism conditions.

The increase of zinc oxide doses increased the values of normal seedling germination in the cold test, with a maximum value of 107.5 g kg^{-1} of Zn in seeds (figure 1, page 100). This fact confirms the beneficial effect of zinc on seeds under adverse conditions of temperature and humidity since this element may have an antioxidant effect, contributing abiotic stresses tolerance or avoidance (7, 24).

In the accelerated aging, when comparing zinc oxide with zinc sulfate, the oxide showed a higher percentage of seedling standards (C1). The same was observed when comparing the absence of zinc in relation to sulphate (C2).

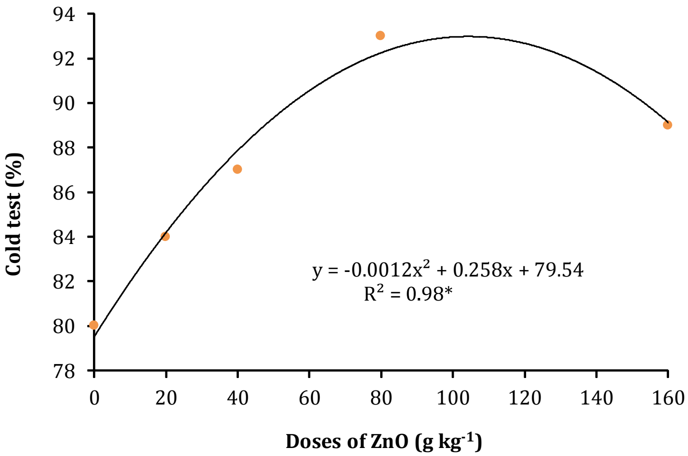
Table 1. Germination percentage (%), cold test, accelerated ageing, seedlings emergence, and speed index of rice seeds emergence in function of coating with different zinc sources and doses.

Tabla 1. Porcentaje de germinación, prueba de frío, envejecimiento acelerado, emergencia de plántulas e índice de velocidad de emergencia de semillas de arroz en función del recubrimiento con diferentes fuentes y dosis de zinc. Estos valores se expresan en porcentaje (%).

Zn source	Zn dose	Germination percentage	Cold test	Accelerated aging	Seedlings emergence	Emergence speed index
g kg^{-1}		(%)				-
-	0	99	80	97	98	16
ZnO	20	99	84	90	90	15
	40	99	87	93	94	15
	80	99	93	92	98	16
	160	98	89	93	96	16
$ZnSO_4$	40	98	80	79	92	15
	CV(%)	4.34	5.08	5.20	5.08	5.66
	F regression	0.15^{ns}	4.73*	0.89^{ns}	1.47^{ns}	1.12^{ns}
	Contrasts	Estimates				
	C1	0.50^{ns}	6.5*	13.50*	2.00^{ns}	0.17^{ns}
	C2	1.00^{ns}	0.5^{ns}	17.50*	6.00^{ns}	0.42^{ns}
	C3	0.50^{ns}	7.00*	4.00^{ns}	4.00^{ns}	0.26^{ns}
	Contrasts					
	ZnO	-	87 a	93 a	-	-
	$ZnSO_4$	-	80 b	79 b	-	-
	0	-	-	97 a	-	-
	$ZnSO_4$	-	-	79 b	-	-
	0	-	80 b	-	-	-
	ZnO	-	87 a	-	-	-

* significant at 5% probability by the t-test; ^{ns} not significant; C1: Zinc oxide (40 g kg^{-1}) x of zinc sulfate (40 g kg^{-1}); C2: absence of Zn x zinc sulfate (40 g kg^{-1}); and C3: absence of Zn x zinc oxide (40 g kg^{-1}).

* significativo al 5% de probabilidad de acuerdo a la prueba t; ^{ns} no significativo; C1: Óxido de zinc (40 g kg^{-1}) x de sulfato de zinc (40 g kg^{-1}); C2: ausencia de Zn x sulfato de zinc (40 g kg^{-1}); y C3: ausencia de Zn x óxido de zinc (40 g kg^{-1}).



* significant at 5% probability by the F test.
* significativo al 5% de nivel de probabilidad por a prueba F.

Figure 1. Cold test of rice seedlings in function of seed coating with increasing doses of zinc oxide.

Figura 1. Prueba en frío de plántulas de arroz en función del revestimiento de semillas con dosis creciente de óxido de zinc.

The absence of this source favored the increase of normal seedlings (table 1, page 99). This test simulates stressful conditions in the seeds due to high temperature and high humidity, and under these conditions, Zn in the form of sulfate impaired their performance.

Seedlings emergence and emergence speed index showed no difference between treatments (table 1, page 99). These results are in agreement with those of Pletsch *et al.* (2014), Smiderle *et al.* (2008), and Tunes *et al.* (2012), which presented no differences for these parameters in canola, bean, and wheat seeds, respectively, using zinc sulfate coating. In some cases, the application of $ZnSO_4$ may decrease the seed germination potential, as observed by Santos *et al.* (2017) and Xavier *et al.* (2016), who evaluated doses of Zn sulfate applied to maize seeds and stylosanthes, respectively.

Results indicated greater root DM for the Zn applied in the form of zinc oxide when compared with sulfate oxide. Moreover, DM values were higher when using the Zn sources than in the absence of Zn (table 2, page 101). In sorghum seeds, the use of zinc oxide increased shoot and root DM, resulting in greater efficiency when using the micronutrient, despite the lower absorption efficiency when compared to the sulfate. In addition, the application of zinc sulfate decreased whole plant DM (14).

An increase in root DM was observed up to the dose of 102.7 g kg^{-1} of Zn (oxide form) in seeds (figure 2b, page 102). Galrão *et al.* (1996) reported different results for the coating of maize seeds, with an increase in root DM with up to 40 g kg^{-1} of Zn in seeds when using oxide. Prado *et al.* (2007b) evaluated doses of up to 40g kg^{-1} of Zn in seeds (zinc oxide and zinc sulfate) in a maize

Table 2. Shoot dry matter, root dry matter, and zinc concentration in the shoots and roots of rice seedlings in function of seed coating with zinc sources and doses.

Tabla 2. Materia seca de la parte aérea, masa seca de raíces y concentración de zinc en la parte aérea y en las raíces de plántulas de arroz en función del revestimiento de semillas con diferentes fuentes y dosis de zinc.

Sources of Zn	Doses of Zn g kg^{-1}	Shoot DM	Root DM	Zn concentration Shoot	Zn concentration Root
		--------- mg per seedlings ---------		--------- mg kg^{-1} ---------	
-	0	4.80	3.15	748.70	268.25
ZnO	20	4.83	3.35	1831.80	1423.75
	40	5.33	3.70	3058.35	2031.30
	80	5.00	3.73	3068.55	2417.15
	160	5.33	3.63	3891.35	3133.40
$ZnSO_4$	40	5.15	3.40	1801.45	1774.50
	CV(%)	4.54	4.13	17.27	12.40
	F regression	8.15*	11.19*	30.76*	85.83*
	Contrasts	Estimates			
	C1	0.18^{ns}	0.30*	1256.90*	256.80^{ns}
	C2	-0.35*	-0.25*	-1052.85*	-1506.25*
	C3	-0.53*	-0.55*	-2213.91*	-1983.15*
	Contrasts				
	ZnO	-	3.70 a	3058.35 a	-
	$ZnSO_4$	-	3.40 b	1801.45 b	-
	0	4.80 b	3.15 b	748.70 b	268.25 b
	$ZnSO_4$	5.15 a	3.40 a	1801.45 a	1774.50 a
	0	4.80 b	3.15 b	748.70 b	268.25 b
	ZnO	5.33 a	3.70 a	3058.35 a	2031.30 a

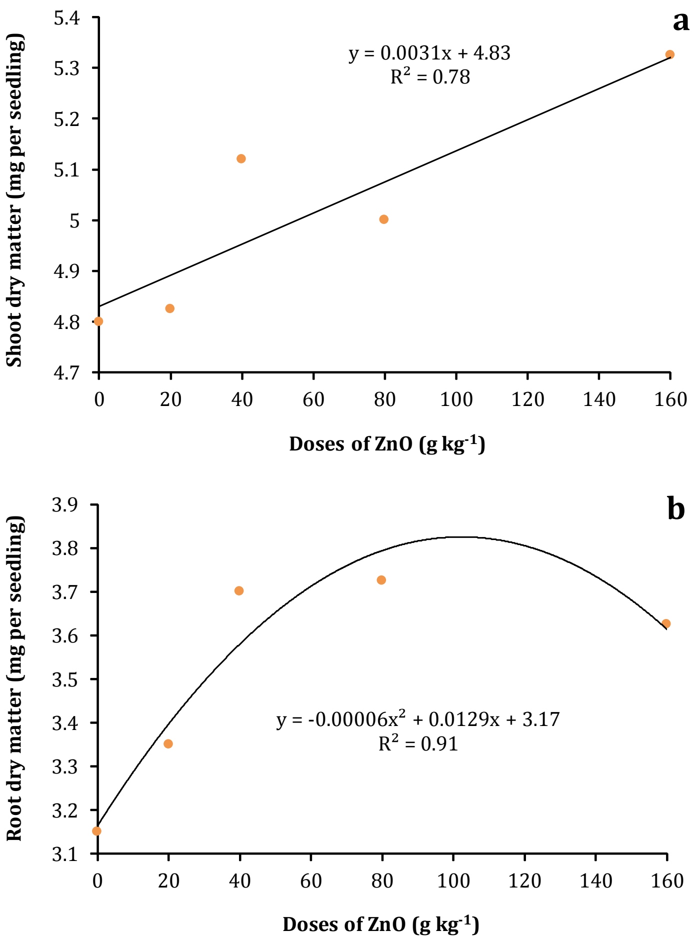
* significant at 5% probability by t-test; ^{ns} not significant; C1: Zinc oxide (40 g kg^{-1}) x zinc sulfate zinc (40 g kg^{-1}); C2: absence of Zn x zinc sulfate (40 g kg^{-1}); and C3: absence of Zn x zinc oxide (40 g kg^{-1}).

* significativo al 5% de probabilidad por la prueba t; ^{ns} no significativo; C1: Óxido de zinc (40 g kg^{-1}) x de sulfato de zinc (40 g kg^{-1}); C2: ausencia de Zn x sulfato de zinc (40 g kg^{-1}); y C3: ausencia de Zn x óxido de zinc (40 g kg^{-1}).

crop and observed a quadratic adjustment for plant DM, with increasing values when using zinc oxide and decreasing values seeds, Prado *et al.* (2008) observed a decreasing linear effect with the use of zinc sulfate and no effect with the use of zinc oxide for DM yield. However, zinc sulfate decreased root DM yield when compared with zinc oxide. Thus, high concentrations of Zn in the plant can be toxic and consequently affect DM yield (10).

For shoot DM, both ZnO and $ZnSO_4$ lead to higher values when compared with the absence of Zn (table 2). An increasing linear adjustment was observed for shoot DM with increasing doses of zinc oxide (figure 2a, page 102). Rozane *et al.* (2008) evaluated zinc oxide and zinc sulfate in rice and verified that sources lead to similar increments of seedlings DM.

The authors also stated that the doses of zinc, regardless of the source, linearly increased whole plant DM. Nevertheless, Prado *et al.* (2008), in a study on sorghum seeds, observed that the doses of zinc quadratic and linearly affected shoot DM yield when using zinc sulfate and zinc oxide, respectively.



* significant at 5% probability by the F test.

* significativo al 5% de nivel de probabilidad por a prueba F.

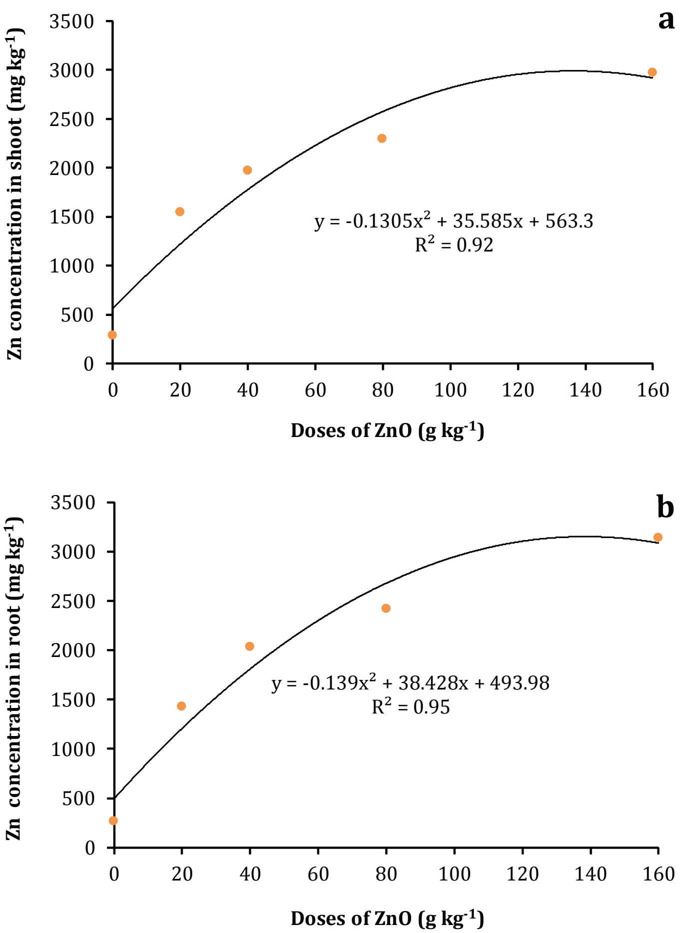
Figure 2. Shoot dry matter (a), and root dry matter (b) of rice seedlings in function of seed coating with doses of zinc oxide.

Figura 2. Materia seca de la parte aérea (a) y raíz (b) de plántulas de arroz en función del revestimiento de semillas con dosis de óxido de zinc.

Zinc sources significantly affected the Zn concentration in shoot DM (table 2, page 101). Zinc oxide showed higher values when compared with zinc sulfate and absence of Zn, with a greater absorption seed of the oxide and greater translocation of Zn to the seedling (table 2, page 101).

Yagi *et al.* (2006) used doses of zinc sulfate in sorghum seeds and observed significant effects on zinc concentrations in shoots. A quadratic regression was observed for zinc concentration in shoots (figure 3a) as a function of Zn doses in seedlings, with maximum Zn concentration of 2988,5 mg kg^{-1} in shoot, corresponding to the application of 136,3 g kg^{-1} of Zn in seedlings. These results are in agreement with those reported by Prado *et al.* (2007c) in maize seeds, that observed a quadratic increase in the Zn concentration in shoots and roots by using zinc sulfate and a linear increase when using zinc oxide.

No difference between the sources was observed in Zn concentration of roots. However, a greater Zn absorption was verified when using zinc oxide. Regarding the absence of Zn, the sources presented greater nutrient concentration (table 2, page 101).



* significant at 5% probability by the F test.

* significativo al 5% de nivel de probabilidad por a prueba F.

Figure 3. Zinc concentration in shoot (a) and root (b) in rice seedlings in function of seed coating with doses of zinc oxide.

Figura 3. Concentración de zinc en la parte aérea (a) y en las raíces (b) en plántulas de arroz en función del revestimiento de semillas con dosis de óxido de zinc.

A positive quadratic effect was observed for zinc concentration in roots of rice seedlings (maximum value of 3.149,9 mg kg^{-1}) when using zinc oxide up to the dose of 138.2 g kg^{-1} in seedlings (figure 3b, page 103). Plants tolerance to zinc excess is related to the complexation of the metal in the cytoplasm of the cells by the exudation of chelating substances in the roots (22).

Rozane *et al.* (2008) reported that the increase in the doses of zinc quadratically increased the zinc concentration and accumulation in the root when using both zinc sulfate and zinc oxide. In maize seeds coated with zinc, Prado *et al.* (2007) observed that the increase in the doses of zinc resulted in a quadratic increase of the nutrient concentration in root when using zinc sulfate; a linear increase of the nutrient concentration when using zinc oxide, and a quadratic increase in Zn accumulation for both sources.

Conclusion

Zinc sources did not change the germination; however, the oxide form provided better seed vigor, except for the highest dose (160 g kg^{-1}). Increases of zinc oxide doses augmented the concentration of this nutrient in rice seedlings DM, when compared with zinc sulfate, which is the traditional source.

References

1. Bataglia, O. C.; Furlani, A. M. C.; Teixeira, J. P. F.; Furlani, P. R.; Gallo, J. R. 1983. Métodos de análise química de plantas. Instituto Agronômico de Campinas, Boletim Técnico. 48 p.
2. Beutler, A. N.; Silva, V. N.; Deak, E. A.; Burg, G. M.; Schmidt, M. R.; Toebe, M. 2014. Zinc doses, sources and application times: seed physiological potential and flooded rice yield. Australian Journal of Crop Science. 8(11): 1517-1525.
3. Dias, M. A. N.; Cicero, S. M. 2016. Effect of copper carbonate and zinc oxide applied to seeds on copper and zinc uptake by maize seedlings. Bragantia. 75(3): 286-291.
4. Farooq, M.; Wahid, A; Siddique, K. H. M. 2012. Micronutrient application through seed treatments - a review. Journal of Soil Science and Plant Nutrition. 12: 125-142.
5. Galrão, E. Z. 1996. Métodos de aplicação de zinco e avaliação de sua disponibilidade para o milho num Latossolo Vermelho-Escuro, argiloso, fase cerrado. Revista Brasileira de Ciência do Solo. 20(2): 283-289.
6. Jianhua, Z.; McDonald, M. B. 1996. The saturated salt accelerated aging test for small seed crops. Seed Science and Technology. 25(1): 123-131.
7. Ma, D.; Sun, D.; Wang, C.; Ding, H.; Qin, H.; Hou, J.; Huang, X.; Xie, Y.; Guo, T. 2017. Physiological responses and yield of wheat plants in zinc-mediated alleviation of drought stress. Frontiers in Plant Science. 8(860): 01-08.
8. Maguire, J. D. 1962. Speed of germination-aid in selection and evaluation for seedling emergence and vigor. Crop Science. 2(2): 176-177.
9. Marcos Filho, J. 1999. Teste de vigor: importância e utilização. In: Krzyzanowski, F. C.; Vieira, R. D.; Neto, J. B. F. Vigor de sementes: conceitos e testes. Londrina. Brasil. Abrates. 1-20.
10. Mengel, K.; Kirkby, E. A. 1987. Principles of plant nutrition. Bern: International Potash Institute. 687 p.
11. Ministério da Agricultura, Pecuária e Abastecimento (MAPA/ACS). 2009. Regras para análise de sementes. Secretaria de Defesa Agropecuária. Brasilia. Brasil. Avaliable in: http://www.agricultura.gov.br/assuntos/insumos-agropecuarios/arquivos-publicacoes insumos/2946_regras_analise__sementes.pdf. Acesso em: 15 set. 2018.

12. Nakagawa, J. 1999. Testes De vigor baseados no desempenho das plântulas. In: Krzyzanowski, F. C.; Vieira, R.; D.; França Neto, J. B. (Eds.). Vigor de sementes: conceitos e testes. Londrina. Brasil. Abrates. 1-24.
13. Pletsch, A.; Silva, V. N. Beutler, A. N. 2014. Tratamento de sementes de canola com zinco. Revista de Ciências Agrárias. 37(2): 241-247.
14. Prado, R. M.; Mouro, M. C. 2007a. Fontes de zinco aplicado em sementes de sorgo cv. BRS 310 e o crescimento inicial. Ciências Agrárias. 28(3): 355-364.
15. Prado, R. M.; Mouro, M. C.; Natale, W. 2007b. Crescimento Inicial e Nutrição de Milho Cultivar P30k75 Submetido à Fertilização de Zinco via semente. Revista Brasileira de Agrociência. 13(4): 495-501.
16. Prado, R. M.; Natale, W.; Mouro, M. C. 2007c. Fontes de Zinco aplicado via semente na nutrição e crescimento inicial do milho cv. Bioscience Journal. 23(2): 16-24.
17. Prado, R. M.; Romulado, L. M.; Rozane, D. E. 2008. Aplicação de zinco em sementes de sorgo cv. BRS 304: efeitos na nutrição e no crescimento inicial. Acta Scientiarum. Agronomy. 30(4): 471-478.
18. Rozane, D. E.; Prado, R M.; Romualdo, L. M.; Simões, R. R. 2008. Resposta de plântulas de arroz cv. BRS - soberana à aplicação de zinco via semente. Ciência e Agrotecnologia. 32(3): 847-854.
19. Santos, A. P.; Morais, O. M.; Prado, R. M.; Leal, A. J. F.; Silva, R. P. 2017. Relation of toxicity in corn seeds treated with zinc and salicylic acid. Communications in Soil Science and Plant Analysis. 48(10): 1123-1131.
20. Smiderle, O. J.; Carvalho, M. V.; Miguel, M. H.; Cicero, S. M. 2008. Tratamento de sementes de feijão com micronutrientes embebição e qualidade fisiológica**.** Agro@mbiente On-line. 2(1): 22-27.
21. Tunes, L. M.; Pedroso, D. C.; Tavares, L. C.; Barbieri, A. P. P.; Barros, A. C. S. A. Muniz, M. F. B. 2012. Tratamento de sementes de trigo com zinco: armazenabilidade, componentes do rendimento e teor do elemento nas sementes. Ciência Rural. 42(7): 1141-1146.
22. Wang, J.; Evangelou, V. P. 1994. Metal tolerance aspects of plant cell wall and vacuole: handbook of plant and cropphysiology. Tucson, The University of Arizona. 325 p.
23. Yagi, R. M.; Simili, F. F.; Araújo, J. C.; Prado, R. M.; Sanchez, S. V.; Ribeiro, C. E. R.; Barretto, V. C. M. 2006. Aplicação de zinco via sementes e seu efeito na germinação, nutrição e desenvolvimento inicial do sorgo. Pesquisa Agropecuária Brasileira. 41(4): 655-66.
24. Yavas, I.; Unay, A. 2016. Effects of zinc and salicylic acid on wheat under drought stress. Journal of Animal and Plant Science. 26: 1012-1018.
25. Yin, H. J; Gao, X. P.; Stomph, T. J.; Li, L. J.; Zhang, F. S.; Zou, C. O. 2016. Zinc concentration in rice (*Oriza sativa* L.) grains and allocation in plants as affected by different zinc fertilization strategies. Communications in Soil Science and Plant Analysis. 47(6): 761-768.

Acknowledgment

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) y Programa Nacional de Cooperação Acadêmica (PROCAD) - Edital 71/2013.

Small farmers' perception of factors influencing regional chemical control of *Diaphorina citri*

Percepción de pequeños productores sobre factores que inciden en el control químico regional de *Diaphorina citri*

Luis Alfredo Pérez-Zarate [1], Juan A. Villanueva-Jiménez [1], Francisco Osorio-Acosta [1*], Laura Delia Ortega-Arenas [2], Lissette C. Bustillo-García [3]

Originales: *Recepción:* 21/11/2017 - *Aceptación:* 30/09/2019

Abstract

Regional Control Areas (RCAs) have been implemented in Mexico as a strategy to delay the spread of *Candidatus* Liberibacter asiaticus, the causal bacterium of the disease known as Huanglongbing (HLB). The implementation of an effective management of the vector insect, *Diaphorina citri* in the RCAs requires the knowledge, acceptance and coordinated engagement of small agricultural producers. This research assessed the perception and knowledge of 62 citrus growers regarding the operational, sociocultural and environmental factors influencing chemical control of *D. citri* in four RCAs within Veracruz State. According to their responses, the following factors have been identified as the operational factors with the highest influence on the effectiveness of insecticides against *D. citri* within RCAs: the lack of knowledge about the use of surfactants, application speed, poor calibration of sprayers and incorrect water quality. The most significant sociocultural factors are the general unawareness of the pest and the safe and proper application of pesticides. The most relevant environmental factors during application: temperature, relative humidity, and wind speed. Sociocultural index correlated with the perception of effectiveness. Therefore, it becomes necessary to consider differences among citrus growers in each region and setting out the most appropriate strategies for vector and disease management.

Keywords

regional control areas • *Candidatus* Liberibacter • chemical control • growers' perception

1 Colegio de Postgraduados. Campus Veracruz, Programa de Posgrado en Agroecosistemas Tropicales. Km. 88.5 Carretera Fed. Xalapa-Veracruz. Manlio F. Altamirano. Veracruz. 91690. México. * fosorioa@colpos.mx

2 Colegio de Postgraduados. Campus Montecillo. Programa en Fitosanidad-Entomología y Acarología. Km. 36.5. Carretera México-Texcoco. Montecillo. Texcoco. Estado de México. 56230. México.

3 Universidad del Zulia. Facultad de Ciencias Veterinarias. Departamento Socioeconómico. Núcleo Agropecuario. Avenida 16. Maracaibo. Venezuela.

Resumen

México ha establecido Áreas Regionales de Control (ARCO) como estrategia para retrasar la dispersión de *Candidatus* Liberibacter asiaticus, bacteria causante de la enfermedad llamada Huanglongbing (HLB). El manejo efectivo de *D. citri* en las ARCO depende del conocimiento, aceptación y participación coordinada de los pequeños productores. Esta investigación evaluó la percepción y el conocimiento de 62 citricultores sobre los factores operacionales, socioculturales y ambientales que inciden en el control químico de *D. citri* de cuatro ARCOs, en el estado de Veracruz. Con base en la respuesta de estos pequeños productores, los factores operacionales que más inciden en la efectividad de insecticidas contra *D. citri* en las ARCO son: desconocimiento sobre uso de adherentes, velocidad de avance del aplicador, deficiente calibración de equipos y calidad de agua utilizada; entre los factores socioculturales destacan: desconocimiento general sobre la plaga, y sobre el buen uso y manejo de plaguicidas, además, los factores ambientales más relevantes al momento de la aplicación son: temperatura, humedad relativa y velocidad de viento. El índice sociocultural se correlacionó con la percepción de la efectividad; por tanto, es necesario considerar las diferencias entre productores de cada región para establecer las estrategias más apropiadas de manejo del vector y la enfermedad.

Palabras clave

áreas regionales de control • *Candidatus* Liberibacter • control químico • percepción de productores

Introduction

Citrus are among the most important horticultural crops in the world (18, 39). However, high yield losses result from pests and diseases. In Mexico, citrus production is affected by the presence of the Asian Citrus Psyllid (ACP) or *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), the primary vector of *Candidatus* Liberibacter asiaticus (CLas). This is the causal bacterium of Huanglongbing (HLB), the most destructive disease of citrus in the world (4, 20). To succeed in getting an evident impact on widespread populations of *D. citri* on citrus orchards, regional control areas (RCAs) have been established as part of the National Campaign against HLB and its Vector. This strategy is expected to delay the spread of CLas in citrus-growing states of Mexico, and to reduce disease severity (41). RCAs management is based on chemical control of the vector, complemented with biological control in suburban areas, use of certified plants, and removal of diseased trees (35).

Most RCAs consist of small farmers' plots. Accordingly, growers' acceptance of management strategies and their engagement are required to implement control actions in a coordinated way, and consequently, to have a better impact on the populations of *D. citri* (29, 42). To succeed in implementing the recommendations made by the Veracruz State Plant Health Committee (CESVVER), it is important to consider: i) citrus growers' perception regarding economic and social advantages of these recommendations;

ii) compatibility with local tradition and knowledge, iii) complexity of the proposed strategy, iv) the possibility of experimenting, and v) visibility of results in the short term (23).

A successful application depends not only on the effectiveness of the insecticidal molecule itself, but also on several factors that may enhance or reduce it, such as: a) the number of resistance genes on pest population (30), b) operational factors during application (29), c) environmental conditions during application (28), and d) growers' knowledge of insecticide management (31). The effectiveness of insecticide applications on RCAs has been assessed in terms of susceptibility at lethal dose level and through biological effectiveness tests (15, 38, 40). However, no consideration has been given to the sociocultural complexity and the specificity of local knowledge on each citrus area, which may determine the effectiveness and acceptance of the HLB Campaign. Therefore, the assessment of citrus growers' perception regarding the factors that could impact on the effectiveness of the insecticides applied in the RCAs is a key aspect. This would allow to propose strategies aimed at performing more efficient applications, and thus, to facilitate reducing the populations of the vector and delaying the dissemination of the bacterium throughout Mexico's main citrus areas.

Perception is regarded as the most basic process of acquiring knowledge, through which people obtain information and codify or classify it into categories delimited by experiences, feelings and thoughts (2, 19). Sociocultural context influences growers' perception about recommendations regarding proper use and management of insecticides. Moreover, inadequate decision-making regarding the rotation of toxicological groups (TGs) and increased application rates have led to the emergence of resistant insect populations. The resistance to some groups of insecticides might result from the high selection pressure, such as that generated in supervised regional applications (SRAs) by CESVVER, in addition to the unsupervised local applications (USLAs), in the area of Martinez de la Torre, Veracruz, and in RCAs in other citrus growing states in Mexico (15, 38). This scenario reduces the lifespan of those insecticides used in the RCAs. Furthermore, the growers' lack of knowledge regarding operational practices, such as equipment calibration, as well as the prevailing weather conditions during applications (high temperatures and wind speed), further limits the maximum potential expression of the insecticides in each application. Poor applications play a role in the spread of CLas within the state, the increase of production costs per agricultural cycle and the reduction of the diversity on beneficial native fauna in each citrus area (40).

Objective

The purpose of this research was to determine the operational, sociocultural and environmental factors that have an impact on the effectiveness of insecticides, according to the perception and knowledge of small farmers within Regional Control Areas located in Martinez de la Torre, Veracruz, Mexico.

Materials and methods

The study was conducted in four RCAs, where coordinated actions have been implemented for the control of *D. citri,* and addressed by the Local Plant Health Board of Totonacapan, associated with CESVVER, in the Municipality of Martínez de la Torre, Veracruz, Mexico. These areas are: RCA 4 [Ejidos "Pueblo Viejo" and "Cartago", along with small adjoining properties (1051.05 ha total)]; RCA 9 [Ejidos "San Antonio Coronado", "Flores Magón", "Paso de Barriles" and "Santa Rosa", along with small adjoining properties (1000 ha total)]; RCA 10 [Ejidos "Valsequillo", "El Insurgente Socialista", "Miguel Hidalgo", "Mesa Chica Nueva el Corcho" and "Augusto Gómez Villanueva" (1000 ha total)] and RCA 11 [(Ejidos "Cañizo", "Flamencos" and "Piedrilla" (1000 ha total)].

Sixty-two growers within the RCAs were interviewed using a structured questionnaire (24, 32). In addition, participatory observation was performed during the meetings of the ejidos or groups integrating the RCAs. The questionnaire was applied from August to December of 2015 during ejido meetings. Interviews were directed to ejido members interested in the HLB campaign, which increased confidence between interviewer and interviewed. To gain additional insight (5), a group of growers who did not attend ejido meetings were also interviewed. All interviews were conducted with RCAs stakeholders and beneficiaries of the Campaign against HLB. The sample represents 10% of the beneficiary population of small farmers in the study area.

The first section of the questionnaire included personal data: name, age, schooling, RCA number, citrus varieties cultivated, orchard land area (ha) and name of the person who sprays the insecticide. The second section included open and closed-ended questions to identify the operational factors of the last application of insecticides, that could have an impact on their effectiveness: insecticides used, dose increment, surfactant use, calibration and type of sprayers used, and their perception about application effectiveness and its relation with water quality, nozzles used and applicator advance speed. The third section focused on identifying sociocultural factors: growers' attendance at meetings, organization, interest in *D. citri* control, knowledge of insecticide management and rotation, protection of natural enemies, training in insecticide management, and their perception on the effectiveness of the RCAs. The fourth section focused on growers' knowledge of the environmental factors to be considered during applications: spraying schedule, wind speed, relative humidity, temperature and rainfall.

A Likert scale was used to categorize the closed-ended responses, and frequencies also were generated with similar open-ended responses. Standardized responses were used to build sub-indexes of operational, sociocultural and environmental factors.

Reference values were developed according to the criteria set out by different authors, including CESVVER technicians and executives' opinion, representing the highest value. These reference values were compared with those obtained. The same procedure was performed with results obtained from each subindex. A Pearson's correlation matrix was developed to compare each subindex (operational, sociocultural and environmental) with the subindex on perception of application effectiveness, using the SAS® PROC CORR procedure (46).

Results and discussion

Description of citrus growers surveyed

The average number of hectares per grower interviewed was 11.8, mostly covered with Persian lime. The majority of growers were men (93.5%), 56.3 years old on average. The level of schooling of most of them was middle-school, 7% had received no schooling at all, and 10% had studied for more than ten years (table 1).

Operational factors

There are two types of operational factors that have an impact on the effectiveness of an application: i) the toxicant applied, and ii) the type of application (29). The values of the operational sub-indexes under assessment were close to the expected maximum in variables related to the toxicant applied, which includes the toxicological group (3 of 3), active ingredient (3 of 3) and applied dose (2 of 2). Therefore, high values could be explained given that these activities are supervised by CESVVER technicians; in addition, only the recommended insecticides are applied on the RCAs. However, those sub-indexes related to management of sprayers and insecticides, such as sprayer calibration (1.71 of 3), surfactant use (2.80 of 3.33), applicator advance speed (1.63 of 3), and water quality (1.65 of 2) had low values in relation to the effectiveness of the RCAs (table 2, page 111).

The HLB Technical Group decides on the insecticides and doses to be used in the RCAs. Also, it determines which is the appropriate rotation of TGs and the use of the minimum effective dose, as well as the crop's phenological stage (9, 41).

In SRAs, most producers claimed they had applied insecticides at the correct time and in the correct way (96%), and denied increasing the doses in any application or having used any mixture of insecticides (both 100%). However, this information is not consistent with previous field assessments (40). Most growers said that since the insecticide provided by CESVVER resulted effective, they kept buying the same TG for USLAs. Organophosphates are the most used insecticides (40). Constant applications of this chemical group and the use of inappropriate doses stimulate the development of resistant populations of *D. citri* and the emergence of secondary pests, as well as a reduction in the lifespan of the insecticides in use (15).

Table 1. Information about citrus growers interviewed in the RCAs of Martinez de la Torre, Veracruz.

Tabla 1. Información de los citricultores entrevistados en las ARCO de Martínez de la Torre, Veracruz.

Variables	Average	S.D. (±)
Area cultivated per grower (ha)	11.80	13.23
Gender (male growers) (%)	93.55	N.A.
Age (years old)	56.30	11.25
Average schooling (years)	7.40	4.60
Main crop	(%)	
Persian Lime	59.00	
Orange	37.00	
Grapefruit	4.00	

S. D. = Standard deviation; N. A. = Not applicable. / S. D. = Desviación estándar; N. A. = No aplica.

Table 2. Maximum expected and obtained values of each subindex for the operational index evaluated in the regional control areas in Veracruz, Mexico.

Tabla 2. Valores máximos esperados y obtenidos de cada subíndice para el índice operacional, evaluado en áreas regionales de control, en Veracruz, México.

Index	Subindex	Maximum expected value	Value obtained in RCA	Reference assigned to maximum value
Operational factors	Toxicological group in SRA	3.00	3.00	(8, CESVVER[1])
	Active ingredient in SRA	3.00	3.00	(8, CESVVER)
	Recommended dose in SRA	2.00	2.00	(8, CESVVER)
	Insecticides in USLA	2.00	1.79	(42)
	Surfactant	3.33	2.80	(26)
	Calibration	3.00	1.71	(15, 23)
	Pump type	2.50	1.56	(49)
	Nozzle type	3.00	1.56	(3)
	Forward speed	3.00	1.63	(27)
	Water quality	2.00	1.65	(12, 17)

[1] Information obtained from CESVVER technicians and executives; SRA = Supervised regional applications; USLA = Unsupervised local applications.

[1] Información obtenida de técnicos y directivos del CESVVER; SRA = Aplicación regional supervisada; USLA= Aplicación local no supervisada.

Correlation of the operational sub-indexes evaluated showed that those small farmers who have a higher perception of SRA effectiveness pay more attention to the quality of water they use in their applications (0.2859, $p < 0.0043$) (table 3, page 112). However, 59% never check the pH of the water used and 11.2% do not check for debris or a strange color. It is known that pH can modify, or even degrade the active ingredient of insecticides. In addition, the presence of organic matter may clog nozzles and accelerate the wearing of the sprayer (26). Leiva (2010) reports that the half-life of organophosphates can be increased from 1 to 35 d by lowering the pH from 8 to 7; recommending to acidify water if it seems cloudy or presents organic matter.

Growers who know that water quality may influence application effectiveness also believe that the use of surfactants (0.3085, $p < 0.0147$) and specific nozzle type (0.4547, $p < 0.0002$) could enhance the effectiveness of insecticides (table 3, page 112). About 24% of growers have never used surfactants in their applications, although those who had used them (76%) do not know the advantages of applying them, and among them, 5% only use surfactants with other agrochemicals, such as herbicides and foliar fertilizers. Our results agree with those of Carvalho *et al*. (2016) in Brazil, who reported that mineral oil is the most commonly surfactant used in combination with insecticide. Once more, 17.9% of interviewed growers use this product without knowing its potential advantages in the mixture. In this sense, Cortez-Mondaca *et al*. (2010) pointed out that using good quality water with surfactants in the mixture can enhance the effect of insecticides.

Growers who consider that applicator advance speed can affect application effectiveness, also believe that an appropriate nozzle type (0.5855, $p < 0.0001$) and a good calibration of equipment (0.4374, $p < 0.0060$) (table 3, page 112) can maximize the effect of any insecticide.

Table 3. Pearson's correlation matrix of the perception of supervised regional application effectiveness subindex, and other operational subindexes.

Tabla 3. Matriz de correlación de Pearson del subíndice de percepción de la efectividad de las aplicaciones regionales supervisadas y otros subíndices operacionales.

	SI-PAE[1]	SI-SU[2]	SI-AAS[3]	SI-NU[4]	SI-WC[5]	SI-SC[6]
SI-PAE		0.1846	0.1812	0.1875	0.2859*	0.0724
SI-SU			0.0055	0.2098	0.3085*	0.2284
SI-AAS				0.5855***	0.1927	0.4375**
SI-NU					0.4547***	0.2926
SI-WC						0.2287
SI-SC						

[1] Perception of application effectiveness subindex; [2] Surfactant use subindex; [3] applicator advance speed subindex; [4] Nozzle use subindex; [5] Water quality subindex; [6] Sprayer calibration subindex.
Significance level of Pearson's linear correlation coefficient: * < 0.05, ** < 0.01, *** < 0.001.

[1] Subíndice de percepción sobre la efectividad de las aplicaciones; [2] Subíndice uso de adherente; [3] Subíndice velocidad de avance; [4] Subíndice uso de boquilla; [5] Subíndice calidad del agua; [6] Subíndice calibración de equipos.
Nivel de significancia del coeficiente de correlación lineal de Pearson: * = <0,05, ** = <0,01, *** = <0,001.

In stark contradiction to this group, 53.2% of the interviewees do not calibrate their equipment and 56.5% do not believe that nozzle type affects effectiveness, even though it has been well established that calibration is one of the most frequently occurring factors causing deficient pest control (50). In general, the RCA strategy along with the recommendations made by CESVVER technicians, have a positive impact on the knowledge of some growers about *D. citri* management. Continuous training of beneficiaries, along with the promotion of their engagement, are required to prevent the spread of CLas in this important citrus area of Veracruz.

Sociocultural factors

Sociocultural factors depend to a great extent on cognitive development, the social environment where each grower was raised, schooling and the level of training in pest management (1, 28). Growers' ideology, perception and knowledge vary among localities and therefore among the RCAs. In most cases, sociocultural sub-indexes obtained low values, particularly in aspects related to organization (1.41 of 2), knowledge about protection of natural enemies (1.86 of 2.5), management and rotation of insecticide (2.27 of 2.71), and growers' perception of SRA effectiveness (2.67 out of 3.67) (table 4, page 113).

Growers who believe that there is greater effectiveness in the RCAs also have a greater knowledge of the pest (0.281, p <0.0270), insecticide management and rotation (0.300, p < 0.0177) (table 5, page 113). This is probably due to a better training, higher investment in inputs such as insecticides, and efficient previous applications. Van-Mele *et al*. (2005) indicate that those growers who actively seek staff training in pest biology and management, might evolve to a more entrepreneurial profile. They also explained that small farmers who strongly depend on a single crop, might be influenced by the advertising of agrochemical suppliers through local retailers, which in turn determine the type of pest management they choose, often based on an exclusive use of insecticides.

Table 4. Maximum expected and obtained values of each subindex integrating the sociocultural index from regional control areas from Veracruz, Mexico.

Tabla 4. Valores máximos esperados y obtenidos de cada subíndice que integra el índice sociocultural en áreas regionales de control de Veracruz, México.

Index	Sub-indexes	Maximum Expected Value	RCA Value Obtained	Maximum Value Reference
Sociocultural factors	Association membership	3	2.21	(42, CESVVER[1])
	Organization	2	1.41	(22, 42, CESVVER)
	Pest	2.67	2.32	(42, CESVVER)
	Knowledge on insecticide management and rotation	2.71	2.27	(7, 30)
	Dose increment	2	1.64	(44, 45)
	Protection of natural enemies	2.5	1.86	(42, 48)
	Perception on insecticide effectiveness	3.67	2.66	(CESVVER)

[1] Information provided by CESVVER technicians and executives.

[1] Información obtenida de técnicos y directivos del CESVVER.

Table 5. Pearson's correlation matrix of the sociocultural indexes with the "perception of supervised regional application effectiveness" subindex.

Tabla 5. Matriz de correlación de Pearson de los subíndices socioculturales con el subíndice "percepción sobre la efectividad de las aplicaciones regionales supervisadas".

	SI-PSRAE [1]	**SI-BC [2]**	**SI-O [3]**	**SI-PK [4]**	**SI-IMR [5]**	**SI-AD [6]**	**SI-NEP [7]**
SI-PSRAE		0.0667	0.1183	0.2809*	0.3003*	-0.0098	0.1695
SI-BC			0.2146	-0.0687	0.3127*	0.3357 **	0.0815
SI-O				-0.1630	0.3149*	0.1749	-0.0255
SI-PK					0.2819*	0.2437	-0.1854
SI-IMR						0.4667***	-0.2578 *
SI-AD							0.4979***
SI-NEP							

[1] Perception of SRA effectivity subindex; [2] Belonging to the campaign subindex; [3] Organization subindex; [4] Pest knowledge subindex; [5] Insecticide management and rotation subindex; [6] Augmentation of dose subindex; [7] Subindex of protection to Natural enemies. Significance level of Pearson's linear correlation coefficient: * < 0.05, ** < 0.01, *** < 0.001. Source: The author's own work.

[1] Subíndice de percepción sobre la efectividad de las SRA; [2] Subíndice de pertenencia a la campaña; [3] Subíndice de organización; [4] Subíndice de conocimiento de la plaga; [5] Subíndice de manejo y rotación de insecticidas; [6] Subíndice de aumento en dosis; [7] Subíndice de protección a enemigos naturales. Nivel de significancia del coeficiente de correlación lineal de Pearson: * = <0,05, ** = <0,01, *** = <0,001. Fuente: Elaboración propia.

The "belong to the campaign" subindex consists of several issues, like growers interest in attending meetings and their perception of possible improvements in ACP control since they became part of the RCA. As this subindex increases, so do the sub-indexes for management and rotation (0.313, $p < 0.0134$) and augmentation of dose (0.336, $p < 0.0076$) (table 5, page 113). In this regard, those growers who are more interested in implementing CESVVER's recommendations are also those that increase to a greater extent the USLA doses and those -ironically- with greater knowledge of insecticide management. Some small farmers believe that they will get better results by increasing insecticide doses, and that these applications should eliminate all types of insects, even if they are not the target pest (-0.258, $p < 0.0431$). In addition, growers who tend to increase USLA doses have a lower perception of protection of natural enemies (-0.498, $p < 0.0001$) (table 5, page 113). This has been by Ruiz-Nájera *et al.* (2011) when studying tomato growers behaviour in Chiapas, Mexico. In this regard, Jallow *et al*. (2017) report that some factors that may explain why small farmers tend to overuse pesticides and increase doses are: i) the degree of formal education (schooling), ii) experience in pest management, iii) training; iv) information sources used when deciding which insecticides to apply; v) access to extension support; and vi) the farmers perception of yield losses due to pests. As aforementioned, it is necessary to increase the availability of information and to properly transfer it through extension services and training, while fostering a culture of protection of natural enemies, as well as good insecticide usage and management (47).

Growers' interest in investing in agricultural inputs and their participation in regional management of *D. citri* might be influenced by factors such as age, schooling, plot size, orchard age, and crop profitability in previous years (9, 36). Growers who are most interested in becoming member of the SRAs, also have more knowledge about insecticide management and rotation (0.315, $p < 0.0127$) and about the pest (0.282, $p < 0.0264$). However, they also apply higher insecticide doses than recommended (0.467, $p < 0.0001$) (table 5, page 113). Most growers with entrepreneurial interest invest more in pest control, since they claime that this action has improved their production volumes.

These growers have a key role within the RCAs, since they can lead by example given that they adopt recommended doses. They need to link their efforts with those of technicians to streamline supervision and regulation of the applied doses, enabling the achievement of proper insecticide resistance management, thereby prolonging lifespan of these molecules (31).

Environmental factors

The value obtained from the environmental sub-indexes, was low in most cases. Some growers of the RCAs believe that certain environmental factors such as wind speed (1.60 of 3.5), relative humidity (2.16 of 3) and temperature (1.21 of 2) have no impact on SRA effectiveness. However, most of them suspend insecticide applications when probability of rainfall is not low (1.90 of 2) (table 6, page 115).

There was no direct correlation between the perception of application effectiveness subindex and the environmental sub-indexes. Most growers indicated they perform applications between 7 a.m. and 11 a.m. (87.1%), whereas all others perform them after 4 pm.

Table 6. Maximum expected and obtained values of each subindex of the environmental index evaluated in regional control areas in Veracruz, Mexico.

Tabla 6. Valores máximos esperados y obtenidos de cada subíndice para el índice ambiental evaluado en áreas regionales de control, en Veracruz, México.

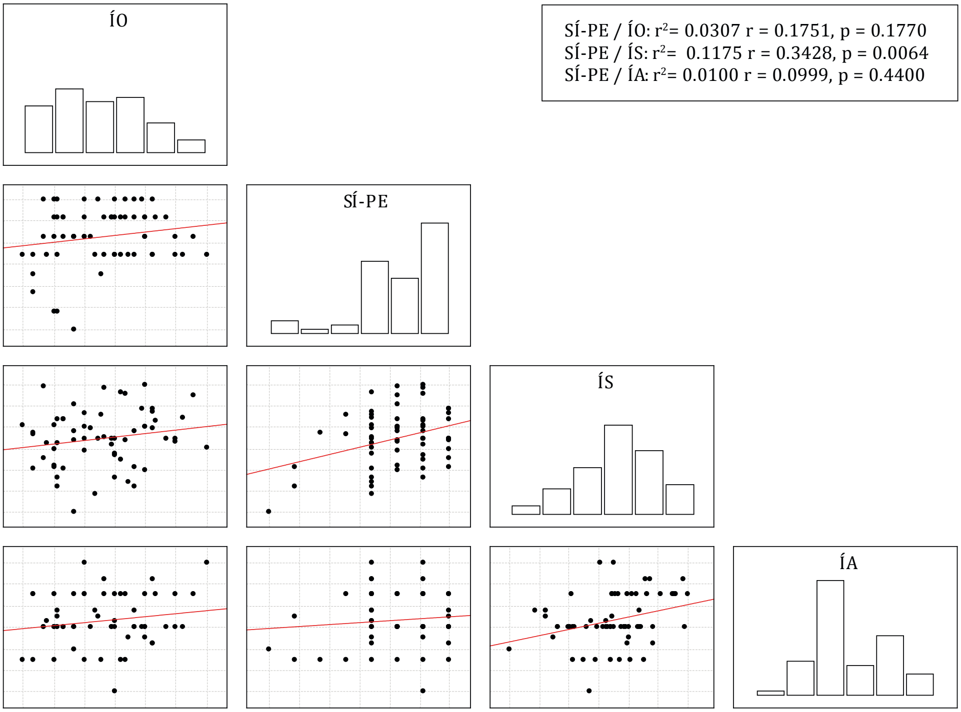
Index	Subindex	Maximum expected value	Value obtained in RCAs	Maximum value references
Environmental Factors	Application schedule	2.33	1.85	(14)
	Wind speed	3.50	1.60	(11, 27)
	Relative humidity	3.00	2.16	(27)
	Temperature	2.00	1.21	(13, 30)
	Precipitation	2.00	1.90	(27)

Source: The author's own work. / Fuente: Elaboración propia.

However, when applications *in situ* were monitored, they started at 8 a.m. on average and some finished after 2 p.m., with temperatures above 30°C (38). According to FAO (2001), the timing of insecticide applications shouldn´t coincide with the feeding times of beneficial insects. When applications are made at noon, insecticides might be lost through evaporation. In addition, the penetration of the insecticide into the foliage is affected by low percentages of relative humidity (26, 48). Some growers believe that wind speed, relative humidity and temperature (79.03%) have no impact on application effectiveness, and only 9.6% have applied insecticides with high rainfall probability. In this regard, Massaro and Fernández (2013) stated that these variables can either directly or indirectly affect the crop, the pest, the product and the sprayer-generated droplets. Gonçalves-Balan *et al*. (2016) reported basic deficiencies in applications when not considering the weather conditions. Pérez-Zarate *et al*. (2016) reported that wind speed had a direct influence on the effectiveness of mineral oil and that the highest mortality percentages of *D. citri* nymphs occurred with relative humidity above 60%. In future SRAs, these factors must be considered to perform efficient insecticide applications, lower production costs, and reduce the aquifer contamination risks and intoxication of people spraying the products.

Perception of SRA effectiveness and its relationship to operational, sociocultural and environmental indexes

A positive correlation (0.3428, $p < 0.0064$) was found between the "perception of application effectiveness" subindex and the sociocultural index (figure 1, page 116). This reflects that the sociocultural context in the RCAs could influence the growers' perception of application effectiveness, decision-making for the management of *D. citri* and the adoption of CESVVER's recommendations in the SRAs. Sarandón and Flores (2014) mention that as agroecosystem administrator, mankind is intimately embedded in a sociocultural context, that determines the way in which the decisions are made. Social acceptance of the strategies to control ACP is essential to prevent the spread of CLas, since with no commitment of growers, technicians, researchers and authorities, citrus-growing areas could be reduced and even disappear, resulting in severe social and economic consequences (29).



SI-PE = Perception of effectiveness subindex in SRA, IO = Operational index, IS = Sociocultural index, IA = Environmental index.

SI-PE = Subíndice de percepción de la efectividad de ARS, IO = índice operacional, ÍS = Índice sociocultural, ÍA = Índice ambiental.

Figure 1. Correlation between the"perception of effectiveness" subindex and the operational, sociocultural and environmental indexes.

Figura 1. Correlación entre el subíndice de "percepción de la efectividad" y los índices operacional, sociocultural y ambiental.

To have a good relationship between technicians and small farmers is important since, due to differences in their knowledge, they approach subjects with different views. In addition, to gain expertise in pest management, technicians must have interpersonal skills to maximize the impact of their recommendations on small farmers (31). Training for technicians should be ongoing and should encourage more participatory and horizontal extension practices. Training should be provided to leading producers, who influence decisions made by the rest of the group regarding the management of *D. citri*. This could increase the chances for the HLB Campaign recommendations to be adopted (5).

Ortiz (2001) concludes that the adoption of new technologies could be achieved by integrating farmers knowledge with technical information. This information should be provided in a gradual and sequenced manner during pest management training, in order to facilitate producers' understanding and to allow them to associate information with local empirical knowledge.

Although both the operational and the environmental indexes showed no relationship with "perception of effectiveness" subindex, in several scenarios these factors have influenced the effectiveness by increasing wind-driven pesticide drift, by decreasing foliar coverage and by causing product evaporation due to high temperatures (13, 26, 33, 44). Growers' decision-making regarding pest management undoubtedly have an impact on SRAs effectiveness. The sociocultural context in the RCAs should be studied and understood to strengthen social relationships between technicians and producers, as well as to facilitate participation and organization, which is a cornerstone of the RCA strategy.

Conclusions

The perception of effectiveness of regional control areas could be influenced by some operational practices that are not being considered by growers in their applications, such as the use of surfactants, machine ground speed, sprayer calibration and quality of water. Sociocultural factors, such as a lack of general knowledge about the pest and its management, as well as on insecticide rotation, were more related to the perception regarding application effectiveness.

Environmental factors did not correlate with the perception of effectiveness on supervised regional applications; indicating that during the application, growers do not always consider weather conditions, such as temperature, wind speed and relative humidity. The sociocultural index correlated to a greater degree with the perception of application effectiveness, which indicates that sociocultural context should be considered in the HLB Campaign as a strategy that considers local knowledge and expedite the adoption of the recommendations made by CESVVER technicians.

References

1. Andrews, K. L.; French, B. J.; Goodell, G. 1989. El contexto socioeconómico del Manejo Integrado de Plagas. In: Manejo Integrado de Plagas Insectiles en la Agricultura. Estado Actual y Futuro. Honduras: El Zamorano. 623 p.
2. Barón, R. A. 1997. Fundamentos de Psicología. Prentice Hall Hispanoamericana. México. 435 p.
3. Bode, L. E. 1981. New pesticide application equipment and techniques. Plant Disease. 65: 32-36.
4. Bové, J. M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology. 88(1): 7-37.
5. Cano-Reyes, O.; Villanueva-Jiménez, J. A.; Reta-Mendiola, J. L.; Huerta-De-la-Peña, A. 2012. Technology transfer networks on papaya production with transitional growers. Tropical and Subtropical Agroecosystems. 15(2): S12-S20.
6. Carvalho, K. F.; Antuniassi, R. U.; Mota, B. A. A.; Chechetto, G. R.; De Carvalho, R. L. 2016. Knowledge on adjuvant properties, disposal of pesticide residues and spray drift occurrence in the state of Mato Grosso. Journal of the Association of Agricultural Engineering. 36(2): 352-360.

7. Cortez-Mondaca, E.; López-Arroyo, J. I.; Hernández, L. M.; Castillo, A. F.; Loera, J. G. 2010. Control químico de *Diaphorina citri* Kuwayama en cítricos dulces en México: Selección de insecticidas y épocas de aplicación. Folleto Técnico No 35. México. INIFAP. 65 p.
8. Cortez-Mondaca, E.; Loera, G. J.; Hernández, F. L.; Barrera, G. J.; Fontes, P. A.; Díaz, Z. U.; Jasso, A. J.; Reyes, R. M.; Manzanilla, R. M.; López, A. J. 2013. Manual para el uso de insecticidas convencionales y alternativos en el manejo de *Diaphorina citri* Kuwayama en cítricos, en México. Folleto Técnico No. 36. México. INIFAP-CIRNO-364 CEVAF. Juan José Ríos. Sinaloa. México. 56 p.
9. Danso-Abbeam, G.; Dope-Setsoafia, E.; Kodwo-Ansah, I. G. 2014. Modelling farmer's investment in agrochemicals: the experience of smallholder cocoa farmers in Ghana. Research in Applied Economics. 6: 16 p.
10. Davis, B. N. K.; Williams, C. T. 1990. Buffer zone widths for honeybees from ground and aerial spraying of insecticides. Environmental Pollution. 63: 247-259.
11. Deer, H. M.; Beard, R. 2001. Effect of water pH on the chemical stability of pesticides. Pesticides Fact Sheet. Electronic Publishing. EP/DF/07-2001. Cooperative Extension Service, Utah State University. Logan. Utah. 3 p.
12. Dong-Soon, K.; Yon-Dong, S.; Kyung-San, C. 2010. The effects of petroleum oil and lime sulfur on the mortality of *Unaspis yanonensis* and *Aculops pelekassi* in the laboratory. Journal of Asia-Pacific Entomology. 13: 283-288.
13. Food and Agriculture Organization of the United Nations (FAO). 2001. Guidelines on good practice for ground application of pesticides. Retrieved on 06/05/2016. Available in: http://www.fao.org/docrep/006/y2767e/y2767e00.htm.
14. Food and Agriculture Organization of the United Nations (FAO). 2008. Prácticas recomendadas para el manejo integrado del cultivo. Retrieved on 10/01/2016. Available in: ftp://ftp.fao.org/docrep/fao/010/a1359s/a1359s05.pdf.
15. García-Méndez, V. H.; Ortega-Arenas, L. D.; Villanueva-Jiménez, J. A.; Sánchez-Arroyo, H. 2016. Susceptibilidad de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) a insecticidas en Veracruz, México. Agrociencia. 50: 355-365.
16. Gómez-Vargas, J. M.; Pitty, A.; Miselem, M. 2006. Efecto del pH del agua en la efectividad de los herbicidas glifosato, fluazifop-p-butil y bentazon. Ceiba. 47(1-2): 19-23.
17. Gonçalves-Balan, M.; Abi-Saab, O. J. G.; do Amaral-Ecker, A. E.; Olivera-Migliorini, G. O. 2016. Description of the application method in technical and scientific work on insecticides. Acta Scientiarum Agronomy. 38: 9-17.
18. Gottwald, T. R. 2010. Current epidemiological understanding of citrus Huanglongbing. Annual Review Phytopathology. 48: 119-139.
19. Guillén, L.; Alcalá de M. D.; Fernández, S.; Pire, A. 2008. Percepción de los agricultores sobre el manejo integrado de plagas en el cultivo de tomate (*Lycopersicum esculentum* Mill.). Revista Facultad de Agronomía (LUZ). 25: 223-242.
20. Halbert, S. E.; Manjunath, K. L. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. Florida Entomologist. 87(3): 330-353.
21. Head, G.; Savinelli, C. 2008. Adapting insect resistance management programs to local needs. In: Onstad, D. W. 2013. Insect resistance management: biology, economics, and prediction. Elsevier Science. 89- 107.
22. Hofman, V.; Solseng, E. 2014. Spray equipment and calibration. North Dakota State University. Available in: https://www.ag.ndsu.edu/pubs/plantsci/crops/ae73.pdf (Retrieved on: October 2015).
23. Hurley, T. M.; Mitchell, P. D. 2008. Insect resistance management: adoption and compliance. In: Onsted, D. W. 2013. Insect resistance management: biology, economics, and prediction. Elsevier Science. 227-253.
24. Hurtado-León, I.; Toro-Garrido, J. 2007. Paradigmas y métodos de investigación en tiempos de cambio, modelos de conocimiento. 5ª Ed. Los Libros de El Nacional. Caracas. Venezuela. 166 p.

25. Hyun, J. W.; Ko, S. W.; Kim, D. H.; Han, S. G.; Kim, G. S.; Kwon, H. M.; Lim, H. C. 2005. Effective usage of copper fungicides for environment-friendly control of citrus diseases. Research in Plant Disease. 11: 115-121.
26. Inostroza-Fariña, J. 2009. Manual de papa para La Araucanía: Manejo y plantación. Boletín INIA (Chile). 193: 85-104.
27. Jallow, A. F. M.; Awadh, G. D.; Albaho, S. M.; Devi, Y. V.; Thomas, M. B. 2017. Pesticide risk behaviors and factors influencing pesticide use among farmers in Kuwait. Science of the Total Environment. 574: 490-498.
28. Jarvis, D. I.; Myer, L.; Klemick, H.; Guarino, L.; Smale, M.; Brown, A. H. D.; Sadiki, M.; Sthapit, B.; Hodgkin, T. 2006. Guía de capacitación para la conservación *in situ* en fincas. Instituto Internacional de Recursos Filogenéticos (IPGRI). Roma. Italia.189 p.
29. Lagunes-Tejeda, A.; Villanueva-Jiménez, J. A. 1994. Toxicología y manejo de insecticidas. Montecillo, México. Colegio de Postgraduados. Montecillo. México. 264 p.
30. Lagunes-Tejeda, Á.; Rodríguez-Maciel, J. C.; De Loera-Barocio, J. C. 2009. Susceptibilidad a insecticidas en poblaciones de artrópodos de México. Agrociencia. 43: 173-196.
31. Landini, F.; Murtagh, S. 2011. Prácticas de extensión rural y vínculos entre saberes locales y conocimientos técnicos. Contribuciones desde un estudio de caso realizado en la provincia de Formosa (Argentina). Ra Ximhai. 7: 263-279.
32. Landini, F. P.; Bianqui, V. 2016. Expectativas de los extensionistas rurales latinoamericanos y contribuciones psicosociales. Psicología & Sociedad. 28(1): 125-134.
33. Leiva, P. D. 2010. Consideraciones generales sobre la calidad del agua para pulverización agrícola. Pergamino (BA) INTA. Retrieved on June 2016. Available in: http://www.sindag.org.br/App_Uploads/trabalhos_tecnicos/32_documents_trabalhos_t ecnicos.pdf.
34. Massaro, R. A.; Fernández, C. 2013. Aplicación de plaguicidas: un concepto poco aplicado. AAPRESID Siembra Directa. (116): 41-46.
35. Mora-Aguilera, G.; Robles-García, P.; López-Arroyo, J. I.; Velázquez-Monreal, J.; Flores-Sánchez, J.; Acevedo-Sánchez, G.; Domínguez-Monge, S.; González-Gómez, R. 2013. Situación actual y perspectivas del manejo del hlb de los cítricos. Revista Mexicana de Fitopatología. 31: 108-116.
36. Onstad, D. W.; Guse, C. A. 2008. Concepts and complexities of population genetics. In: Onsted, D. W. 2013. Insect resistance management: biology, economics, and prediction. Elsevier Science. New York. 69- 88.
37. Ortiz, O. 2001. La información y el conocimiento como insumos principales para la adopción del manejo integrado de plagas. Manejo Integrado de Plagas (Costa Rica). (61): 12-22.
38. Osorio-Acosta, F.; Villanueva-Jiménez, J. A.; Ortega-Arenas, L. D.; García-Méndez, V. H.; Díaz-Zorrilla, U.; Pérez-Zarate, L. A.; Martínez-Hernández, R. 2015. Determinación de susceptibilidad a insecticidas de *Diaphorina citri* en Veracruz, Yucatán, Colima, Puebla y Michoacán, México. Informe técnico. Retrieved on April 2016: CESVVER-CP. Available in: https://drive.google.com/file/d/0B6bOl59pynbtNmd1b3BHcjFZY3c/view.
39. Peña, J. H. M. 2003. Eslabonamientos productivos entre la industria y el sector primario, una propuesta de medición: El caso de la cadena naranja-jugo concentrado en México. Claridades Agropecuarias. 119: 42-49.
40. Pérez-Zarate, L. A.; Osorio-Acosta, F.; Villanueva-Jiménez, J. A.; Ortega-Arenas, L. D.; Chiquito-Contreras, R. G. 2016. Factores que inciden en el control químico de *Diaphorina citri* Kuwayama en Áreas Regionales de Control. Southwestern Entomologist. 41(4): 1037-1050.
41. Robles-García, P. 2012. Protocolo para establecer áreas regionales de control del Huanglongbing y el psílido asiático de los cítricos (ARCOs). SENASICA. Retrieved on February 2016. Available in: http://www.siafeson.com/sitios/simdia/docs/protocolos/ProtocoloparaestablecerAreas RegionalesARCOSDICIEMBRE2012.pdf.
42. Robles-García, P. 2014. Áreas Regionales de Control (ARCOs) del Psílido Asiático de los Cítricos (*Diaphorina citri*). In: X Simposio Internacional Citrícola y 2° Simposio Internacional Sobre HLB en Cítricos Ácidos. Manzanillo. Colima. México.

43. Rozman K.; Doull, J. 2003. Scientific foundations of hormesis. Part 2. Maturation, strengths, and possible applications in toxicology, pharmacology, and epidemiology. Critical Reviews in Toxicology. 33: 451-462.
44. Ruiz-Nájera, R. E.; Ruiz-Nájera, J. A.; Guzmán-González, S.; Pérez-Luna, E. de J. 2011. Manejo y control de plagas del cultivo de tomate en Cintalapa. Chiapas. México. Revista internacional de Contaminación. 27: 129-137.
45. Sarandón, S. J.; Flores, C. C. 2014. Agroecología: bases teóricas para el diseño y manejo de agroecosistemas sustentables. La Plata: Universidad Nacional de la Plata, Argentina. 466 p.
46. SAS Institute Inc. 2013. What's New in SAS 9.4. Cary, USA. SAS Institute Inc. 200 p.
47. Timprasert, S.; Datta, A. A.; Ranamukhaarachchi, S. L. 2014. Factors determining adoption of integrated pest management by vegetable growers in Nakhon Ratchasima Province, Thailand. Crop Protection. 62: 32-39.
48. Urzúa-Soria, F. 2002. Técnicas de aplicación de plaguicidas. In: Martínez, N. B.; Gómez, O. D. 2001. Bases para realizar estudios de efectividad biológica de plaguicidas. Colegio de Postgraduados, Campus Veracruz. 148 p.
49. Van-Mele, P.; Salahuddin, A.; Magor, N. P. 2005. Innovations in Rural Extension: Case studies from Bangladesh. CABI Publishing, Wallingford. 311 p.
50. Vega-Gutiérrez, M. T.; Rodríguez-Maciel, J. C.; Díaz-Gómez, O.; Bujanos-Muñiz, R.; Mota-Sánchez, D.; Martínez-Carrillo, J. L.; Lagunes-Tejeda, A.; Garzón-Tiznado J. A. 2008. Susceptibilidad a insecticidas en dos poblaciones Mexicanas del Salerillo *Bactericera cockerelli* (Sulc.) (Hemiptera: Triozidae). Agrociencia. 42: 463-471.

Acknowledgements

The authors wish to thank CONACYT for the financial support provide to the first author, the technicians and managers of the *Comité Estatal de Sanidad Vegetal de Veracruz* (Veracruz State Plant Health Committee, CESVVER) and the *Junta Local de Sanidad Vegetal del Totonacapan* (Local Plant Health Board of Totonacapan) for their logistical support, and the growers of RCAs 4, 9, 10 and 11 of Veracruz, for their kindness and time provided.

Rev. FCA UNCUYO. 2020. 52(1): 121-127. ISSN (en línea) 1853-8665.

Sorghum silage production in the northern oasis of Mendoza, Argentina

Producción de sorgos sileros en el oasis norte de Mendoza, Argentina

Leandra Ibarguren, Cecilia Rebora, Alejandra Bertona, Carlos Antonini

Originales: *Recepción:* 06/06/2018 - *Aceptación:* 06/07/2019 *Nota científica*

Abstract

The study and use of preserved forages in the northern oasis of the province of Mendoza arise from the expansion of intensive farming in the area combined with the limited supply of forage due to the seasonal nature of perennial pasture production. Forage-type sorghums are a silage option because they adapt well to limiting edaphoclimatic conditions and have high forage yields. This paper evaluates four sorghum hybrids for whole-plant silage (ACA 558, ACA 715, ACA 740, Silero Inta-Pemán) cultivated in Mendoza's northern oasis (33°00'38" S and 68°52'28" O) during the 2015-2016 and 2016-2017 crop cycles. Genotypes were characterized according to green matter (GM) and dry matter (DM) yields, plant height, and to the panicle dry weight/whole-plant dry weight ratio. No significant differences ($p \geq 0.05$) were observed in GM or DM yields among the hybrids in the crop cycles under study. In the 2015-2016 crop cycle, the average GM and DM yields were 110,024 kg/ha^{-1} and 30,914 kg/ha^{-1}, respectively, whereas during the 2016-2017 crop cycle, the average GM and DM yields were 115,122 kg/ha^{-1} and 30.752 kg/ha^{-1}, respectively. The results obtained confirm that whole-plant sorghum silage is an interesting forage resource for intensive cattle farming in Mendoza.

Keywords
forage • intensive cattle farming • arid areas

Universidad Nacional de Cuyo. Facultad de Ciencias Agrarias. Departamento de Producción Agropecuaria. Cátedra de Agricultura Especial. Almirante Brown 500. Chacras de Coria - Luján de Cuyo. CPA M5528AHB. Mendoza. Argentina.
libarguren@fca.uncu.edu.ar

Resumen

El crecimiento de la ganadería intensiva en Mendoza y la falta de alimento debido a la estacionalidad de las pasturas durante el año, motivó el uso y estudio de forrajes conservados en la Provincia. Los sorgos forrajeros son una alternativa para ensilar, debido a su reconocida adaptación a condiciones edafoclimáticas limitantes y su elevada producción de forraje. En este trabajo se evaluaron cuatro híbridos de sorgo para silaje (ACA 558, ACA 715, ACA 740, Silero Inta-Pemán) cultivados en el oasis norte de Mendoza (33°00'38" S y 68°52'28" O), durante las campañas 2015-2016 y 2016-2017. Se caracterizaron los genotipos evaluados de acuerdo con: producción de materia verde (MV) y de materia seca (MS), altura de plantas y relación peso seco panoja/peso seco planta entera. No se observaron diferencias significativas en el rendimiento de MV ni en MS entre los híbridos en las campañas evaluadas. En la campaña 2015-2016 la MV promedio fue de 110.024 kg/ha y la MS de 30.914 kg/ha. En la siguiente campaña (2016-2017) se obtuvieron 115.122 kg/ha de MV promedio y 30.752 kg/ha de MS. Los resultados obtenidos confirman que el silaje de sorgo es un recurso forrajero interesante para intensificar la ganadería en la Provincia.

Palabras clave

forraje • producción ganadera intensiva • zonas áridas

Introduction

Known for a long time, silage preserves high moisture forage by storing it for fermentation, which takes place through the activity of anaerobic bacteria on the sugar content of cells. The process makes it possible to maintain a reduced pH under anaerobic conditions throughout forage preservation (5).

In Argentina, corn (*Zea maiz*) is the quintessential silage cereal; however, sorghum (*Sorghum bicolor* L.), because of its low water requirement, tolerance to high temperatures and highly efficient use of fertilizers, has recently become popular (6). Several characteristics of sorghum, although without the prestige of other cereal grains, make it a necessary option in areas with edaphoclimatic constraints. Sorghum does well in a broad ecological area extending approximately from 22° S to 40° S and delimited in the west by the 500 mm isohyet of annual precipitation. The southern boundary is determined by a period of 180 frost free days and by the 14°C mean annual isotherm (3).

According to Torrecillas (2006), the main advantages of silage sorghum production are the following: 1- Compared to corn, sorghum has a lower water requirement, higher nutrient uptake efficiency, and greater tolerance to drought and degraded soils. 2- Some sorghum genotypes are able to remain in a state of latency for prolonged periods of drought and then resume growth (although they do not reach full yield potential). 3- The vegetative component of many sorghum genotypes is of high nutritional quality. 4- Sorghum adapts well to low-fertility, saline and floodable soils.

Sorghum has a low planting cost

For whole plant sorghum silage to be of high nutritional quality special attention should be paid to some of the plant traits, such as digestibility. Parts of the plant, such as the lignin in the cane, are sacrcely digestible, reducing its nutritional value; therefore, lowering the cane ratio increases digestibility (6). BMR (Brown Mid Rib) sorghums are characterized by little lignin and high digestibility. The panicle/whole-plant ratio should also be high, as it is indicative of a high proportion of grains and, consequently, of the higher energy content of whole- plant silage. Another characteristic to be taken into consideration is the dry matter content of sorghum for silage, which should be at least 30%. Otherwise, nutrients are lost by leaching while clostridium growth and butyric acid production are promoted, increasing losses and reducing silage quality. Good whole-plant silage is important to enhance use efficiency of forage resources and profitability in cattle-raising systems. Successful silage begins with grain cultivation, continues with silo construction, preservation and supply, and ends with animal yield (meat or milk) (2).

The expansion of agriculture in Argentina, which has displaced cattle raising to extra-Pampean regions, has given the province of Mendoza the opportunity to engage in intensive cattle farming. Suitable agro-climatic conditions for the cultivation of forage crops, the availability of almost 100,000 hectares with irrigation rights among other strengths contribute to the potential for cattle farming in Mendoza's irrigated oases (9).

One of the main problems cattle farms are confronted with is the lack of feed due to the seasonal nature of pasture. For that reason, preserved forages are used as they ensure the supply of high quality and yield forage that meets beef cattle requirements (1). Forage sorghum, given its well-known adaptability to limiting soil and its climate conditions and high forage yield (10), is a good silage option for Mendoza. Moreover, in adverse conditions, sorghum usually does better than corn as forage (7, 8, 10).

Through various trials conducted within the framework of this study, regional information was obtained on the production of silage sorghum under the growing conditions prevailing in Mendoza's northern oasis. Specific objectives were to compare green matter (GM) and dry matter (DM) yields per hectare of four sorghum genotypes; and to characterize the genotypes assessed according to plant height and the panicle dry weight (DW)/whole-plant dry weight ratio.

The hypothesis is that dry matter yields per hectare vary among the different sorghum hybrids.

Materials and Methods

During the 2015-2016 and 2016-2017 crop cycles four sorghum hybrids - ACA 558, ACA 715, ACA 740, and Silero INTA-Pemán- were grown at the San Antonio agricultural experiment station of the Facultad de Ciencias Agrarias de la Universidad Nacional de Cuyo, Mendoza (33°00'38" S and 68°52'28" W). Its alluvial soil has a clay-loam texture, the mean annual temperature is 15.7°C and the mean annual rainfall is 248.4 mm (*Estación Meteorológica Chacras de Coria*, 1959-2013). The experimental plots had three 10 m-long furrows 0.60 m apart; and 15 seeds per linear meter were hand sown

(234,400 plants/ha). The experimental design consisted of randomly selected plots with three replications (4 hybrids times 3 replications = 12 experimental plots).

The plants were harvested when the grain was at the milk-wax stage of ripeness. At pre-harvest the number of plants was counted, and the height of 10 plants in the central row of each experimental plot was measured.

The central row of each plot was harvested (with scissors) to estimate GM yield. Three plants from each plot were weighed while green and separated to calculate the percentage of dry matter, panicle dry weight, and whole-plant dry weight.

Analysis of variance and comparison of means (Tukey test) were performed on the variables under study. Plot management is described in table 1.

Results and Discussion

The GM and DM yields of the hybrids evaluated in both crop cycles are shown in tables 2 and 3 (page 125). No significant differences ($p \leq 0.05$) in yields among genotypes were detected in both crop cycles. In the 2015-2016 crop cycle average GM and DM yields were 110,024 kg/ha^{-1} and 30,914 kg/ha^{-1}, respectively, whereas during the 2016-2017 crop cycle average GM and DM yields were 115,122 kg/ha^{-1} and 30,752 kg/ha^{-1}, respectively.

Table 1. Cultural management of the experimental plots- 2015-2016 and 2016-2017 crop cycles.

Tabla 1. Manejo cultural de las parcelas experimentales, campañas 2015-2016 y 2016-2017.

	2015-2016 crop cycle	**2016-2017 crop cycle**
Soil preparation	Cross harrowing & furrowing	Cross harrowing & furrowing
Sowing date	11/17/2015	10/25/2016
Fertilization 18-46-0 (at sowing)	150 kg	150 kg
Weed control	Manual	Manual
Water depth applied	Thirteen 30 mm irrigations: 390 mm Rainfall: 308 mm Total: 698 mm	Twelve 30 mm irrigations: 360 mm Rainfall:226.6 mm Total: 586.6 mm
Harvest date	03/17/2016	03/9/2017

Table 2. Green matter (GM) and dry matter (DM) yields, and percentage of dry matter (% DM) of the sorghum hybrids included in the trial. 2015-2016 crop cycle, Luján de Cuyo, Mendoza, Argentina.

Tabla 2. Producción de materia verde (MV), materia seca (MS) y porcentaje de materia seca (% MS) de los distintos híbridos de sorgo ensayados. Campaña 2015-2016, Luján de Cuyo, Mendoza, Argentina.

Hybrid	Plants at harvest (No.)	GM yield (kg/ha^{-1})	DM yield (kg/ha^{-1})	% DM
ACA 558	149,400±12,938 **a***	94,620±13,260 **a**	27,611±2,362 **a**	29.48±4.12 **a**
ACA 715	132,800±21,959 **a**	105,709±42,190 **a**	29,068±10,498 **a**	27.74±1.01 **a**
ACA 740	146,633±9,584 **a**	117,583±29,591 **a**	30,799±4,074 **a**	26.72±3.15 **a**
Inta Pemán	136,950±9,130 **a**	122,184±37,198 **a**	36,175±13,009 **a**	29.35±2.04 **a**

* Means followed by the same letter are not statistically different (Tukey's HSD).

* Medias con letras iguales indican que no hay diferencias significativas (Prueba de Tuckey).

Table 3. Green matter (GM) and dry matter (DM) yields, and percentage of dry matter (% DM) of the sorghum hybrids included in the trial. 2016-2017 crop cycle, Luján de Cuyo, Mendoza, Argentina.

Tabla 3. Producción de materia verde (MV), materia seca (MS) y porcentaje de materia seca (%MS) de los distintos híbridos de sorgo ensayados. Campaña 2016-2017, Luján de Cuyo, Mendoza, Argentina.

Hybrid	Plants at harvest (No.)	GM yield (kg/ ha^{-1})	DM yield (kg/ ha^{-1})	% DM
ACA 558	156,704±10,435 **a***	90,676±55,701 **a**	24,739±15,272 **a**	27.39±1.00 **a**
ACA 715	129,314±19,167 **a**	103,925±28,036 **a**	27,826±7,502 **a**	26.89±2.47 **a**
ACA 740	170,980±14,754 **a**	146,468±24,729 **a**	36,902±3,742 **a**	25.39±1.92 **a**
Inta Pemán	140,214±22,392 **a**	119,419±28,383 **a**	33,538±5,576 **a**	28.39±2.00 **a**

* Means followed by the same letter are not statistically different (Tukey's HSD).

* Medias con letras iguales indican que no hay diferencias significativas (Prueba de Tuckey).

Experiences in other parts of the country, though under rainfed conditions, show lower yields per hectare: 10,789 kg of DM/ha^{-1} and 43,754 kg of GM/ha^{-1} on average for 19 sorghum hybrids tested at the AER INTA Manfredi, province of Córdoba (3); and 12,250 kg of DM/ha^{-1} on average in tests carried out at EEA INTA Corrientes (4). With regards to dry matter percentage of the whole plant at harvest, no differences among hybrids were detected in none crop cycle: the average value was 28.32% for the first harvest and 27.01% the following year. Leiva *et al.* (2012) obtained a DM average of 25.61%.

With regard to plant height, there were significant differences ($p \leq 0.05$) among hybrids in both crop cycles, as shown in table 4. In the first harvest ACA 715, ACA 740 and Inta Pemán showed significant differences only with ACA 558. In the second harvest, ACA 715 and Inta Pemán exhibited no differences between them but with ACA 558 and ACA 740, which also differed among themselves.

The panicle dry weight/whole-plant dry weight ratio showed the same difference among hybrids in both crop cycles.

Table 4. Plant height, and panicle DW/ whole-plant DW ratio of the sorghum hybrids included in the trial. 2015-2016 and 2016-2017 crop cycles, Luján de Cuyo, Mendoza, Argentina.

Tabla 4. Altura de planta y relación PS panoja/PS planta entera de los distintos híbridos de sorgo ensayados. Ambas campañas, Luján de Cuyo, Mendoza, Argentina.

	2015-2016		**2016-2017**	
Hibryd	**Plant height (m)**	**Panicle DW / whole-plant DW (%)**	**Plant height (m)**	**Panicle DW / whole-plant DW (%)**
ACA 558	1.54±0.07 **a***	19.52±2.70 **c**	1.62±0.10 **a**	16.70±0.93 **c**
ACA 715	2.81±0.29 **b**	3.80±0.36 **a**	2.52±0.07 **b**	3.45±1.14 **a**
ACA 740	2.72±0.11 **b**	13.50±3.19 **bc**	3.07±0.03 **c**	10.42±4.43 **bc**
Inta Pemán	2.50±0.09 **b**	9.27±2.81 **ab**	2.7±0.09 **b**	7.39±1.66 **ab**

* Means followed by the same letter are not statistically different (Tukey's HSD).

* Medias con letras iguales indican que no hay diferencias significativas (Prueba de Tuckey).

Conclusions

It is feasible to achieve high yields from silage sorghum in Mendoza's northern oasis. Although the hybrids tested exhibited significant differences in plant height and in the panicle dry weight/ whole-plant dry weight ratio, their performance with regard to green matter yield (kg/ha^{-1}) and dry matter yield (kg/ ha^{-1}) showed no differences. Therefore, our working hypothesis is rejected.

The results obtained confirm that sorghum silage is an interesting and feasible forage resource for intensive cattle farming in the province of Mendoza. In future trials it would be desirable to determine forage quality by analyzing crude protein (CP) and acid detergent fiber (ADF) and, from the results then obtained, to calculate digestibility (Dig) and energy concentration (EC) of the chopped forage.

References

1. Carrillo, J. 2003. Manejo de Pasturas. EEA INTA Balcarce. 457 p.
2. Flores, J. 2015. Claves de un buen silo de sorgo. Avaliable in: http://marcaliquida.com.ar/claves-de-un-buen-silo-de-sorgo/ (Accessed May 2016).
3. Giorda, L. 2017. Variedades de sorgo para cada utilización y su manejo. Avaliable in: https://inta.gob.ar/sites/default/files/inta_manfredi_y_el_sorgo_variedades_de_sorgo_para_cada_utilizacion_y_su_manejo.pdf (Accessed May 2019).
4. Leiva, M.; Clausen, A.; Clausen, S. 2012. Evaluación de sorgos sileros en el noroeste de Córdoba. INTA, Cartilla digital Manfredi. Avaliable in: http://inta.gob.ar/sites/default/files/script-tmp-evaluacion_sorgos_sileros.pdf (Accessed May 2018).
5. Peiretti, J. 2019. Silajes: claves para incrementar la calidad en la presente campaña. Avaliable in: https://inta.gob.ar/sites/default/files/silaje_claves_para_incrementar_calidad.pdf (Accessed April 2019).
6. Pereyra, M.; Roig, H.; Acosta, F. 2011. Silo de sorgo: análisis económico de una estrategia de alimentación. Sitio argentino de producción animal. Avaliable in: http://www.produccion-animal.com.ar/produccion_y_manejo_reservas/reservas_silos/173-Silo_sorgo.pdf (Accessed May 2018).
7. Rebora, C.; Ibarguren, L. 2018. Producción de maíces y sorgos sileros en Mendoza. Suplemento Fincas Diario Los Andes. Avaliable in: https://losandes.com.ar/article/produccion-de-maices-y-sorgos-sileros-en-mendoza (Accessed May 2018).
8. Rebora, C.; Ibarguren, L.; Barros, A.; Bertona, A.; Antonini, C.; Arenas, F.; Calderón, M.; Guerrero, D. 2018. Corn silage production in the northern oasis of Mendoza, Argentina. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 369-375.
9. Redacción diario Los Andes. 2011. Ganadería bovina: desafío y oportunidad para Mendoza en la próxima década. Print edition 11/20/2011.
10. Redacción diario Los Andes. Suplemento Fincas 2017. Avaliable in: http://www.losandes.com.ar/article/maiz-forrajero-crece-la-superficie-cultivada-en-mendoza (Accessed February 2018).
11. Romero, A. 2004 Silaje del Sorgo. Avaliable in: http://www.produccion-animal.com.ar/produccion_y_manejo_reservas/reservas_silos/08-silaje_sorgo.pdf (Accessed May 2018).
12. Torrecillas, M. 2006. Sorgo para silo. Avaliable in: http://www.produccion-animal.com.ar/produccion_y_manejo_reservas/reservas_silos/60-sorgo_para_silo.pdf (Accessed May 2018).

Analysis of the implementation of the "Man and the Biosphere" programme in the biosphere reserves of Andalusia

Análisis de la implantación del programa "Hombre y la Biosfera" en las reservas de biosfera de Andalucía

Paula Andrea Castaño-Quintero *, María Victoria Gil-Cerezo,
Carmen Galán Soldevilla, Eugenio Domínguez-Vilches

Originales: *Recepción:* 18/04/2018 - *Aceptación:* 04/05/2019

Abstract

The Spanish Committee for the Man and the Biosphere (MaB) Programme runs a programme that monitors the Spanish Network of Biosphere Reserves. The monitoring programme features a system of indicators enabling the degree of implementation and the territorial integration of the Biosphere Reserves (BRs) located on Spanish territory. This paper sets out a statistical analysis based on the results of the degree of implementation for the BRs of Andalusia (Spain) obtained for the period 2008-2014. The analysis allows the identification of the indicators that have had the most influence on the degree of implementation in the Andalusian BRs, as well as the factors that may be strengthened in order to enhance the degree of implementation. The effectiveness of the indicator system will be improved if additional work is done to redefine those conceptual aspects that tend to generate discrepancies in the interpretation of compliance with the requirements of the variables. To improve the management of the Andalusian BRs, complementary studies to allow the evaluation of the impact of the initiatives related to the implementation of the MaB Program, should be carried out.

Keywords
Andalusia • degree of implementation • monitoring • participation in management • UNESCO MaB Programme

Universidad de Córdoba. España. Departamento de Botánica, Ecología y Fisiología Vegetal. Carretera Nacional IV, km 396. Código Postal 14014. Córdoba. España. * paulacastanoquintero@gmail.com

Resumen

El Comité Español del Programa MaB cuenta con el Programa de Seguimiento de la Red Española de Reservas de Biosfera. El programa dispone de un sistema de indicadores que permiten estudiar el grado de implantación y la integración territorial de las Reservas de Biosfera (RBs) del territorio español. En este trabajo se presenta el análisis estadístico efectuado a los resultados obtenidos del grado de implantación de las RBs de Andalucía (España) durante el período 2008-2014. El análisis ha permitido identificar los indicadores que mayor influencia han tenido en el grado de implantación de las RBs, así como los factores que podrían ser fortalecidos para incrementar este grado de implantación. La efectividad del sistema de indicadores se verá reforzada si se realiza un trabajo adicional para redefinir aquellos aspectos conceptuales que tienden a generar discrepancias en la interpretación del cumplimiento de los requisitos de las variables. Para mejorar la gestión de las RBs de Andalucía, se deberían llevar a cabo estudios complementarios que permitan la evaluación del impacto de las iniciativas relacionadas con la implantación del Programa MaB.

Palabras clave
Andalucía • grado de implementación • monitoreo • participación en la gestión • Programa MaB UNESCO

Introduction

The Biosphere Reserves (BRs) of the UNESCO MaB Programme are areas in which *"methods for managing natural resources are put to the test while simultaneously fostering economic development"* (41). However, the mere designation of an area as a Biosphere Reserve (BR) does not guarantee the real or full implementation of the concept (1, 8, 16, 43). At the 1995 International Conference of Seville, it was established that the competent authority would review the situation of each BR every ten years and submit a report based on the fulfilment of the criteria upon which they were designated (40). By means of such evaluation, it would be possible to assess the effectiveness of their management, thereby helping to determine the potential that such areas possess in terms of achieving their goals, identifying opportunities and threats, and encouraging stakeholders to adapt to changing conditions (31). This evaluation must contemplate an integral and multidimensional view of sustainability and be complemented with a systemic approach both in the conceptualization and in its operational component (37).

Various authors (29, 31) suggest that the 10-year interval between the periodic reviews is excessively long, posing challenges for the monitoring of BRs. Such challenges affect the efficiency of the periodic review process as an effective mechanism for ensuring their quality and degree of implementation (3, 30). The alternatives suggested in order to overcome the challenges include: establishing provisional mechanisms for submitting reports; reducing the time between periodic reviews, and the establishment of an information system with mechanisms and indicators enabling the state and effectiveness of the implementation of the BRs to be reviewed, being much more closely linked to the periodic review process (29, 30, 31).

The Spanish Network of Biosphere Reserves (Spanish acronym: RERB) comprises 48 areas, covers 10.9% of the total surface area of Spain (more than 5.5 million hectares) and encompasses a population of nearly two million inhabitants (4.12% of the total). The Spanish Committee for the MaB Programme is coordinated by the Autonomous Organisation of National Parks (Spanish acronym: OAPN) and runs a RERB Monitoring Programme. The RERB Monitoring Programme is one of various results obtained from the Montseny Plan of Action (Spanish acronym: PAMO) for the RERB (38). The PAMO was the adaptation carried out in the Spanish context of the Madrid Action Plan (Spanish acronym: PAM) for the RERB (26, 41). This Programme was designed to gather information about the state and evolution of the Spanish BRs and assess the attainments achieved in terms of the challenges set by the MaB Programme. Within the framework of the Monitoring Programme, the Reserves have been assessed over three periods: 2008-2010, 2010-2013 and 2013-2014.

In its initial stages, the RERB Monitoring Programme relied on a total of 17 indicators, each of them being a synthesis of a range of variables. Seven of these indicators were designed to provide information about the degree of consolidation of the RERB. The ten remaining indicators were designed to provide information about two fundamental aspects of Spanish BRs: their degree of implementation (fulfilment of the basic requirements stemming from the BR concept) and their territorial integration. Those who developed the indicator system (OAPN and TRAGSA) decided that the Spanish BRs' degree of implementation would be assessed by six indicators, and their territorial integration would be assessed by means of four indicators (38). In 2013 OAPN and TRAGSA presented a new system to assess the implementation of Spanish BRs by means of eight indicators (39). One of the reasons for carrying out these adjustments could have been probably, not wanting to lose sight of the ecological and social elements on which the BR's are based. These elements that go beyond the promotion of a practice or set of practices, must be understood by the researchers and local actors, who form the main basis for the construction and even more, the evaluation and transformation of the BR's and their agro-ecological systems (25). Since 2013, the methodology used, the results obtained and the appropriateness of the indicators themselves, have been analysed on an ongoing basis by the Management Council and by the Scientific Council, both advisory bodies to the Spanish Committee for the MaB Programme.

At the time of writing, the degree of implementation of the BRs that make up the RERB is assessed by eight indicators. Some of these indicators contain "lock" variables, a concept that had not been considered at the initial stages of the Monitoring Programme. Lock variables are those that, when they accrue a score of 0 for total non-compliance, render the indicator to which they correspond non-assessable due to non-fulfilment of a basic requirement of the MaB Programme (28). In order to obtain the final assessment of the BR, the non-assessable indicator is assigned a value of 0. A non-assessable indicator reduces the score obtained by the BR and is taken as evidence that, at its next periodic review, the BR may be subjected to a recommendation from the MaB Programme's International Coordination Council. In the event of this not being addressed, it may result in the triggering of the withdrawal mechanism of the World Network of Biosphere Reserves (WNBR).

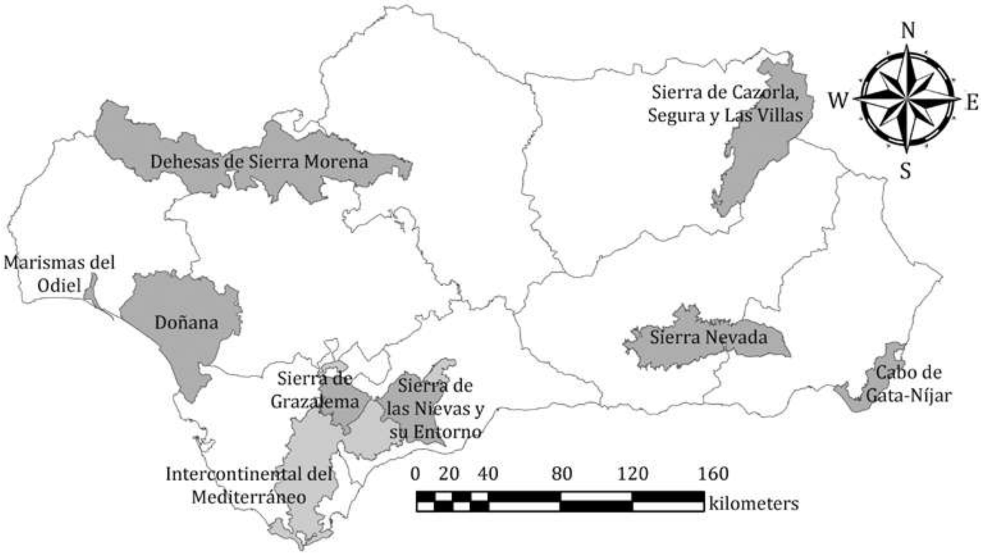
Based on the results obtained through the Monitoring Program of the Spanish Network of Biosphere Reserves, the objectives of this article are: i) to identify the behavior of the implementation of the Andalusian BRs over the 2008-2014 period and ii) to identify the incidence of the indicator "Participation in management" in the levels of implementation achieved by these BRs.

The hypotheses of this work are: i) In the three follow-up evaluations of the Spanish Network of Biosphere Reserves carried out during the period 2008 - 2014, the Biosphere Reserves of Andalusia obtained the same degree of implementation and ii) the indicator "Participation in management" is the indicator that has the greatest impact on the degree of implementation achieved by the BRs of Andalusia.

Materials and methods

Area of study

Nine of the 48 Spanish BRs are located in the Autonomous Community of Andalusia (southern Spain), and are known as the Andalusian Biosphere Reserve Network (Spanish acronym: RRBA). The RRBA (figure 1) comprises a significant part of the overall RERB in Spain, both in terms of number and in terms of designated surface area (table 1, page 132).



Source: Authors' own compilation, with information from the Environmental Information Network of Andalusia.
Fuente: Elaboración propia con información de la Red de Información Ambiental de Andalucía.

Figure 1. Geographical distribution of Andalusian Biosphere Reserves.

Figura 1. Distribución geográfica de las Reservas de Biosfera de Andalucía.

Table 1. Andalusian Biosphere Reserves.

Tabla 1. Reservas de Biosfera de Andalucía.

Andalusian Biosphere Reserves	Year of creation	Area (ha)	Provinces	N° municipalities (partial and/or total territorial integration)
Sierra de Grazalema (henceforth RBSG)	1977	51,695	Cádiz and Málaga	14
Doñana (RBDÑ)	1980	268,293	Huelva, Seville and Cádiz	14
Sierras de Cazorla, Segura y las Villas (RBCSV)	1983	217,000[a]	Jaén	26
Marismas del Odiel (RBMO)	1983	7,185	Huelva	4
Sierra Nevada (RBSNV)	1986	172,238	Almería and Granada Granada	60
Sierrra de las Nieves y su Entorno (SNyE)	1995	93,930	Málaga	11
Cabo de Gata-Níjar (RBCGN)	1997	49,624	Almería	3
Dehesas de Sierra Morena (RBDSM)	2002	427,400	Huelva, Seville and Córdoba	43
Intercontinental del Mediterráneo (IM)	2006	907,185.02 (423,535 in Andalusia)	Cádiz and Málaga (Andalusia) and Tétouan, Chefchaouen, Larache and Tangier (Morocco)	109[b]

Source: Authors' own compilation of data from the Environmental and Territorial Planning Council of the Andalusian Regional Government (Junta de Andalucía).

[a] Extended in 2014 as a response to the recommendations made by the ICC for the MaB Programme after its periodic review in 2003.

[b] 66 municipalities in Andalusia are included, 22 in the province of Cádiz and 39 in Málaga, while 48 municipalities are involved in Morocco, of which 23 belong to Tétouan, 17 to Chefchaouen, seven to the province of Larache and one to the province of Tangier.

Fuente: Elaboración propia con datos de la Consejería de Medio Ambiente y Ordenación del Territorio de la Junta de Andalucía (Junta de Andalucía).

[a] Ampliada en el año 2014 para dar respuesta a las recomendaciones que el CIC del Programa MaB emitió tras su revisión periódica del año 2003.

[b] En Andalucía participan 66 municipios, 22 de la provincia de Cádiz y 39 de Málaga, mientras que en Marruecos participan 48 municipios, de los que 23 pertenecen a la provincia de Tetuán, 17 a Chefchaouen, siete a la provincia de Larache, y uno a la provincia de Tánger.

Table 2. Implementation indicators of the RRBA BRs, expressed as percentages (2008-2014).

Tabla 2. Indicadores de implantación de las RBs de la RRBA, expresada en porcentajes (2008-2014).

INDICATOR	BR									RRBA
	SG*	DÑ*	CSV*	SNV*	MO*	SNyE*	CGN*	DSM*	IM*	
2008-2010 PERIOD										
Zoning	23.33	43.33	23.33	55.00	43.33	56.67	66.67	56.67	66.67	48.33
Management body	53.33	48.33	53.33	48.33	53.33	46.67	53.33	38.33	86.67	53.52
Participation in management	76.67	76.67	76.67	76.67	76.67	65.00	76.67	0.00	45.00	63.64
Planning and management tools	33.33	33.33	33.33	33.33	33.33	66.67	33.33	16.67	50.00	50.00
Available resources	50.00	50.00	50.00	50.00	50.00	100.00	50.00	50.00	33.33	53.70
Initiatives for the fulfilmentof functions	48.00	48.00	48.00	48.00	48.00	40.67	48.00	48.00	48.00	47.19
SYNTHESIS OF THE PERIOD	47.44	49.94	47.44	51.89	50.78	51.50	54.67	34.95	54.94	49.28
2010-2013 PERIOD										
Zoning	100.00	100.00	100.00	100.00	56.67	90.00	100.00	100.00	100.00	94.07
Management body	100.00	100.00	100.00	100.00	93.33	41.67	100.00	93.33	83.33	90.18
Participation in management	76.67	86.67	90.00	100.00	66.67	66.67	66.67	86.67	66.67	78.52
Management plan (and action programme)	76.67	76.67	76.67	100.00	76.67	53.33	76.67	76.67	86.67	77.78
Initiatives for fulfilment of the conservation function	93.33	100.00	93.33	100.00	76.67	83.33	83.33	90.00	93.33	90.37
Initiatives for the fulfilment of the development function	93.33	100.00	93.00	100.00	60.00	76.67	86.67	86.67	93.33	87.78
Initiatives for the fulfilment of the logistics support function	71.67	68.33	80.00	75	66.67	65.00	71.67	58.33	45.00	66.85
Participation in networks	90.00	90.00	90.00	100.00	90.00	100.00	90.00	90.00	90.00	92.22
SYNTHESIS OF THE PERIOD	87.71	90.21	90.42	96.88	73.33	72.08	84.38	85.21	82.29	84.72
2013-2014 PERIOD										
Zoning	100.00	100.00	33.33	100.00	0.00	33.33	33.33	33.33	100.00	59.26
Management body	100.00	100.00	73.33	100.00	65.00	75.00	73.33	73.33	73.33	81.48
Participation in management	100.00	100.00	100.00	100.00	80.00	76.67	66.67	86.67	56.67	85.19
Management plan (and action programme)	86.67	100.00	100.00	100.00	53.33	56.67	76.67	80.00	76.67	81.11
Initiatives for fulfilment of the conservation function	93.33	100.00	100.00	100.00	66.67	93.33	76.67	100.00	93.33	91.48
Initiatives for the fulfilment of the development function	93.33	100.00	93.00	100.00	60.00	86.67	86.67	86.67	86.67	88.15
Initiatives for the fulfilment of the logistics support function	65.00	75.00	80.00	66.67	55.00	65.00	71.67	71.67	58.33	67.59
Participation in networks	93.33	93.33	93.33	100	93.33	100	93.33	100	93.33	95.55
SYNTHESIS OF THE PERIOD	91.46	96.04	84.17	95.83	59.17	73.33	72.29	78.96	79.79	81.23

Source: Authors' own compilation from RERB Monitoring Programme reports (2011-2015).

Fuente: Elaboración propia a partir de los informes del Programa de Seguimiento de la RERB (2011-2015).

*.- Key to abbreviations: BR (Biosphere Reserves), SG (Sierra de Grazalema), DÑ (Doñana), CSV (Cazorla, Segura y las Villas), SNV (Sierra Nevada), MO (Marismas del Odiel), SNyE (Sierra de las Nieves and its surroundings), CGN (Cabo de Gata Níjar), DSM (Dehesas de Sierra Morena), IM (Mediterranean Intercontinental), RRBA (Andalusian Network of Biosphere Reserves).

*.- Clave de abreviaciones: BR (Reserva de Biosfera), SG (Sierra de Grazalema), DÑ (Doñana), CSV (Cazorla, Segura y las Villas), SNV (Sierra Nevada), MO (Marismas del Odiel), SNyE (Sierra de las Nieves y su entorno), CGN (Cabo de Gata Níjar), DSM (Dehesas de Sierra Morena), IM (Intercontinental del Mediterráneo), RRBA (Red de Reservas de Biosfera de Andalucía).

Sources for data used

The data used in this article are derived from the results of evaluating the implementation indicators of the RRBA BRs over the period 2008-2014. These data were obtained by the Spanish Committee for the MaB Programme, via application of the RERB Monitoring Programme (28, 38, 39). The main data used are set out in table 2 (page 133).

Calculation of new indicators

Owing to the fact that the indicators for the 2008-2010 period differ from those of the other two periods (2010-2013, 2013-2014), a series of statistical inferences (atypical values, jumps or discontinuities, concentrations of values, variable of those that make up the indicator, possible response options to the variable, percentage contribution of the variable in the final value of the indicator) were made to obtain the data that would enable a statistical analysis of the whole period (2008-2014).

When checking the methodology used to calculate the indicator of "Initiatives for the fulfilment of functions" for the period 2008-2010 (38), it was noted that it comprised variables that were the equivalent to some of the new indicators included in the reports for the 2010-2014 period (29, 39). Thus, bearing in mind the contribution of each indicator, its corresponding value for the 2008-2010 period was calculated. The variable-indicator equivalences were the following:

- "Number of initiatives that fundamentally contribute to fulfilment of the conservation function" was considered to be equivalent to the indicator "Initiatives for fulfilment of the conservation function".
- "Number of initiatives that fundamentally contribute to fulfilment of the development function" was considered to be equivalent to the indicator "Initiatives for fulfilment of the developments function".
- "Number of initiatives that fundamentally contribute to fulfilment of the logistics support function" was considered to be equivalent to the indicator "Initiatives for fulfilment of the function for logistics support".

This was done because the variables used to calculate the indicators are basically descriptive. For each, there were four possible options, so, the possible responses of these variables were like the ones that make up the indicator for which equivalence was proposed.

In addition, indicator 8, "Participation in networks", included in 2010-2013 and 2013-2014, was not considered in the report for the 2008-2012 period. For its calculation, quantitative and qualitative analyzes of each of the variables that make up each of the indicators of the three periods analyzed, were carried out, according to the methodology proposed by the OAPN (28), combining this with multiple regression techniques applied to all the BRs in Spain. Finally, indicator 3, "Instruments for planning and management", for the 2008-2010 period was deemed to be the exact equivalent of indicator 4, "Management plan (and action programme)", in the other two periods.

The indicators for the whole period (2008-2014) as well as the abbreviations used in the statistical analysis are shown in table 3 (page 135).

Table 3. Indicators analysed for the RRBA (2008-2014).
Tabla 3. Indicadores analizados para la RRBA (2008-2014).

Indicator	Abbreviations for the statistical analysis
Zoning	IND 1
Management body	IND 2
Participation in management	IND 3
Management plan (and action programme)	IND 4
Initiatives for fulfilment of the conservation function	IND 5
Initiatives for fulfilment of the development function	IND 6
Initiatives for fulfilment of the logistics support function	IND 7
Participation in networks	IND 8

Methodology for the statistical analysis of the data

An exploratory analysis of the data was carried out using the ANOVA (21) procedure. This enabled the normality and the homogeneity of the indicator variances to be assessed to a 95% level of confidence.

The variance analysis and the test of means were carried out using the GLM procedure (36) with a level of significance of 0.05. For the analysis of variance, the three periods being studied (2008-2010, 2010-2013 and 2013-14) were assumen as treatments. And for the means test, Tukey's studentized range was used (36).

As well as providing the mean for each period, the test of means enabled identification of the minimum significant differences. These two analyses (the analysis of variances and test of means) were used in conjunction to compare the means of the indicators over the different periods of study. The comparison made it possible to determine the differences between the three periods in terms of two basic aspects: i) the performance of each indicator and ii) the degrees of implementation achieved. All these enabled to establish the importance of one or more periods of time.

In addition, canonical discriminant analysis (CDA) (12) was used to identify the influence that each of the indicators had on the degrees of implementation achieved in the RRBA. The CDA groups correspond to the periods under study. The statistical analysis was based on the following multivariate lineal model:

$$yijkh = \mu h + BRih + Pjh + \varepsilon ijkh$$

where:

yijkh = multivariate vector of the k observation relating to the h variable for the BRi and period j.

μh = multivariate vector of general means relating to the h variable.

BRih = multivariate vector of the effects of the RBi on the h variable.

Pjh = multivariate vector of the period j on the h variable.

εijkh = multivariate vector for random errors associated with the observations vector yijkh.

(In the present study, the multivariate vector of the effects of interaction between BRi and the period j on the h variable was not included in the model, because no repetitions were present).

With the standard variables obtained from the analysis, a canonical discriminant graph was drawn up (20).

The Minitab (Minitab Inc., State College, Pennsylvania) and SAS version 9.4 (SAS Inst., Inc., Cary, North Carolina) programs were used to manage the data and perform the calculations.

Results

Table 4 shows the values of the indicators for the period 2008-2010 that were used for the analysis of the entire 2008-2014 period. The values of indicators 1, 2 and 3 correspond to those obtained with the RERB Monitoring Program. The values of indicators 4, 5, 6, 7 and 8 correspond to those calculated in this article.

Exploratory analysis of the indicator data for the period 2008-2014 showed that none of the indicators presented any significant deviation regarding the assumptions of normality and homogeneity of the variances to a degree of 95% of confidence. This fact revealed an absence of limitations for conducting the ANOVA.

Table 4. New implementation indicators for RRBA 2008-2010 in percentages.
Tabla 4. Nuevos indicadores de implantación para la RRBA 2008-2010 en porcentajes.

INDICATOR	BR									RRBA
	SG	DÑ	CSV	SNV	MO	SNyE	CGN	DSM	IM	
1. Zoning	23.33	43.33	23.33	55.00	43.33	56.67	66.67	56.67	66.67	48.33
2. Management body	53.33	48.33	53.33	48.33	53.33	46.67	53.33	38.33	86.67	53.52
3. Participation in management	76.67	76.67	76.67	76.67	76.67	65.00	76.67	0.00	45.00	63.64
4. Management plan (and action programme)	33.33	33.33	33.33	33.33	33.33	66.67	33.33	16.67	50.00	37.04
5. Initiatives for fulfilment of the conservation function	66.66	66.66	66.66	33.33	66.66	0.00	66.66	66.66	33.33	51.85
6. Initiatives for fulfilment of the development function	33.33	33.33	33.33	66.66	33.33	66.66	33.33	33.33	66.66	44.44
7. Initiatives for fulfilment of the logistics support function	66.66	66.66	66.66	66.66	66.66	66.66	66.66	66.66	66.66	66.66
8. Participation in networks	64.64	64.64	64.64	76.49	64.64	76.49	64.64	64.64	76.49	68.59
SYNTHESIS OF THE PERIOD	52.24	54.12	52.24	57.06	54.74	55.6	57.66	42.87	61.44	54.22

The ANOVA of the indicators, to a 0.05 degree of significance, highlighted significant differences in indicators 1, 2, 4, 5, 6 and 8, but not in indicators 3 or 7 (table 5).

The results of Tukey's test of means are shown in table 6 (page 138). It is noticeable that seven of the eight indicators show no significant differences for the periods 2010-2013 and 2013-2014. The 2008-2010 period is significantly different from the other two periods for five of the eight indicators. The three periods analysed do not show significant differences for indicators 3 and 7. The 2010-2013 period exhibits significant differences with respect to the other two for indicator 1.

The multivariate analysis of the data using CDA revealed a significant effect ($\alpha = 0.05$) for the result of Wilks's multivariate Lambda test (table 7, page 138). The value obtained by running the CDA test with this statistic (0.008 with $P < 0.0001$), indicates that the multivariate contrast that explains the relationship between the values of the nine reserves' eight indicators in the three periods analyzed, is significant ($\alpha = 0.05$). This statistic also revealed that there is separation between groups and a supposition of multivariate error normality.

The CDA results for the relationship between the effects of BR indicators and P, demonstrate that this relationship needs only two dimensions in order to be represented (table 8, page 138). However, out of these two dimensions, only the first is significant ($\alpha = 0.05$), entailing that the relation is one-dimensional. The eigenvalue proportion (or the proportion of explained variability) of the first canonical variable (Can 1) is 0.991, which indicates that the first canonical function represents 99.1% of the total variation of the relationship between the effects of the RBs and P (table 8, page 138). The second canonical variable (Can 2) only accounts for 0.9% of the said variation.

Figure 2 (page 139), shows the canonical discriminant structure of the three periods analyzed.

Table 5. ANOVA for the degree-of-implementation indicators of the RRBA BRs, 2008-2014.

Tabla 5. Análisis de varianza para los indicadores del nivel de implantación de las RB de la RRBA (período 2008-2014).

Indicator	Sum of squared error	Mean squared error	Value of F	Pr
IND1	16,708.88	696.20	7.40	0.0031
IND2	5,940.99	247.54	13.34	0.0001
IND3	8,843.78	368.49	3.06	0.0653 *
IND4	5,232.49	218.02	24.86	<0.0001
IND5	6,324.63	263.53	17.40	<0.0001
IND6	4,646.16	193.59	29.36	<0.0001
IND7	1,342.20	55.92	0.04	0.9617 *
IND8	448.02	18.67	322.43	<0.0001

$^*Pr > \alpha = 0.05$.

Table 6. Means test for the degree of implementation indicators of the RRBA BRs, 2008-2014.

Tabla 6. Prueba de medias para los indicadores del nivel de implantación de las RB de la RRBA 2008-2014.

Tukey's studentised range test (HSD) (α = 0.05)				
VARIABLE	**2008-2010**	**2010-2013**	**2013-2014**	**df**
IND1	48.220 b	94.070 a	59.260 b	31.062
IND2	53.517 b	90.184 a	81.480 a	18.522
IND3	63.336 a	78.521 a	85.187 a	22.598
IND4	37.036 b	77.780 a	81.112 a	17.382
IND5	51.847 b	90.369 a	91.481 a	19.111
IND6	44.440 b	87.778 a	88.149 a	16.380
IND7	66.660 a	66.852 a	67.593 a	8.804
IND8	49.189 b	92.222 a	95.553 a	5.086

Values with different letters in the same row differ significantly between periods.

Valores con letras diferentes en la misma fila difieren significativamente entre periodos.

Table 7. Statistics of multiple variables and F approximations.

Tabla 7. Estadísticos de múltiples variables y aproximaciones F.

Statistic	**Value**	**F-Value**	**Num DF**	**Den DF**	**Pr > F**
Wilks's Lambda	0.008	21.30	16	34.0	<.0001
Pillai trace	1.382	5.03	16	36.0	<.0001
Hotelling-Lawley trace	73.107	74.64	16	24.5	<.0001
Roy's largest root	72.453	163.02	8	18.0	<.0001

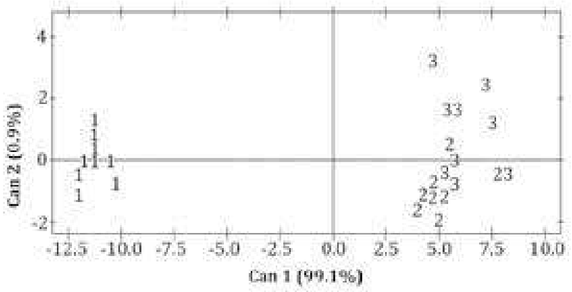
The F statistic for Roy's largest root is an upper limit. The F statistic for Wilks's Lambda is exact.

El estadístico F para la raíz mayor de Roy es un límite superior. El estadístico F para Lambda de Wilks es exacto.

Table 8. Summary of CDA for the relationship between the effects of the BR indicators and P.

Tabla 8. Resumen del ADC para la relación entre los efectos de los indicadores de las RBs y P.

Canonical variable	**Canonical correlation**	**Eigenvalue**	**Proportion eigenvalue**	**Accumulated proportion**	**Value of probability**
Can 1	0.99	72.453	0.991	0.991	<.0001
Can 2	0.63	0.654	0.009	1.000	0.1764



Note: A hidden observation.
Nota: Una observación oculta.
Key to abbreviations: 1 (BRi in the period 2008-2010); 2 (BRi in the period 2010-2013); 3 (BRi in the period 2013-2014).
Clave para las abreviaturas: 1 (RBi en el periodo 2008-2010); 2 (RBi en el periodo 2010-2013); 3 (RBi en el periodo 2013-2014).

Figure 2. Canonical discriminant structure plot of the three periods analysed.
Figura 2. Estructura discriminante canónica de los tres periodos analizados.

The impact of both canonical axes is observable in 100% of the total variability. In the case of the first factorial plane (Can 1 *vs.* Can 2), 99.1% of the variation between the periods being analyzed is accounted by the first canonical dimension (Can 1), whereas the second canonical dimension (Can 2) only accounts for 0.9% of variability. Can 1 is mainly aligned with the second and third periods (2012-2013 and 2013-2014). Can 2 is determined mainly by the first period (2008-2010).

Table 9, shows the coefficients of total canonical structure (also known as the correlation structure or canonical discriminant weights), which indicate the correlations between the indicators and the canonical functions. From this table it may be observed that Can 1 is strongly dominated indicator 8, followed by indicators 6 and 4 and to a lesser extent by indicators 5 and 2, all of them positive.

Table 9. Coefficients of total canonical structure.

Tabla 9. Coeficientes de estructura canónica total.

Variable	Total canonical structure	
	Can1	**Can2**
IND1	0.403	**-0.748**
IND2	**0.702**	**-0.319**
IND3	0.440	0.178
IND4	**0.827**	0.022
IND5	**0.774**	-0.033
IND6	**0.847**	-0.059
IND7	0.040	0.065
IND8	**0.989**	0.020

Can 2 is dominated by indicators 1 and 2, both with negative values and not as large as those of the first canonical axis. Thus, it is evident that the total variation in the relationship between the effects of the BR indicators and P is due, principally, to indicators 8, 6 and 4, and therefore, these three indicators are responsible for a major portion of the discrimination between the combinations of the BR indicators and P.

Discussion

Implementation performance

Two clearly differentiated phases, 2008-2010 and 2010-2014, become apparent after the analysis of the implementation period of the RRBA BRs (2008-2014). In the first phase there was a lesser degree of implementation than in the second, as shown by the values obtained in the test of the indicators' means (table 6, page 138). The differences between these two phases may be explained by i) the fact that the periodic application of the indicators has served as a learning tool for the Andalusian BRs (31), something that also emerges from the approval of all these reserves' periodic review reports over the last ten years, some of them without receiving any type of recommendation from the MaB Programme ICC; ii) the effort expended by the Spanish Committee for the MaB Programme to improve the understanding, the differences in criteria and the application of the indicators; iii) the managerial improvements in these areas instituted by the competent body of the Andalusian Regional Government.

The indicators that recorded improved results in the second phase were: Indicator 2, "Management body", Indicator 4, "Management plan and action programme", Indicator 5, "Initiatives for fulfilment of the conservation function", Indicator 6, "Initiatives for fulfilment of the development function" and Indicator 8, "Participation in networks".

The management body for Andalusian BRs is the Environment Department of the Andalusian Regional Government, which it delegates responsibility for action on the ground to the manager of each BR (2). The improvements in indicator 2, "Management body", may be related to the increase and/or advances in: i) the consultation-participation and decision-taking mechanisms; ii) the representation of various public administrations with territorial faculties; iii) the ability to promote and implement an integrated, participatory and sustainable management plan; iv) the fulfilment of the Andalusian Biosphere Reserves Committee's functions. (The Andalusian Biosphere Reserves Committee is an advisory and coordination body reporting to the Andalusian Regional Government on the subject of Biosphere Reserves, which has, among other functions, the task of supporting managing coordination Andalusian BRs).

The management plans of the Andalusian BRs have been the main instruments for the management and planning of the protected areas that constitute them (6). In this context, the progress shown by indicator 4, "Management plan (and action programme)", may be due to: i) improvements and adaptations of the contents of the management tools of the protected areas that constitute the RRBA. It is expected that within the management and planning instruments of Andalusia's protected areas, the goals and functions of the BRs to which they belong are explicitly set out. ii) the design of specific management schemes for one or more of Andalusia's BRs; iii) drafting and/or improvements in the action programmes. The action programmes should i) incorporate the goals and the three functions of the BRs, ii) have the resources needed for their application and iii) make provisos for a research and monitoring programme.

It is no surprise that Indicator 5, "Initiatives for fulfilment of the development function", exhibited improved results in the second phase. The Autonomous Community of Andalusia has been characterised by its interest, commitment and dynamism in environmental matters, particularly the protection of natural resources (22, 33). This is demonstrated by the numerous regional programmes and schemes geared towards the conservation, protection and recuperation of various aspects of natural heritage that complement those derived from national and international origins.

Meanwhile the improved results for indicator 6, "Initiatives for fulfilment of the development function", may reflect the effort made by the Andalusian Regional Government to offer local inhabitants opportunities to improve their quality of life and welfare by making sustainable use of natural resources. It is acknowledged that the protected areas currently comprising the Andalusian BRs, have become places where the environment is appreciated as a basic productive resource for sustained economic growth, thus turning the business sector into a key part of sustainable development (11). In keeping with this, it may be supposed that the Andalusian BRs have made headway in learning to master the rational exploitation and conservation of natural heritage and encouraging integrated human development, which are basic goals of all BRs (9).

Indicator 3, "Participation in management", and Indicator 7, "Initiatives for fulfilment of the logistics support function", present a very consistent trajectory over the three periods analysed, probably due to the fact that they are components in which Andalusian BRs have exhibited particular strengths, and hence no major effort has been made to improve them, and/or they have been assigned secondary priority in the management of these reserves.

Indicator 1, "Zoning", is the indicator that presents the most inconsistent pattern. The mean value of this indicator during the second period of study, is significantly greater than the first and third periods. In other words, there was considerable improvement in the second period with regards to the first, but the mean decreased considerably in the third period compared to the second, regressing to the values obtained in the initial period. This performance may be explained by: i) more thorough reviews of zoning, which led to falls in this parameter in some of the BRs; ii) differences of criteria in the way the indicators were applied over the course of the three periods; iii) the influence of "lock" variables on the annulment of this indicator in some BRs; iv) the alteration of the indicators, revealing cases of basic non-fulfilment of the MaB Programme.

Influence of the indicators on the degree of implementation

The indicators that have the greatest influence on the degree of implementation achieved by the BRs of Andalusia, are Indicator 8, "Participation in networks", Indicator 6, "Initiatives for fulfilment of the development function", and Indicator 4, "Management plan (and action programme)". These results differ from the findings reported by the Vietnam BRs (4).

Stakeholders in the latter reserves, perceive that the key factors most impinging on their management, and thus , that explain the successful implementation of the MaB Programme, are: Participation and collaboration; Governance; Finance and resources; Awareness and communication; and Management and implementation (4).

It appears that the degrees of implementation achieved by the BRs of Andalusia may be explained by: i) the exchange of knowledge and experiences using national and international environmental networks; ii) the relatively large population residing within them, and the actions carried out by the Managing Body to encourage and investigate sustainable development, and to integrate it into conservation; iii) the contents of the management plans, the degree to which policies are integrated into these and their corresponding action programmes. Bearing all these results in mind, there is a case to be made for the RRBA bringing forward improvement and strengthening initiatives for the management plan and the development function, since it may be possible to increase the degree of implementation of the MaB Programme at such Reserves.

The indicator with the least influence on the degrees of implementation achieved by Andalusian Biosphere Reserves appears to be Indicator 7, "Initiatives for fulfilment of the logistics support function", followed by Indicator 1, "Zoning" and Indicator 3, "Participation in management". According to the results obtained, initiatives related to the investigation and management of knowledge, to communication and to territorial visibility have not been determining factors in the implementation of these reserves. As far as Schliep and Stoll-Kleemann (2010) are concerned, the weaknesses existing in the implementation of the BR concept may be corrected by improving communication between the interested parties and encouraging the development of capabilities. Meanwhile, as far as the MaB Programme is concerned, people and organisations should be equipped with the ability to address the functions and designation criteria of BRs (42). To this end, it would be worth carrying out studies and action plans in order to strengthen the logistics support function in Andalusian BRs, in spite of the fact that to date, it may not have been a key factor in their implementation.

Indicator 3, "Participation in management", appears to have had little influence on the degrees of implementation of Andalusian BRs over the 2008-2014 period. Thus, it may be said that the current levels of participation achieved by these reserves, have not been determining factors in the outcomes of the MaB Programme, which may be an indication of the participatory processes not having been completely developed (35). This indicator provides information on the organ of participation, on the representativeness of social stakeholders, the level of participation and the social stakeholders capacity to influence. However, this indicator is difficult to rate because there are still conceptual and methodological gaps with regard to participation in the management of a BR and its assessment (28). Moreover, the indicator does not allow for assessment of the effectiveness of the dynamics of the participatory processes, the level of organic and functional representation, nor applications that would involve new stakeholders in the management of the BR (5).

It is important to emphasize that the participatory process of a BR can help to correct those aspects that hinder its implementation (34) possibly due to social learning, the building of relationships and the improvement in the understanding of other participants perspectives that this process generates (27). In this context, the results support the recommendation made by Schultz *et al*. (2011) on the desirability of carrying out further in-depth studies that would allow other factors, related to participation, to be analysed, such as governance structures and management practices. And those made by Hernandez-Hernandez *et al.* (2018), related to the essential strengthening of the links between the actors. Otherwise, the territory will continue to suffer the effects of the disarticulation in space and time, where local actors can take effective measures to build a territory socially fair, economically viable and harmoneous (14). Studies of this sort might contribute to the management and implementation of BRs in general, and particularly in Andalusia.

Monitoring indicators and the impact of BRs

The system of indicators used for the RERB has been useful in reviewing the state and the effectiveness of the implementation of BRs in Andalusia, enabling the basic MaB Programme requirements to be measured. Nevertheless, the system does not allow for assessment of the quality and characteristics of the initiatives carried out to comply with the functions and designation criteria of the BRs, nor their impact on sustainable development. In other words, the system does not allow for the attainment of the goals set out in their management assessed instruments.

In general terms, when a protected area, and particularly a BR, is effectively managed and administered, it becomes a secure site for the conservation of biodiversity and for the provision of ecosystems services that in turn contribute to humanity welfare (7, 10, 13, 15, 17, 44). There is thus a need, as other authors have acknowledged, to identify performance indicators for BRs enabling the effectiveness of attainment of its goals to be evaluated, and hence its contribution to global targets for conservation and sustainability (19).

The evaluation of learning and the progress made towards a BR's sustainable development is a major challenge that needs to be addressed by those in charge of its periodic review (31). The creation of a methodological process enabling the headway made by these Andalusian BRs to be evaluated in terms of the attainment of sustainable development, would improve their periodic review processes. Such an evaluation would provide information about the management impacts of Andalusian BRs, confirming whether such territories are fulfilling the goal of becoming exemplary places for the testing and demonstration of sustainable development methods at a regional level (29, 40). The information obtained could foster a process of ongoing learning accompanied by reflection and innovation, and allowing the creation of appropiate policies and strategies for the territory, giving effective responses to the current context of global socio-ecological change (18). These policies and strategies must be carefully planned, considering all the actors involved in benefit of the environment, natural resources and inhabitants of the BRs. In addition, the aforementioned policies and strategies should prioritize the rational use of resources and the regional economic benefit, whose primary objective should be to maintain the integral sustainability of the territory by contextualizing social, economic and environmental benefits (23, 24, 32).

Conclusions

The study of the performance of values obtained from the Monitoring Programme indicators of the RERB has enabled different phases in the implementation of Andalusian BRs to be established. The statistical methods used have enabled identification of those aspects of the management of Andalusian BRs that need to be strengthened in order to increase their levels of implementation. Nevertheless, there are still many aspects that need to be estudied in terms of each of the factors that shape the way the BRs of Andalusia are implemented.

The system of indicators used by the RERB has enabled the degree of fulfilment of the basic requirements of the MaB Programme in the Andalusian BRs, to be evaluated. Although the system has helped to address the periodic review process of these Reserves in a positive manner, its effectiveness in evaluating implementation will be enhanced if further work is done on redefining those conceptual aspects that tend to lead to discrepancies of interpretation among the interested that participate in assessing the indicators. These interpretation discrepancies could also be reduced if: i) the evaluation was carried out jointly between the manager of the BR and a member of the Scientific Council; ii) a checklist of compliance with the requirements and conditions of the assessment assigned to each variable was completed; and iii) the respective evidence supporting the valuation assigned to each variable was provided. Complementary studies enabling in-depth investigation of the characteristics, the quality and impact of the initiatives taken in the BRS of Andalusia in terms of the fulfilment of designation criteria, and the functions befitting BRs, should also be carried out. Some of these complementary studies could be the evaluation of the level of compliance with the Sustainable Development Goals and the assessment of the management of the BR by the local communities.

References

1. Bridgewater, P. 2016. The Man and Biosphere programme of UNESCO: rambunctious child of the sixties, but was the promise fulfilled? Current Opinion in Environmental Sustainability. 19: 1-6.
2. Castaño-Quintero, P. A. 2015. Tercer informe de revisión periódica de la Reserva de Biosfera Sierras de Cazorla, Segura y las Villas. Descripción del proceso de elaboración. Trabajo de Fín de Máster en el Máster "Cambio Global: Recursos Naturales y Sostenibilidad" de la Universidad de Córdoba. Accessed August 10 2017. Avaliable in: http://hdl.handle.net/10396/14087.
3. Coetzer, K. L.; Witkowski, E. T.; Erasmus, B. F. 2014. Reviewing Biosphere Reserves globally: effective conservation action or bureaucratic label? Biological Reviews. 89(1): 82-104.

4. Cuong, V. C.; Dart, P.; Dudley, N.; Hockings, M. 2017. Factors influencing successful implementation of Biosphere Reserves in Vietnam: Challenges, opportunities and lessons learnt. Environmental Science & Policy. 67: 16-26.
5. Domínguez-Vilches, E.; Gil-Cerezo, M. V.; González-Barrios, A. J.; León, Y.; Domínguez, J. 2012. Validación del Sistema de Indicadores de la RERB para las Reservas de Biosfera Andaluzas. Unpublished report.
6. Domínguez-Vilches, E.; Gil-Cerezo, M. V.; González, A. J.; Castaño-Quintero, P. A. 2014. Informe Decenal de la Reserva de Biosfera Sierras de Cazorla, Segura y las Villas. Programa de Seguimiento de la Red Española de Reservas de Biosfera. Comité Español del Programa MaB. Unpublished report.
7. Edgar, G. J.; Stuart-Smith, R. D.; Willis, T. J.; Kininmonth, S.; Baker, S. C.; Banks, S.; Barrett, N. S.; Becerro, M. A.; Bernard, A. T. F.; Berkhout, J.; Buxton, C. D. 2014. Global conservation outcomes depend on marine protected areas with five key features. Nature. 506(7487): 216.
8. Fernández-Galiano, E. 1984. Las Reservas de la Biosfera. Montes. 1: 26-29.
9. García, F. 1995. El programa MaB (Hombre y la Biosfera) en España. Bios. 1: 18-19.
10. Geldmann, J.; Barnes, M.; Coad, L.; Craigie, I. D.; Hockings, M.; Burgess, N. D. 2013. Effectiveness of terrestrial protected areas in reducing habitat loss and population declines. Biological Conservation. 161: 230-238.
11. Gessa, A.; Toledano, N. 2011. Turismo, emprendimiento y sostenibilidad en los espacios naturales protegidos: El caso de Andalucía-España. Estudios y perspectivas en turismo. 20(5): 1154-1174.
12. Hair, J. F.; Black, W. C.; Babin, B. J.; Anderson, R. E.; Tatham, R. L. 1998. Multivariate data analysis Uppersaddle River. Multivariate Data Analysis (5th ed) Upper Saddle River.
13. Halpern, B. S. 2014. Conservation: making marine protected areas work. Nature. 506(7487): 167.
14. Hernández Hernández, M. L.; Gallardo Cobos, R.; Dios-Palomares, R.; Martínez Pellégrini, S. E. 2018. An application of network theory to territorial analysis: The case of Yaqui Valley networks (2006 and 2016). Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 311-329.
15. Hutchings, J. A.; Côté, I. M.; Dodson, J. J.; Fleming, I. A.; Jennings, S.; Mantua, N. J.; Peterman, R. M.; Riddell, B. E.; Weaver, A. J.; VanderZwaag, D. L. 2012. Is Canada fulfilling its obligations to sustain marine biodiversity? A summary review, conclusions, and recommendations. Environmental Reviews. 20(4): 353-361.
16. Ishwaran, N. 2012. Science in intergovernmental environmental relations. 40 years of UNESCO's Man and the Biosphere (MAB) Programme and its future. Environmental Development: 1(1): 91-101.
17. Juffe-Bignoli, D.; Burgess, N. D.; Bingham, H.; Belle, E. M. S.; De Lima, M. G.; Deguignet, M.; Bertzky, B.; Milam, A. N.; Martinez-Lopez, J.; Lewis, E.; Eassom, A.; Wicander, S.; Geldmann, J.; Van Soesbergen, A.; Arnell, A. P.; O'Connor, B.; Park, S.; Shi, Y. N.; Danks, F. S.; MacSharry, B.; Kingston, N. 2014. Protected planet report 2014. UNEP-WCMC. Cambridge. Accessed June 26 2017. Avaliable in: http://www.unep-wcmc.org/system/dataset_file_fields/files/000/000/289/original/Protected_Planet_Report_2014_01122014_EN_web.pdf
18. Kristjanson, P.; Harvey, B.; Van Epp, M.; Thornton, P. K. 2013. Social learning and sustainable development. Nature Climate Change. 4(1): 5.
19. Matar, D. A.; Anthony, B. P. 2017. UNESCO Biosphere Reserve management evaluation: where do we stand and what's next? International Journal of UNESCO Biosphere Reserves. 1(1): 37-52.
20. Medina, J. L.; Orellana, Y. G.; Pino, M. M.; Diaz, L. 2014. Análisis discriminante canónico con técnicas gráficas multivariadas aplicado a un diseño con dos factores. Avances Investigación en Ingeniería. 11(2): 38-47.
21. MINITAB. 2004. Minitab Release 14.02.0. Satatistical Software. State College. P. A.
22. Mulero, M. A. 2001. Los espacios naturales protegidos en Andalucía: evolución, caracterización geográfica y singularidades. Ería. (54-55): 141-157.

23. Navarrete-Molina, C.; Meza-Herrera, C.; Ramirez-Flores, J. J.; Herrera-Machuca, M. A.; Lopez-Villalobos, N.; Lopez-Santiago, M.A.; Veliz-Deras, F.G. 2019a. Economic evaluation of the environmental impact of a dairy cattle intensive production cluster under arid lands conditions. Animal, 1-9. In press. https://doi.org/10.1017/51755173111900048X.
24. Navarrete-Molina, C.; Meza-Herrera, C.; Herrera-Machuca, M. A.; Lopez-Villalobos, N.; Lopez-Santos, A.; Veliz-Deras, F. G. 2019b. To beef or not to beef: Unveiling the economic environmental impact generated by the intensive beef cattle industry in an arid region. Journal of Cleaner Production. 231:1027-1035. Avaliable in: https://doi.org/10.1016/j.jclepro.2019.0.5.267.
25. Noguera-Talavera, A.; Salmerón, F.; Reyes-Sánchez, N. 2019. Bases teórico-metodológicas para el diseño de sistemas agroecológicos. Revista de la Fcultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 273-293.
26. Oficina del Programa MaB de la UNESCO en España & Organismo Autónomo Parques Nacionales (OAPN). 2009. Plan de Acción Montseny de la Red de Reservas de Biosfera Española. Accessed Jun 26, 2017. Avaliable in: http://rerb.oapn.es/images/PDF_publicaciones/oapn_mab_planaccion_montseny_tcm7-45387.pdf.
27. Onaindia, M.; Ballesteros, F.; Alonso, G.; Monge-Ganuzas, M.; Peña, L. 2013. Participatory process to prioritize actions for a sustainable management in a biosphere reserve. Enviromental Science & Policy. 33: 283-294.
28. Organismo Autónomo de Parques Nacionales (OAPN). 2015. Programa de Seguimiento de la Red Española de Reservas de Biosfera. Aplicación del sistema de indicadores de la Red Española de Reservas de Biosfera 2013-2014. Unpublished report.
29. Price, M. F. 2002. The periodic review of biosphere reserves: a mechanism to foster sites of excellence for conservation and sustainable development. Environmental Science & Policy. 5(1): 13-18.
30. Price, M.; Park, J.; Bouamrane, M. 2010. Reporting progress on internationally designated sites: the periodic review of biosphere reserves. Enviromental science & policy. 13: 549-557.
31. Reed, M.; Egunyu, F. 2013. Management effectiveness in UNESCO Biosphere Reserves: Learning from Canadian periodic reviews. Enviromental science & policy. 25: 107-17.
32. Ríos-Flores, J.L.; Rios-Arredondo, B. E.; Cantú-Brito, J. E.; Rios-Arredondo, H. E.; Armendáriz-Erives, S.; Chávez-Rivero, J. A.; Navarrete-Molina, C.; Castro-Franco, R. 2018. Análisis de la eficiencia física, económica y social del agua en espárrago (*Asparagus officinalis* L.) y uva (*Vitis vinífera*) de mesa del DR-037 Altar-Pitiquito-Caborca, Sonora, México 2014. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 101-122.
33. Rubio, J. 1990. La gestión de los espacios naturales andaluces. Revista de Estudios Andaluces. 14: 41-56.
34. Schliep, R.; Stoll-Kleemann, S. 2010. Assessing governance of biosphere reserves in Central Europe. Land Use Policy. 27(3): 917-927.
35. Schultz, L.; Duit, A.; Folke, C. 2011. Participation, adaptive co-management, and management performance in the world network of biosphere reserves. World Development. 39(4): 662-671.
36. Statistical Analysis System Institute (SAS). 2012. SAS Version 9.4 TS Level 1M1. Cary. NC. USA.
37. Tonolli, A. J. 2019. Propuesta metodológica para la obtención de indicadores de sustentabilidad de agroecosistemas desde un enfoque multidimensional y sistémico. Revista de la Fcultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(2): 381-399.
38. Tragsatec and Organismo Autónomo de Parques Nacionales (OAPN). 2011. "Programa de Seguimiento de la Red Española de Reservas de Biosfera: Primer Informe de Seguimiento para el trienio 2008-2010". Unpublished report.
39. Tragsatec and Organismo Autónomo de Parques Nacionales (OAPN). 2013. "Programa de seguimiento de la Red Española de Reservas de Biosfera. Aplicación del sistema de indicadores período 2010-2013". Unpublished report.

40. United Nations Educational, Scientific and Cultural Organization (UNESCO). 1996. Biosphere Reserves: The Seville Strategy and the Statutary Framework of the Word Network. Accessed June 26 2017. Avaliable in: http://unesdoc.unesco.org/images/0010/001038/103849Eb.pdf.
41. United Nations Educational, Scientific and Cultural Organization (UNESCO). 2008. Madrid Action Plan for Biosphere Reserves (2008-2013). Accessed June 26 2017. Avaliable in: http://unesdoc.unesco.org/images/0016/001633/163301e.pdf.
42. United Nations Educational, Scientific and Cultural Organization (UNESCO). 2013. Periodic review format for Biosphere Reserves. Accessed June 26 2017. Avaliable in: http://www.unesco.org/fileadmin/MULTIMEDIA/HQ/SC/pdf/Periodic_review_form_english_2013.pdf
43. Walker, R. T.; Solecki, W. D. 1999. Managing Land Use and Land-Cover Change: The New Jersey Pinelands Biosphere Reserve. Annals of the Association of American Geographers, 89(2): 220-237.
44. Watson, J. E. M.; Dudley, N.; Segan, D. B.; Hockings, M. 2014. The performance and potential of protected areas. Nature. 515(7525): 67-73.

Spatial and temporal synchronicity in the phenological events of *Prosopis flexuosa* in the Central Monte Desert

Sincronización espacial y temporal de los eventos fenológicos de *Prosopis flexuosa* en el Desierto del Monte Central

Guillermo Debandi [1*], Bertilde E. Rossi [2], Pablo E. Villagra [3], María A. Giantomasi [3], Nancy G. Mantován [4]

Originales: *Recepción:* 25/02/2019 - *Aceptación:* 23/05/2019

Abstract

Some desert plant species are capable of using underground water and are therefore independent of rainfall events. Species of the genus *Prosopis* are thought to be facultative phreatophytes, since they have deep and shallow roots that allow them explore water from underground layers and from sub-surface soil horizons. We created a seven-year series of phenological data in order to make comparisons between two natural Reserves of Mendoza province (Ñacuñán and Telteca) with different rainfall regimes and accessibility of *Prosopis flexuosa* trees to water. Percentage of trees in each phenological phase, date of maximum expression, and intensity of each phenological phase were recorded. We found that the trees had a similar date for leafing and flowering across years and sites, even with very different rainfall regimes. However, pod maturation dates varied significantly, ocurring 37 days sooner in Telteca. A second peak of leaves and flowers were recorded at both sites, being highly variable and non-synchronous in most cases, suggesting a quick response to rainfall events. The ability of *P. flexuosa* to respond to unpredictable rainfall pulses could be an important adaptation to keep ecosystem services functioning, even though associated pollinators and seed dispersers could get decoupled from changes in phenological events.

Keywords
phenological pattern • fruit set • phenological intensity • blooming pattern • synchronization • facultative phreatophyte

1 Instituto Nacional de Tecnología Agropecuaria (INTA) - EEA Junín, Isidoro Busquet s/n (5572). Junín. Mendoza. Argentina. * debandi.guillermo@inta.gob.ar
2 IADIZA-CONICET - CC 507 (5500) - Museo de Ciencias Naturales y Antropológicas Juan Cornelio Moyano. Mendoza. Argentina.
3 Universidad Nacional de Cuyo. Facultad de Ciencias Agrarias. CONICET. IANIGLA C. C. 330 (5500). Mendoza. Argentina.
4 IADIZA-CONICET – CC 507 (5500). Mendoza. Argentina.

Resumen

Algunas plantas desérticas pueden utilizar agua subterránea volviéndose independientes de los eventos de lluvia. Se cree que las especies de *Prosopis* son freatófitas facultativas ya que tienen raíces profundas y superficiales que les permiten explorar capas subterráneas y sub-superficiales del suelo en busca de agua. Creamos una serie de datos fenológicos de siete años para comparar dos Reservas naturales de la provincia de Mendoza (Ñacuñán y Telteca) con diferentes regímenes de precipitación y accesibilidad de *Prosopis flexuosa* al agua. Se registraron: porcentaje de árboles en cada fase fenológica, fecha de máxima expresión, e intensidad de cada fase fenológica. El inicio del desarrollo de hojas y flores fue similar a través de años y sitios, incluso con diferentes regímenes de lluvia. La fecha de maduración de los frutos sin embargo, fue significativamente (37 días) más corto en Telteca. Una segunda cohorte de hojas y flores, muy variable y no sincrónica en la mayoría de los casos, se registró en ambos sitios, sugiriendo una rápida respuesta a pulsos de lluvia. Esta capacidad de respuesta de *P. flexuosa* puede jugar un papel importante al mantener funcionando los servicios ecosistémicos, aunque los polinizadores y dispersores de semillas asociados podrían desacoplarse de los eventos fenológicos.

Palabras clave

patrones fenológicos • producción de frutos • intensidad fenológica • patrón de floración • sincronización • freatófito facultativo

Introduction

Water availability is one of the main factors that determines productivity in the Monte Desert, triggering a new flush of leaves in most perennial woody plants and germination of annual herbs and grasses (34). Some plant species are capable of using underground water (phreatophytes and other deep rooted plants), and are therefore independent of rainfall events. These plants can produce fruits and seeds during dry years, when most other species barely survive. Species of the genus *Prosopis* are thought to be facultative phreatophytes, because they are characterized by deep roots that allow them to obtain water from deep soil layers, as well as from shallow soil horizons (3, 14, 17, 18). Thus, *Prosopis* trees can maintain their maximum leaf area during the hottest and driest months of the year (26).

Prosopis flexuosa D.C. is the most important tree growing in the central Monte (2). Trees of *P. flexuosa* are very important for subsistence of native people from the Monte, where wide temperature fluctuations between winter and summer (-10°C to 48°C) are frequent, providing shade to domestic animals and people, wood for house and corral construction, and fire for cooking and heating. However, one of the most important services of *P. flexuosa* is fruit production. Fruits are consumed as food and used to prepare beverages. In addition, they are important source of feed for domestic animals when there is no enough grass (19). *Prosopis flexuosa*, like other species of the genus, has stable water potential values, which indicates that this species could be independent of rainfall (7). This idea is reinforced by the fact that its roots reach a depth of more than 10 meters, allowing this species to use underground water (17, 18). Studies carried out on this species for two years at Andalgalá (Catamarca) demonstrated high synchronization in foliage and flowering dates between years (24, 35). In spite of this, research on the development of phenological events in *P. flexuosa* are very scarce, and long series of phenological data on this tree in the Monte desert are lacking.

The humanity is facing climatic change events that have intensified over the last years. Knowing wich factors drive phenological events in perennial plants is useful for testing how these plants may respond to these events.

Objectives

Our objectives were to compare phenological data sets across various years and, in more detail, between two natural reserves with different water availability for *Prosopis* trees. We used data from a seven-year series of two sites in order to describe the phenological pattern of this species. Moreover, a more detailed four-year data series taken simultaneously at both reserves is analysed, in search for similarity on the intensity of each phenological phase. According to the phreatophytic condition of *Prosopis* trees, we expected high synchronization of the beginning of leafing and flowering among years, in each reserve. However, we also expected differences in the degree of expression of phenological events attributable to opportunistic water use from local sporadic rains.

Materials and methods

Study sites

The study was carried out at two natural reserves in the Monte Desert:

Ñacuñán Biosphere Reserve (12 300 ha; 34°02' S, 67°58' W; 540 m a. s. l.)

Situated in the centre of the Monte Desert, it has been excluded from grazing and logging for more than 40 years. The climate is dry and temperate, with cold winters. Annual rainfall averages 337.6 mm (1973-2005 average), however 75% of the rain concentrates in spring and summer. Mean monthly temperature of the coldest month (July) is 6.9°C and 22.4°C for the warmest (January), whereas mean annual temperature is 15.6°C, with an absolute maximum of 42.5°C and an absolute minimum of -13.0°C (12). The vegetation is characterized by an open woodland with sparse trees dominated by *Prosopis flexuosa*, a layer of shrubs (*Larrea divaricata*, *Lycium chilense*, *Junellia aspera*, *Condalia microphylla*, and *Capparis atamisquea*) and a grass layer composed mostly of perennial species (*Pappophorum caespitosum*, *Trichloris crinita*, *Aristida mendocina*, *Digitaria californica*, *Setaria leucopila*, and *Sporobolus cryptandrus*). Several annual grasses and herbs grow in the summer after rain events (33).

Telteca Provincial Reserve (38 500 ha; 32°23' S, 67°54' W; 500 m a. s. l.)

Located within the Monte Desert, is characterized by hot rainy summers and cold dry winters. The absolute maximum temperature reaches 48°C in summer, while the absolute minimum in winter is -10°C, with a mean annual temperature of 18.5°C. Rainfall is variable, ranging between 50 and 200 mm, with a mean annual rainfall of 156 mm (1972-2007 average). The landscape is characterized by a system of dunes and scrublands dominated by *Larrea divaricata*, *Tricomaria usillo*, and *Bulnesia retama*. The herbaceous layer is dominated by perennial (*Aristida mendocina, Panicum urvilleanum*) and annual grasses (*Bouteloua aristidoides, Aristida adscencionis*), with large areas of bare soil between shrubs. Open woodlands of *P. flexuosa* occur mainly in the low-lying areas between dunes, with a shrub layer dominated by *B. retama*, *Capparis atamisquea*, and *Lycium tenuispinosum* (1).

Data collection

Three series of phenological data from Ñacuñán Biosphere reserve and two series from Telteca reserve were used in order to obtain the longest phenological pattern possible. These series were discontinued in time. All trees used in the series were randomly selected and of similar size. At Ñacuñán, ten trees, were selected for the first group of data, and phenological observations were gathered from October 1993 to April 1995. The second group of data was comprised of observations from 10 trees, from October 1998 to May 1999. The last group of data was obtained from a more detailed phenological study, where three sites, each with four trees, and located at least 1 km apart, were selected. This group of trees differs from previous series. The objective was to assess three sites (and two in Telteca, see below) getting a wider representation of spatial variability in phenology. Observations for the last series were made from October 2000 to January 2004. At the Telteca Reserve, two sites with ten trees were observed from October 2000 to January 2004. This series is analogous in detail to that collected at the Ñacuñán reserve during the same period. The second series comprises a three-year study from October 2005 to January 2008, working on two sites, at least 1.5 km apart, with eight trees each.

Series of climatological data are also scant and mostly incomplete in the Monte Desert. We had a good representation of data for Ñacuñán for all years assessed. However, for Telteca we only had data starting in 2001, with some gaps in 2001 and 2004. All data were gathered from meteorological stations located at each Reserve.

Definition of variables and data analysis

Phenological patterns

Percentages of trees were calculated, showing different reproductive phenological phases along the growing season: flower buds, inflorescences, juvenile pods, mature pods, and vegetative growth. In this last phase, we only considered non-expanded leaves. Presence of those plant components was enough to consider that a tree was going through a phenological phase, without considering its intensity. For this variable, we used all the available data, seven years for each site that overlapped during 2000-2004 reproductive periods.

Date of phenological maximum expression

We used the date in which most trees demonstrated a phenological event, expressed as Julian day. The phenological phases observed were: flower buds, inflorescences, juvenile pods, and mature pods. We tested differences in the time at which most important phenological events occurred between Ñacuñán and Telteca. Since Julian day was also used as a variable, differences between sites were tested using Generalized Linear Models (GLM) with Poisson error, taking years as replicates. Analyses of GLM were done under the R environment (30), and testing significance of interactions was done by likelihood ratio tests (LRT), comparing models with and without interaction (*i.e.*, nested models) (9).

Intensity of phenological events

Previously used by Debandi *et al.* (2002), this variable accounts for the proportion of the canopy covered by a phenological event in each individual plant. The percentage recorded was assigned to a numerical value according to the following scale: 1 (1-20%), 2 (21-40%), 3 (41-60%), 4 (61-80%), and 5 (81-100%). The mean of each phenological event across all trees was calculated for each date. The phenological phases considered were: non-expanded leaves, flower buds, inflorescences, juvenile pods (not yet mature, greenish pods), mature pods (brownish to yellow fruits, still on the tree), and fallen pods (mature fruits fallen on the ground).

Results

Phenological patterns

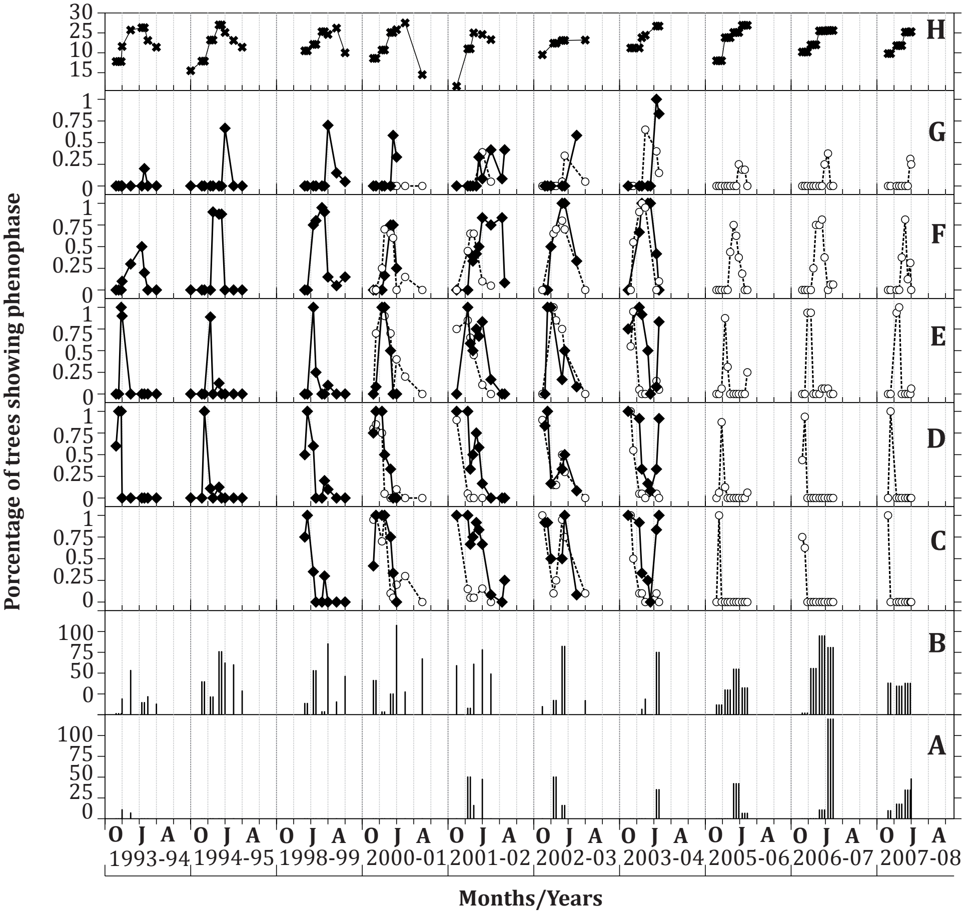
Vegetative growth of *P. flexuosa*, measured by a new flush of leaves, started in early October, with the highest percentage of trees by mid-October (figure 1C, page 153). These leaves quickly expand and all trees are fully covered by new, recently expanded leaves by late October (data not shown). These events were very similar at both sites and for all assessed years. At the time when new non-expanded leaves appear, flower buds are also observable, peaking by mid-October (figure 1D, page 153), and becoming fully flowered trees by late October (figure 1E, page 153).

In some years a second peak of leaves and flowers was recorded at both reserves; being, however, more frequent at Ñacuñán. This was more evident by mid December 2002 at both sites, and by mid-January 2004 at Ñacuñán (figure 1C, D, E; page 153). There was a different number of trees fructifying across the years assessed, and the duration of the period of pods maturation was also variable. In a similar way, the peak of trees fructifying was different among years and between sites. To find a coincidence in the date and percentage of trees between sites was only possible during the period 2003-2004. Pods maturation begins by the end of December or mid-January at Ñacuñán, and some weeks earlier at Telteca. Data from complementary phenological series (those data not assessed simultaneously at both sites), were very useful to confirm that the appearance of leaves and flowers occurs at very similar dates, regardless of rainfall.

Date of phenological maximum expression

Maximum expression of phenological phases resulted in subtle differences between sites. On average, new leaves had their maximum expression around October 14th (± 9 days) at Telteca, and around October 20th (± 10 days) at Ñacuñán (figure 2, page 154), having no statistical differences between sites (χ^2 = 0.28; df = 1; P = 0.59). Flower buds reached their maximum, on average, four days sooner at Telteca (October 19th ± 7 days) than at Ñacuñán (October 23th ± 3 days), showing no statistical differences (χ^2 = 0.23; df = 1; P = 0.62). Flowering reached its maximum by November 5th (± 7 days) at both reserves (χ^2 = 0.0009; df = 1; P = 0.97), while immature pods reached their maximum by December 4th (± 8 days) at Telteca and eight days later at Ñacuñán (December 12th ± 21 days), which is not significantly different (χ^2 = 0.72; df = 1; P = 0.39).

Date of maximum expression of mature fruits however, did vary significantly, occurring on average 37 days sooner at Telteca (December 27th ± 8 days) than at Ñacuñán (February 1st ± 17 days; χ^2 = 11.77; df = 1; P = 0.0006; figure 2, page 154).

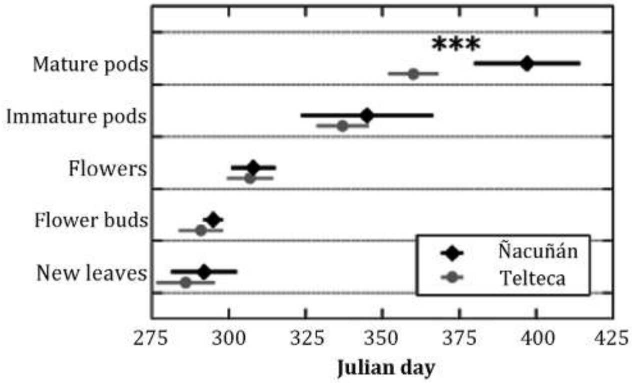


A: monthly rainfall at Telteca; B: monthly rainfall at Ñacuñán; C: non-expanded leaves; D: flower buds; E: inflorescences; F: immature pods; G: mature pods; H: temperature (°C) recorded at the Mendoza meterological station. On the x axe, each reproductive period is shown from October (O) of one year to April (A) of the next year.

A: Lluvia mensual en Telteca; B: lluvia mensual en Ñacuñán, C: hojas no expandidas; D: yemas florales; E: inflorescencias; F: vainas inmaduras; G: vainas maduras; H: temperatura (°C) registrada en la estación meteorológica de Mendoza. En el eje x se muestra los períodos reproductivos desde octubre (O) de un año a abril (A) del siguiente.

Figure 1. Percentage of *Prosopis flexuosa* trees showing different phenological phases along ten reproductive periods at two sites: Ñacuñán reserve (continuous lines and filled markers) and Telteca reserve (dashed lines and empty markers).

Figura 1. Porcentaje de árboles de *Prosopis flexuosa* mostrando diferentes fases fenológicas durante diez períodos reproductivos en dos sitios: Reserva Ñacuñán (línea continua y marcador lleno) y Reserva Telteca (línea discontinua y marcador vacío).



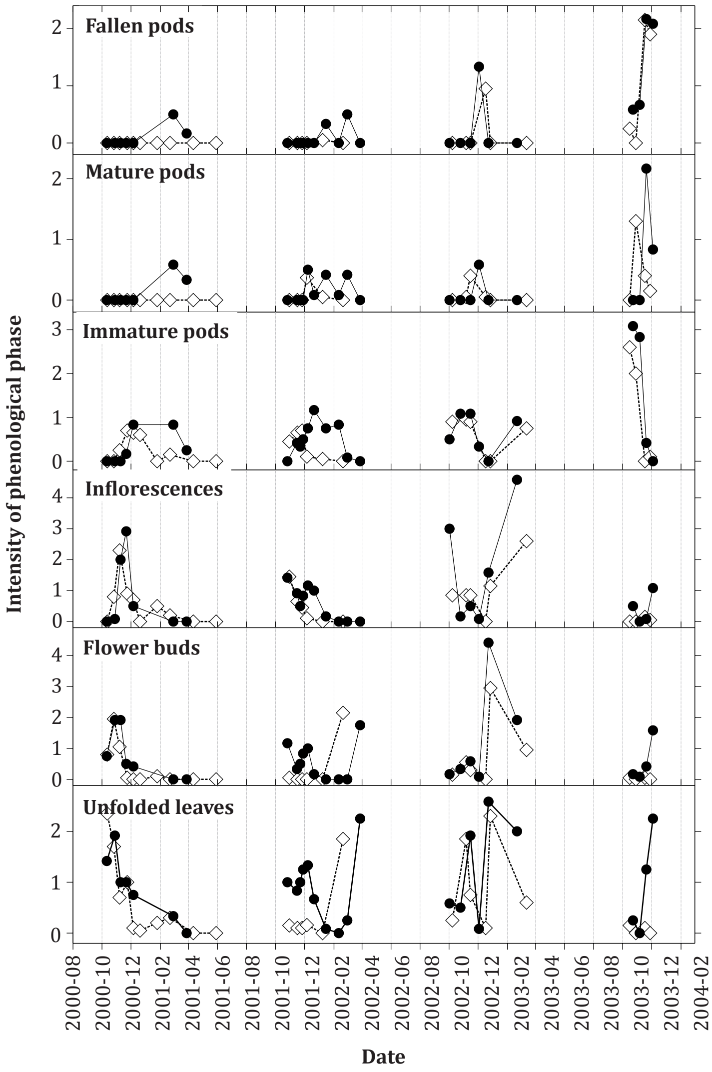
Asterisks (***) indicate significant differences in developing time between sites (probability at $P < 0.001$).
Los asteriscos (***) indican diferencias significativas en el tiempo de desarrollo entre sitios (con probabilidad $P < 0,001$).

Figure 2. Development of phenological phases of *Prosopis flexuosa* along the year. Values are means for seven years of Julian days (± SD) when maximum expression was detected (see text for details), taken at two sites of the Monte Desert.

Figura 2. Desarrollo de las fases fenológicas de *Prosopis flexuosa* a lo largo del año. Los valores representan el día juliano cuando se detectó la máxima expresión, promediado a lo largo de siete años de observación (± SD), en cada uno de los dos sitios estudiados del desierto del Monte.

Intensity of phenological events

Non-expanded leaves reached their peak with high intensity at the beginning of the reproductive season in concordance with bud burst. However, they quickly expanded before the inflorescences began to open. A second peak of non-expanded leaves was observable in some years, which seems to be a response to local conditions, since secondary peaks were different in intensity at both sites. In Telteca, during the 2001-2002 and 2003-2004 periods, no new flush of new leaves ocurred after the main peak of this event, but in Ñacuñán we registered a second peak of non-expanded leaves, that reached the magnitude of the first peak (for example February 2004, figure 3, page 155). In 2002-03 there was a second peak of non-expanded leaves with similar intensity at both sites. Flower buds and inflorescences reached similar intensities in their first peak during the 2000-01 and 2001-02 periods at both sites, but had a higher intensity in Ñacuñán during the last two years recorded (figure 3, page 155). Likewise, a second peak was recorded during the 2001-2002 and 2003-2004 periods with higher magnitude at Ñacuñán (figure 3, page 155). Juvenile pods had a similar intensity during the first three years, but increased considerably in the last year, at both sites (figure 3, page 155). This increase in intensity during the 2003-04 period was subsequently translated into higher intensities of mature and fallen pods, since the magnitude of these events was higher than in previous years.



Intensity follows a subjective scale with values of 0-5 (see text for details).

Los valores de intensidad están expresados acorde a una escala subjetiva de 0-5 (ver texto para detalles).

Continuous lines with filled circles indicate events at Ñacuñán reserve, and dotted lines with empty diamonds indicate events at Telteca reserve.

Líneas continuas con círculos llenos indican eventos en la Reserva de Ñacuñán, mientras que líneas discontinuas con rombos vacíos indican eventos en Reserva Telteca.

Figure 3. Mean intensity of phenological events of *Prosopis flexuosa* at two sites of the Monte Desert.

Figura 3. Intensidad promedio de los eventos fenológicos de *Prosopis flexuosa* en dos sitios del Desierto del Monte.

We observed a gradual increase in fallen pods across the considered years, from 2000 to 2004.

Discussion

Initiation of vegetative and reproductive cycles of *P. flexuosa* can be considered independent from rainfall events, since the observed periods of leafing and flowering always occurred before the rainy season. This pattern had already been observed, for this species by different authors (26, 35, 36), and in other phreatophyte species of this genus: *Prosopis alpataco* (6), *P. articulata* (23), *P. caldenia* (11), *P. chilensis* (35, 36), *P. glandulosa* (25), and *P. laevigata* (29). This independence from rainfall events is possible, at least in part, due to underground water availability. At Telteca, low annual rainfall should not allow the development of *Prosopis* woodlands (16). However, the presence of this tree could be explained by the fact that underground saline water can be found at a depth of 5 to 15 meters (4, 18). By contrast, at Ñacuñán, underground water can be found at a depth of 70-80 meters. According to Roig (1993), this species could likely obtain water from intermediate-depth humid sandy-clay layers that can be observed frequently across the soil profile. Thus, water availability constrains the distribution of mesquite trees and allows them to synchronize their phenological phases over large geographic areas.

In addition to the independence from rainfall events, other local conditions can trigger phenological events. According to Solbrig and Cantino (1975), the photoperiod is an important sign for leafing and flowering initiation in *P. velutina* and *P. chilensis*; while Goen and Dahl (1982) found a relationship between bud-burst and temperature rising during spring in *P. glandulosa*. These environmental conditions can generate in some cases a latitudinal cline (5), indicating an adjustment to local temperature and/or photoperiod, and to the length of the growing season (36). The assessed sites for this study are located 184 km apart from each other (almost 1.5° in latitude), and the date of maximum expression of most phenological events was very similar at both sites, or a bit earlier at Telteca, the northernmost site. Leafing started by early to mid-October. However in northern latitudes like Andalgalá (27°34' S; 66°18' W-Catamarca, Argentina (35)) and Chancaní (31°24' S; 65°27' W-Córdoba, Argentina) (28), leafing in *P. flexuosa* began in early spring (September) while in southern latitudes it began in early November (38°45' S; 63°45' W-La Pampa) (11). In a similar way, flowering started in mid-to-late October at both Reserves, and 15-30 days earlier in northern latitudes (Andalgalá and Chancaní), while in southern latitudes flowering was recorded in late November-early December (La Pampa).

Although there were similarities in bud burst and flowering dates, reaching their maximum expression at very similar times at both study sites, differences begin to be observable when trees start fruiting. Immature pods appear, in average, sooner at Telteca, with similar maximum expression dates across the years. However, this difference becomes more evident when fruits reach maturity, as the time needed to reach maturity is significantly longer in the southernmost site. This difference may be due to climatic reasons, since Telteca has a hotter and dryer climate than Ñacuñán. It might also be due to genetic differences, at is has been demonstrated that the genus *Prosopis* maintains a genetically fixed phenological pattern when individuals from separate geographical areas are grown under uniform conditions (8, 22, 24).

While it is clear that *P. flexuosa* can flower independently from rainfall events, the main blooming period was very short. At both sites, mesquite bloomed from October to December, although bloom never lasted for the three months. Long periods in which *P. flexuosa* have visible flowers, have been attributed to early and late flowering individuals that overlap their blooms for many weeks (28). However, in our case, blooms were very short in time and a second and discrete bloom was observable, especially in Ñacuñán, in most of the study years. The flush of new leaves and flowers could be correlated to the cambial growth found by Giantomasi *et al.* (2012). According to this finding, *P. flexuosa* has an immediate growth response to short-term rainfall, evidenced by the production of new derivative cells linked to rains occurring a few days before any manifestation of cambial cell division (13). What is not clear is the advantage of these low intensity secondary peaks, especially those related to reproduction. Of the 10 reproductive periods assessed, only in 2003-04 at Ñacuñán, the secondary bloom was of high intensity. The 2003-04 period also had the highest fruit intensity (juvenile, mature, and fallen pods). On average, fallen fruits had an intensity equivalent to 20-40% of the ground beneath the trees, regularly covered by pods. This last reproductive period studied, yielding the highest pod production, also had the lowest annual rainfall of the last 10 years prior to 2004, in Ñacuñán. This is in accordance with Nilsen *et al.* (1991), and Lee and Felker (1992). Their findings for *P. glandulosa*, recorded an increase of flowers per branch and pod production as water stress increased. This lack of rainfall during the main bloom of mesquite has an important positive effect in the reproductive success of this species. Many authors have documented this relationship (11, 21, 35), also well known by local people.

Finally, we intend to point out the importance of long series studies on phenology, given that they provide valuable data for inferring phenological responses to environmental conditions. In this paper, we present the first descriptive phenological aspect of *P. flexuosa* in the central Monte Desert. Moreover, some surprising events like the intensity of flower buds in Ñacuñán during January 2004, open a series of questions about water use by *P. flexuosa* trees. Is it possible that mesquite trees use superficial water to produce the secondary flush of leaves and flowers? This question has been party answered by the cambial growth research done by Giantomasi *et al.* (2012). However it is still necessary to focus on the effect of rainfall events and other environmental conditions that promote a large flush of new flowers during summer, *i.e.*, after the main reproductive event of spring, and to determine the fate of these flowers. The response of cambial growth to short-term rainfall events should be viewed as an adaptation to the sporadic and convective nature of rains in this region (13, 32). Undoubtedly, the ability to take advantage of rains in a desert must be important for these plants. However, if this adaptation is beneficial from a reproductive point of view is not clear, given the low production efficiency of mature fruits.

In the context of climate change in the Monte Desert (20), facultative phreatophytes, like *P. flexuosa*, can play an important role in ecosystem response and continue to provide ecosystem services. They, not only contribute with a surplus of productivity in arid environments, but also have the capacity to respond to the unpredictable pulses in water availability. In the future, we expect higher frequency and magnitude of secondary pulses of leafing, flowering, and fruiting that will extend these phenological events in the growing season. If pollinators and seed dispersers will adapt to these changes and take advantage of them, remains to be know.

Conclusions

Prosopis flexuosa can flower independently from rainfall. During all assessed years flowering initiated before rainfall events. The main blooming period was very short at our study sites, with high synchronicity of leafing and flowering among trees at each site, as well as among sites. Differences in the extension of flowering periods can be attributable to secondary peaks related to rainfall events. The highest pod production year, also had the lowest annual rainfall of the 10 years prior to 2004, corroborating the relationship of high pod production with rainfall decreases.

References

1. Alvarez, J. A.; Villagra, P. E.; Cony, M. A.; Cesca, E.; Boninsegna, J. A. 2006. Estructura y estado de conservación de los bosques de *Prosopis flexuosa* D.C. en el Noreste de Mendoza, Argentina. Revista Chilena de Historia Natural. 79:75-87.
2. Alvarez, J. A.; Villagra, P. E. 2009. *Prosopis flexuosa* DC. (Fabaceae, Mimosoideae). Kurtziana. 35: 49-63.
3. Ansley, R. J.; Jacoby, P. W.; Cuomo, G. J. 1990. Water relations of honey mesquite following severing of lateral roots: influence of location and amount of subsurface water. Journal of Range Management. 43: 436-442.
4. Araníbar, J. N.; Villagra, P. E.; Gomez, M. L.; Jobbágy, E.; Quiroga, M.; Wuilloud, R. G.; Monasterio, R. P.; Guevara, A. 2011. Nitrate dynamics in the soil and unconfined aquifer in arid groundwater coupled ecosystems of the Monte desert, Argentina. Journal of Geophysical Research. 116: 1-12.
5. Balboa, O.; Parraguez, J. M.; Arce, P. 1988. Phenology studies of *Prosopis* species growing in Chile. In: Habit, M. A.; Saavedra, J. C. (Eds.). The current state of knowledge on *Prosopis juliflora*. II International Conference on *Prosopis*. Brasil, Food and Agriculture Organization of the United Nations, Plant Production and Protection Division. 259-268.
6. Campanella, M.; Bertiller, M. 2008. Plant phenology, leaf traits and leaf litterfall of contrasting life forms in the arid Patagonian Monte, Argentina. Journal of Vegetation Science. 19: 75-85.
7. Cavagnaro, B.; Passera, C. B. 1993. Relaciones hídricas de *Prosopis flexuosa* (algarrobo dulce) en el Monte, Argentina. In: Roig, F. A.; Trione, S. O.; Cavagnaro, B. (Eds.). Conservación y mejoramiento de especies del género *Prosopis*. Contribuciones mendocinas a la quinta reunión regional para América Latina y el Caribe de la red de forestación del CIID. Argentina. IADIZA-CRICYT-CIID. 73-78.
8. Cony, M. A. 1996. Genetic variability in *Prosopis flexuosa* D.C., a native tree of the Monte phytogeographic province, Argentina. Forest Ecology and Management. 87: 41-49.
9. Crawley, M. J. 2013. The R Book. 2nd Ed. UK. John Wiley & Sons.
10. Debandi, G.; Rossi, B. E.; Araníbar, J.; Ambrosetti, J. A.; Peralta, I. E. 2002. Breeding system of *Bulnesia retama* (Gill. ex Hook.) Gris. (Zygophyllaceae) in the Central Monte Desert (Mendoza, Argentina). Journal of Arid Environments. 51: 141-152.
11. Distel, R. A.; Peláez, D. V. 1985. Fenología de algunas especies del distrito del Caldén (*Prosopis caldenia* Burk.). IDIA [Setiembre-Diciembre]: 35-40.

12. Estrella, H.; Boshoven, J.; Tognelli, M. 2001. Caractersticas del clima regional y de la Reserva de Ñacuñán. In: Claver, S.; Roig-Juñent, S. (Eds.). El desierto del Monte: La Reserva de Biosfera de Ñacuñán. Argentina. IADIZA-MAB-UNESCO. 25-33.
13. Giantomasi, M.; Roig-Juñent, F.; Patón-Domínguez, D.; Massaccesi, G. 2012. Environmental modulation of the seasonal cambial activity in *Prosopis flexuosa* DC trees from the Monte woodlands of Argentina. Journal of Arid Environments. 76: 17-22.
14. Giordano, C. V.; Guevara, A.; Boccalandro, H. E.; Sartor, C.; Villagra, P. E. 2011. Water status, drought responses and growth of *Prosopis flexuosa* trees with different access to the water table in a warm South American desert. Plant Ecology. 212: 1123-1134.
15. Goen, J. P.; Dahl, B.E. 1982. Factors affecting budbreak in honey mesquite in west Texas. Journal of Range Management. 35: 533-534.
16. González-Loyarte, M.; Rodeghiero, A. G.; Buk, E.; Trione, S. 2000. Análisis comparativo de dos comunidades en el bosque de *Prosopis flexuosa* DC. del NE de Mendoza, Argentina. Multequina. 9: 75-89.
17. Guevara, A.; Giordano, C. V.; Aranibar, J.; Quiroga, M.; Villagra, P. E. 2010. Phenotypic plasticity of the coarse root system of *Prosopis flexuosa*, a phreatophyte tree, in the Monte Desert (Argentina). Plant and Soil. 330: 447-464.
18. Jobbágy, E. G.; Nosetto, M. D.; Villagra, P. E.; Jackson, R. B. 2011. Water subsidies from mountains to deserts: Their role sustaining groundwater-fed oases in a sandy landscape. Ecological Applications. 21: 678-694.
19. Karlin, U.; Díaz, R. 1984. Potencialidad y Manejo de Algarrobos en el Árido Subtropical Argentino. Argentina, SECYT. 69 p.
20. Labraga, J. C.; Villalba, R. 2009. Climate in the Monte Desert: past trends, present conditions, and future projections. Journal of Arid Environments. 73: 154-163.
21. Lee, S. G.; Felker, P. 1992. Influence of water/heat stress on flowering and fruiting of mesquite (*Prosopis glandulosa* var. *glandulosa*). Journal of Arid Environments. 23: 309-319.
22. Mantován, N. G. 2005. Variabilidad intra-especifica de *Prosopis flexuosa* DC. var. *flexuosa* en el Monte. Su estudio morfo-fisiologico. Tesis doctoral en Biología. PROBIOL. Universidad Nacional de Cuyo. Mendoza, Argentina. 179 p.
23. Maya, Y.; Arriaga, L. 1996. Litterfall and phenological patterns of the dominant overstorey species of a desert scrub community in north-western Mexico. Journal of Arid Environments. 34: 23-35.
24. Mooney, H. A; Simpson, B. B.; Solbrig, O. T. 1977. Phenology, morphology, physiology. In: Simpson, B. B. (Ed.). Mesquite: Its biology in two desert ecosystem. USA, Hutchinson & Ross. Inc. (US/IBP Synthesis Series 4). 26-43.
25. Nilsen, E. T.; Sharifi, M. R.; Rundel, P. W.; Jarrell, W. M.; Virginia, R. A. 1983. Diurnal and seasonal water relations of the desert phreatophyte *Prosopis glandulosa* (honey mesquite) in the Sonoran Desert of California. Ecology. 64: 1381-1393.
26. Nilsen, E. T.; Sharifi, M. R.; Virginia, R. A.; Rundel, P. W. 1987. Phenology of warm desert phreatophytes: seasonal growth and herbivory in *Prosopis glandulosa* var. *torreyana* (honey mesquite). Journal of Arid Environments. 13: 217-229.
27. Nilsen, E. T.; Sharifi, M. R.; Rundel, P. W. 1991. Quantitative phenology of warm desert legumes: seasonal growth of six *Prosopis* species at the same site. Journal of Arid Environments. 20: 299-311.
28. Parizek, B.; de la Reta, M.; Catalán. L.; Balzarini, M.; Karlin, U. 2000. Observaciones fenológicas del algarrobo negro (*Prosopis flexuosa* D.C.) y del algarrobo blanco (*Prosopis chilensis* (Mol.) Stuntz) en el Chaco Árido, Argentina. Multequina. 9: 135-146.
29. Pavón, N.; Briones, O. 2001. Phenological patterns of nine perennial plants in an intertropical semi-arid Mexican scrub. Journal of Arid Environments. 49: 265-277.
30. R Development Core Team. 2016. R, a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available in: http://www.R-project.org (Accessed December 2016).

31. Roig, F. A. 1993. Informe Nacional para la Selección de Germoplasma en Especies del Género *Prosopis* de la República Argentina. In: Roig, F. A.; Trione, S. O.; Cavagnaro, B. (Eds.). Conservación y mejoramiento de especies del género *Prosopis*. Contribuciones mendocinas a la quinta reunión regional para América Latina y el Caribe de la red de forestación del CIID. Argentina. IADIZA-CRICYT-CIID. 1-36.
32. Roig, F. A.; Roig, F. A. 1998. Wood anatomy of geo and phytodinamic indicators in the Province Fitogeográfica del Monte, Argentina. Bamberger Geographische Schriften. 15: 181-209.
33. Rossi, B. E. 2004. Flora y vegetación de la Reserva de Biosfera de Ñacuñán después de 25 años de clausura. Heterogeneidad espacial a distintas escalas. Tesis doctoral en Biología. PROBIOL. Universidad Nacional de Cuyo. Mendoza. Argentina.
34. Rundel, P. W.; Villagra, P. E.; Dillon, M. O.; Roig-Juñent, S.; Debandi, G. 2007. Arid and semi-arid ecosystems. In: Veblen, T. T.; Young, K.; Orme, A. (Eds.). The Physical Geography of South America. UK. Oxford University Press. 158-183.
35. Simpson, B. B.; Neff, J. L.; Moldenke, A. R. 1977. *Prosopis*. Flowers as a resource. In: Simpson, B.B. (Ed.). Mesquite. Its Biology in Two Desert Scrub Ecosystems. USA. Dowden, Hutchinson & Ross. Inc. (US/IBP Synthesis Series 4). 84-107.
36. Solbrig, O. T.; Cantino, P. D. 1975. Reproductive adaptations in *Prosopis* (Leguminosae, Mimosoideae). Journal of the Arnold Arboretum. 56: 185-210.

Acknowledgments

We want to thank Juan Alvarez, Gualberto Zalazar, Erica Cesca, Valeria Corbalán and Mariano Cony for field assistance. Nelly Horak and Anabela Bonada for assistance in the English version, the Dirección de Recursos Naturales Renovables of Mendoza, and IADIZA for allowing us to conduct the studies at Telteca and Ñacuñán reserves, respectively.

This research was supported by Agencia Nacional de Promoción Científica y Tecnológica under grant PICT 03220-PICTR 2002 00200; and IAI - Interamerican Institute for Global Change Research under grant CRN3.

Rev. FCA UNCUYO. 2020. 52(1): 161-174. ISSN (en línea) 1853-8665.

Soil compaction caused by the impact of machinery traffic during corn (*Zea mays*) harvest

Compactación del suelo causado por el tránsito de maquinarias en la cosecha de maíz (*Zea mays*)

Ramón Jesús Hidalgo [1*], Oscar Rubén Pozzolo [2, 3], José Fabio Domínguez [1],
Laura Giménez [1], Guido Fernando Botta [4]

Originales: *Recepción:* 25/03/2018 - *Aceptación:* 15/06/2019

Abstract

The aim of this work was to study soil compaction caused by the traffic of two types of combine harvesters and a tractor plus a grain cart with two loading conditions (fully loaded hoppers-empty hoppers) during corn harvest on vertic argiudoll soil by means of direct seeding system. Soil penetration resistance (PR) and soil bulk density (BD) were measured to a depth of 40 cm in five sampling sites. Response variables determinations were also analyzed. The tractor and the fully loaded grain cart traffic caused greater soil compaction in all the sampling depths, exceeding 60 cm on both sides of the footprint center. What is more, the values obtained from PR and BD measurements were higher than those values considered suitable for normal root growth. This was only evident in the tread width of the tires during the passing of the two fully loaded combine harvesters. The analysis of inflation pressures and tire loads used, indicated in some cases, poor concordance between these variables. The analysis also indicated that the tires were inflated to the limit of resistance.

Keywords
combine harvester • tractor • grain cart • penetration resistance • bulk density

1 Universidad Nacional del Nordeste (UNNE). Facultad de Ciencias Agrarias. Sargento Cabral 2131. (3400). Corrientes. rj_hidalgo@yahoo.com.ar
2 Estación Experimental INTA. Concepción del Uruguay. RP39. Concepción del Uruguay. Entre Ríos.
3 Universidad Nacional de Entre Ríos (UNER). Facultad de Ciencias Agropecuarias. RP11 Km. 10.5. E3101 Oro Verde. Entre Ríos. Argentina.
4 Universidad Nacional de Luján. Ruta Nacional 5 y Avenida Constitución. Luján. Provincia de Buenos Aires C. P. 6700. Argentina.

Resumen

El objetivo del trabajo fue estudiar la compactación causada por el tráfico de dos tipos de cosechadoras y equipo tractor más carro granelero con dos condiciones de carga (tolvas vacías-tolvas llenas) en la cosecha de maíz sobre un suelo argiudol vértico en sistema de siembra directa. Las determinaciones de las variables respuestas, resistencia a la penetración (RP) y densidad aparente (Da), se hicieron hasta los 40 cm de profundidad y en cinco sitios de muestreo. El tránsito del equipo tractor más carro granelero lleno fue el que mayor compactación causó en todas las profundidades de muestreo superando los 60 cm a ambos lados del centro de huella con datos de RP y Da superiores a los considerados crítico para el normal desarrollo radicular. Esta situación, en el pasaje de las dos cosechadoras en condiciones llenas, solamente se dio en el ancho de pisada de los neumáticos. El análisis de las presiones de inflado y cargas de los neumáticos usados indicaron, en algunos casos, una escasa concordancia entre estas variables y que las ruedas se encontraban en el límite de resistencia a las presiones utilizadas.

Palabras claves

cosechadora • tractor • carro granelero • resistencia a la penetración • densidad aparente

Introduction and background

Machinery traffic has a detrimental effect on soil. One of the major problems is soil compaction which leads to a variation in soil pore size and distribution since soil particles are tightly packed due to a reduction in the air volume with a consequent reduced rate of water infiltration and drainage. Consequently, there is a density and penetration resistance increase (3, 16)

Several factors are involved in soil compaction caused by tires: type, size, tire carcass type (conventional or radial) and its flexibility, load applied, contact pressure, soil moisture and number of passes (13).

Agricultural tires cause soil deformation at the soil-tire interface (19). This leads to a certain degree of compaction where the highest values are generally found at the tire footprint surface and below the tire footprint surface at its center (9). However, a single determination of bulk density at the footprint center at a single depth is probably not representative of the maximum value at each site located horizontally or vertically, in terms of conditions of low bearing capacity soils and the usage of R2 tires with deep tread (8).

Another important factor influencing soil compaction is tire inflation pressure since bulk density increases with high levels of inflation pressure (10). Studies carried out by Schjønning *et al.* (2008) examined two radial tires with a constant load on the tire but with inflation pressures that were below, equal to and higher than the pressure, recommended by tire manufacturers. These studies found that over inflated tires caused greater surface compaction.

Studies indicate that the specific pressure in the tire-soil contact area, closely linked to inflation pressure, causes ground strata compaction (17). Apart from that, loading accumulation causes compaction of the deepest soil layers at a depth of more than 30-50 cm regardless of the extent of the surface on which it is distributed (22).

Two methods can be used to determine compaction caused by farm equipment traffic considering bulk density (BD) and penetration resistance (PR). There are studies that determine certain critical BD values suitable for normal root growth: 1.45 Mg m^{-3} for clayey-textured soil horizons; 1.55 Mg m^{-3} for medium-textured soil horizons; and 1.65 Mg m^{-3} for sandy-textured soils (18). Corn is one of the major field crops prone to suffer root damage and suffer yield decreases, owed to compaction. This occurs since corn is sensitive to water stress, rising temperatures and factors resulting from compaction, that have a profound impact on crop yield and quality (20).

Studies carried out by Botta *et al.* (2018), regarding PR, analyzed the effect of compaction on crop yields. Those studies determined that soil-surface compaction and increasing penetration resistance, play a major role in crop yield decline. Penetration resistances exceeding values of 1.5 MPa in the first 20 cm depth inhibits normal root development. Besides, if the value is above 2.5 MPa, roots may stop growing.

To date, there is little information on the effect of combine harvesters, tractors and grain carts tires on compaction during corn harvest, mainly on vertic argiudoll soils. Hence, research on this problem would considerably benefit from rigorous studies.

Objective

To analyze the compaction effect caused by the passage of two types of combine harvesters and a tractor plus a grain cart with two loading conditions (fully loaded hoppers-empty hoppers) during corn harvest.

Hypothesis

The greater size and load capacity of machines and equipment used in harvesting increase the mass per axle, influencing the surface and subsurface compaction of the soil, even when using more buoyant radial tires.

Materials and methods

The study was carried out near the town of Sauce in the province of Corrientes, Argentina. The soil type is vertic argiudoll belonging to the series Paraje Francisco Gómez (11).

Morphological description: The soil horizons sequence is: Ap - A - Btss - BCss - Ck. It presents a 40 cm arable horizon which is thick, loam-textured, black, and porous. This horizon has strong biological activity but it is weakly structured owing to the action of tillage implements (discs) in the first 18 cm. The argillic horizon is visible at a depth of 40 cm to 68 cm. It is clayey, dark grey/black, strongly structured, plastic and sticky. From 68 cm, the clayey matrix (BCss) turns greyish-brown. The presence of calcium carbonate concretions is visible from 88 cm. This series presents high levels of organic matter and calcium, wich forms different salts.

Studies were carried out to evaluate the compaction effect caused by the combine harvesters and the tractor plus the grain cart traffic during the corn harvesting stage.

The experimental design was randomized complete block design with three repetitions where the blocks were constituted by the passes of the three machines under study. An analysis of variance was conducted and differences between means were determined by means of a Duncan test with a significance level of $p<0.05$.

The treatments were: Control treatment (T1); Combine harvester 1, John Deere 1450, empty (T2); Combine harvester 1, fully loaded (T3); Combine harvester 2, Claas Lexion 660, empty; Combine harvester 2, fully loaded (T5); Tractor Agco Allis 6.220A plus grain cart Cestari 20550 4R, empty (T6); Tractor Agco Allis 6.220A plus fully loaded grain cart (T7). Dry bulk density (BD) and penetration resistance (PR) were determined. Bulk density (BD) was measured directly after Hidalgo (2003) through the weighing of a known volume of soil taken with cylinders specially adapted to obtain the samples.

Penetration resistance determinations (PR) were made by using the ASAE standard penetrometer with the denominations S3133.2 (1). Samples for the two variables were taken at four depth ranges: 0-10; 10-20; 20-30; 30-40 cm, and at five sites: footprint center; 30-40 cm left and right of the footprint center coinciding with the tread edge; 60-80 cm to the left and to the right of the footprint center (figure 1). The first distance for tires with narrow tread width combined 1 front tires, tractor rear tires and grain cart tires. The second distance for the tire with the widest tread width combine harvester 2 front tires.

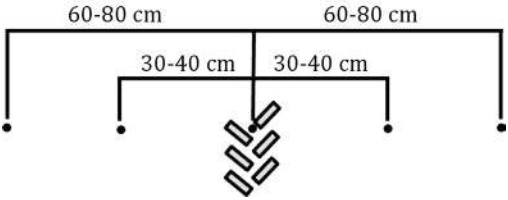


Figure 1. Sampling site for penetration resistance and bulk density.

Figura 1. Lugar de toma de muestra de resistencia a la penetración y densidad aparente.

Three trenches were dug by means of treatment and repetition for the collection of bulk density samples. This was done transversely to each pass. The trenches were 1.30 m and 1.70 m long for tires with a tread width of 60 and 80 cm respectively. The trenches had a width of 50 cm and a depth of approximately 50 cm.

Soil moisture was close to field capacity under test conditions. An additional study of this variable was carried out since it influences PR and BD values. Moisture data were also evaluated at all sampling sites when developing the method for determining the latter variable. An average was determined for each machine and loading condition as no clear differences were recorded in the values obtained.

The characteristics of the machines used are described in table 1 (page 165).

Table 1. Characteristics of the machinery used in the trial.
Tabla 1. Características de la maquinaria usada en el ensayo.

Machinery	Empty weight (kN)	Gross weight/ with ballast (kN)	Tire	Inflation pressure (kPa)	Recommended inflation pressure (kPa)	Difference of pressure (%)
Combine harvester 1 ED	91.2	132	24.5-32	206.8	200	+3
Combine harvester 1 ET	23	35	16.9-24	165.5	170	-3
Combine harvester 2 ED	116	184	800/70R32	220.6	240	-8
Combine harvester 2 ET	29	46	18.4-26	137.9	110	+20
Tractor ED	---	30.45	16.9-28	193.1	110	+43
Tractor ET	---	37.22	24.5-32	165.5	110	+33
Grain cart	44.13	189.76	23.1-30 R3	241.3	210*	+13

* Maximum pressure recommended by manufacturer. / * Presión máxima recomendada por fabricante.
ED front axle. ET rear axle. / ED eje delantero. ET eje trasero.

The method proposed by McKyes, 1985 cited by O' Sullivan *et al.* (1998), was used to determine tire-soil contact area and to estimate the contact pressure from the weight of the machine. This method has the advantage of its simplicity for data collection since it uses the measurements provided by the manufacturer, following this equation:

$$A = b * d / X$$

where:
A = contact area
X = constant whose value varies between 4 for rigid soils and 2 for loose soils. Coefficient 3 was used in this case
b = tire width;
d = tire diameter.

However, it is important to consider that this method underestimates the actual contact area as determined by Palancar *et al.* (2009). This may lead to an overestimation of the pressures exerted by the tires. The recommended pressures were obtained using the Tire-pressure-inflation-calculator (6).

Results and discussion

Contact areas: The exerted pressures exerted were estimated as shown in table 2 (page 166) by using this model the measurements provided by the manufacturers (4, 7).

Table 2. Tire dimensions and ground pressures.

Tabla 2. Medidas de los neumáticos y presiones ejercidas sobre el suelo.

Machines/ tires	Tire model	Tire width (mm)	Tire diameter (mm)	Contact surface (m^2)	Contact pressure (kPa)
C1 F	24.5-32	620	1826	0.3773	174.93
C1 R	16.9-34	429	1318	0.1885	92.84
C2 F	800/70R32	793	1936	0.5117	179.79
C2 R	18.4-26	467	1450	0.2257	101.90
Tractor F	16.9-28	429	1367	0.1955	155.75
Tractor R	24.5-32	620	1826	0.3773	97.94
Grain Cart	23.1-30	587	1707	0.3340	142.03

C1 F, combine harvester 1 front tire. C1 R, combine harvester 1 rear tire. C2 F, combine harvester 2 front tire. C2 R, combine harvester 2 rear tire. Tractor F, Tractor front tire. Tractor R, tractor rear tire.

C1 F, cosechadora 1 neumático delantero. C1 R, cosechadora 1 neumático trasero. C2 F, cosechadora 2 neumático delantero. C2 R, cosechadora 2 neumático trasero. Tractor F, Tractor neumático delantero. Tractor R, tractor neumático trasero.

It is important to note that both the combine harvester and the tractor had a high contact pressure at full load. These results are likely to provide an overestimation error derived from the use of the method proposed by McKyes 1985, as already mentioned.

Regardless, it is important to point out that the size of the tire used was close to the permitted load limits in the case of the C1 harvester. There is only one tire type, a 16 ply tire, capable of bearing the weight exerted on the front axle, at speeds of less than 10 $km.h^{-1}$. This tire is not nationally manufactured.

A similar situation occurred with the fully loaded grain cart and the use of tires that exceeded, by approximately 25%, the maximum load limit, recommended by the manufacturer. This situation was evidenced after finding that the used pressures, were 13% higher than the recommended maximum pressures.

The pressures used did not coincide with those recommended by the manufacturer, obtained through the calculation program (5).

The combine harvester 1 is the only machine whose values were close to optimum values. The other machines used pressures that differ from the pressures they should have been using by approximately 18%.

Penetration resistance

Significant differences were observed between machines and loading conditions as well as between sampling sites when analyzing the effect caused by the passage of the combine harvesters and the tractor plus the grain cart. The passing of the combine harvesters and the tractor plus the grain cart with both loading conditions (empty and fully loaded) caused greater penetration resistance in the center of the footprint. This contradicts other authors who point out that the greatest compaction using conventional tires occurs in the center of the footprint (9).

Machinery traffic caused greater compaction effect of 60-80 cm on both sides of the center of the footprint with values greater than the control treatment in the heaviest load condition. The increase in mass per axle in addition to pressures different from those recommended by the manufacturer may have been the cause of this effect. This coincides with studies carried out by Schjønning *et al.* (2008), who described that surface compaction was recorded only with pressures higher than those recommended by the manufacturers.

On the contrary, the passage of empty machinery did not generate compaction effects on the soil at the greatest distance (60-80 cm) on neither side of the center of the footprint, at depths of 0 to 10 cm (table 3). It is important to point out that, in the surface (0 to 10 cm), the values determined through penetrometer could be affected towards both sides of the center of the footprint by a lateral soil displacement effect. These values may be even lower values than the control treatment (15, 17).

At greater depths, the values were more consistent and always superior to the control treatment, even at 30-40 cm of depth (table 3; table 4, page 168; tables 5 and 6, page 169). These results would explain the increase in PR values even at the most distant sampling sites from the center of the footprint. This is directly related to the increase in machine masses by storing harvested grain in their hoppers.

Table 3. Depth 0-10 cm. Statistical analysis of penetration resistance. PR values expressed in MPa. Moisture values expressed as a percentage.

Tabla 3. Profundidad 0-10 cm. Análisis estadístico de resistencia a la penetración. Valores de RP expresados en MPa. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	19.7	0.840 p	1.130 jkl	1.270 g	1.150 hijk	0.880 o
CH 1 F	20.0	1.120 kl	1.380 e	1.470 d	1.380 e	1.190 h
CH 2 E	20.2	0.930 n	1.160 hij	1.390 e	1.170 hi	0.980 m
CH 2 F	19.6	1.090 l	1.540 c	1.610 b	1.540 c	1.140 ijk
T + GC E	20.4	0.980 m	1.310 f	1.48 0 d	1.300 fg	0.980 m
T + GC F	20.0	1.180 h	1.630 b	1.750 a	1.620 b	1.176 hi
Control Treatment	20.3	1.000 m	1.000 m	1.000 m	1.000 m	1.000 m

+Different letters present statistical differences. Duncan test $p < 0.05$.

+ Diferentes letras presentan diferencias estadísticas. Test de Duncan $p < 0,05$.

The highest values determined through the use of the penetrometer were observed in the passage of the tractor plus the grain cart, and in the harvester 2 in fully loaded hopper conditions (table 3, page 167; table 4, page 168; tables 5 and 6, page 169), coinciding with the highest contact pressures calculated for each machine.

Even with these increases, the data were inferior to the 2 MPa considered critical for root development in the first 20 cm of depth according to (2) (table 3, page 167 and table 4). It is noticeable how the effect of the successive passes, even using machines with low mass, turns important. This resulted in the highest compaction values evaluated with the penetrometer.

Bulk density

BD results, after the passes of the combine harvesters and the tractor plus the grain cart, resembled PR results. Statistical differences were observed between machines, loading conditions and sampling sites.

The passage of the combine harvesters and the tractor plus the fully loaded grain cart caused greater densification in the center of the footprint with a negative trend towards the sides. Nonetheless, it was higher than the control treatment value even at a distance of 60-80 cm on both sides of this sampling site. The maximum values were recorded with the tractor plus the grain cart (center of footprint, 30 cm on both sides), throwing data similar or superior to 1.45 - 1.46 Mg m^3.

These data were considered critical for root development of gramineae in clayey soils according to studies of Reinert *et al.* (2008) and Sadras and Calviño (2001) despite the fact that this equipment has the smallest mass.

Table 4. Depth 10-20 cm. Statistical analysis of penetration resistance. PR values expressed in MPa. Moisture values expressed as a percentage.

Tabla 4. Profundidad 10-20 cm. Análisis estadístico de resistencia a la penetración. Valores de RP expresados en MPa. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	20.8	1.268 qr	1.471 no	1.572 l	1.461 o	1.254 rs
CH 1 F	20.6	1.483 mn	1.737 fg	1.777 e	1.734 g	1.489 m
CH 2 E	20.3	1.241 s	1.491 m	1.669 h	1.493 m	1.241 s
CH 2 F	20.8	1.600 k	1.839 d	1.936 b	1.832 d	1.600 k
T + GC E	20.5	1.283 pq	1.610 k	1.753 f	1.628 j	1.279 pq
T + GC F	20.3	1.654 hi	1.878 c	1.974 a	1.874 c	1.635 ij
Control Treatment	20.4	1.291 p	1.291 p	1.291 p	1.291 p	1.291 p

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan $p < 0,05$.

Table 5. Depth 20-30 cm. Statistical analysis of penetration resistance. PR values expressed in MPa. Moisture values expressed as a percentage.

Tabla 5. Profundidad 20-30 cm. Análisis estadístico de resistencia a la penetración. Valores de RP expresados en MPa. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60cm L	T+GC 30cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	20.6	1.502 q	1.587 no	1.699 l	1.594 n	1.508 q
CH 1 F	20.8	1.704 l	1.814 gh	1.870 e	1.834 fj	1.664 m
CH 2 E	21.0	1.533 p	1.732 k	1.856 ef	1.757 ij	1.507 q
CH 2 F	21.2	1.745 jk	1.846 f	2.000 b	1.852 ef	1.752 ijk
T + GC E	20.7	1.774 i	1.798 h	1.900 d	1.812 gh	1.751 ijk
T + GC F	21.3	1.853 ef	1.967 c	2.080 a	1.962 c	1.857 ef
Control Treatment	20.8	1.363 r	1.363 r	1.363 r	1.363 r	1.363 r

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan $p < 0,05$.

Table 6. Depth 30-40 cm. Statistical analysis of penetration resistance. PR values expressed in MPa, moisture values expressed as a percentage.

Tabla 6. Profundidad 30-40 cm. Análisis estadístico de resistencia a la penetración. Valores de RP expresados en MPa. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	21.5	1.396 p	1.470 n	1.572 jk	1.507 m	1.357 q
CH 1 F	20.8	1.507 m	1.630 hi	1.727 g	1.612 i	1.532 l
CH 2 E	21.3	1.403 p	1.564 k	1.624 hi	1.567 k	1.400 p
CH 2 F	21.4	1.610 i	1.820 e	1.943 a	1.813 e	1.613 i
T + GC E	21.5	1.424 o	1.630 hi	1.774 f	1.638 h	1.421 o
T + GC F	21.0	1.850 cd	1.874 b	1.953 a	1.858 bc	1.830 de
Control Treatment	21.6	1.429 o	1.429 o	1.429 o	1.429 o	1.429 o

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan $p < 0,05$.

This situation indicates that the double-passage effect on the same footprint, *i.e.* the 4-axis passage, was more important than the mass involved, in a similar way to that observed with a penetrometer. These effects were evident for all evaluated depths (table 7, tables 8 and 9, page 171 and table 10, page 172).

Combines 1 and 2 affected the soil bulk density differently in both full and empty conditions at all depths measured at the sides of the center of the footprint, even though estimated contact pressure values were not very different (table 2, page 166). This indicates that contact pressure value alone, cannot predict compaction risks due to traffic.

The greatest effects of densification caused by traffic were at 20-30 cm depth and on both sides of the center of the footprint (table 9, page 171). A negative trend towards the sides was observed for all cases following the same trends evaluated with the penetrometer.

Table 7. Depth 0-10 cm. Statistical analysis of bulk density. BD values expressed in Mg m^{-3}. Moisture values expressed as a percentage.

Tabla 7. Profundidad 0-10 cm. Análisis estadístico de densidad aparente. Valores de Da expresados en Mg m^{-3}. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	19.7	0.977 m	1.231 i	1.272 h	1.192 j	1.056 l
CH 1 F	20.0	1.178 j	1.328 f	1.380 de	1.329 f	1.173 j
CH 2 E	20.2	1.131 k	1.270 h	1.420 c	1.270 h	1.120 k
CH 2 F	19.6	1.316 fg	1.382 de	1.472 b	1.387 de	1.314 fg
T + GC E	20.4	1.050 l	1.280 h	1.367 e	1.290 gh	1.070 l
T + GC F	20.0	1.383 de	1.464 b	1.531 a	1.454 b	1.409 cd
Control Treatment	20.3	1.124 k	1.124 k	1.124 k	1.124 k	1.124 k

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan p < 0,05.

Table 8. Depth 10-20 cm. Statistical analysis of bulk density. BD values expressed in Mg m^{-3}. Moisture values expressed as a percentage.

Tabla 8. Profundidad 10-20 cm. Análisis estadístico de densidad aparente. Valores de Da expresados en Mg m^{-3}. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30 cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	20.8	1.170 q	1.267 p	1.331 m	1.282 o	1.171 q
CH 1 F	20.6	1.302 n	1.457 g	1.506 f	1.440 h	1.279 o
CH 2 E	20.3	1.167 q	1.360 l	1.441 h	1.379 k	1.164 q
CH 2 F	20.8	1.400 j	1.553 d	1.582 c	1.538 e	1.418 i
T + GC E	20.5	1.143 r	1.378 k	1.466 g	1.393 j	1.151 r
T + GC F	20.3	1.461 g	1.578 c	1.694 a	1.603 b	1.460 g
Control Treatment	20.4	1.170 q	1.170 q	1.170 q	1.170 q	1.170 q

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan $p < 0,05$.

Table 9. Depth 20-30 cm. Statistical analysis of bulk density. BD values expressed in Mg m^{-3}. Moisture values expressed as a percentage.

Tabla 9. Profundidad 20-30 cm. Análisis estadístico de densidad aparente. Valores de Da expresados en Mg m^{-3}. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30 cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	20.6	1.342 q	1.404 n	1.500 i	1.398 n	1.358 p
CH 1 F	20.8	1.384 o	1.550 h	1.61 f	1.549 h	1.399 n
CH 2 E	21.0	1.354 p	1.469 l	1.562 g	1.483 jk	1.336 q
CH 2 F	21.2	1.426 m	1.627 e	1.691 c	1.647 d	1.407 n
T + GC E	20.7	1.406 n	1.570 g	1.631 e	1.568 g	1.384 o
T + GC F	21.3	1.479 k	1.702 b	1.768 a	1.711 b	1.492 ij
Control Treatment	20.8	1.262 r	1.262 r	1.262 r	1.262 r	1.262 r

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan $p < 0,05$.

Table 10. Depth 30-40 cm. Statistical analysis of bulk density. BD values expressed in Mg m^{-3}. Moisture values expressed as a percentage.

Tabla 10. Profundidad 30-40 cm. Análisis estadístico de densidad aparente. Valores de Da expresados en Mg $^{m-3}$. Valores de humedad expresados en porcentaje.

Machines	Moisture (%)	C1 60 cm L	C1 30 cm L	Center	C1 30cm R	C1 60 cm R
		C2 80 cm L	C2 40 cm L		C2 40cm R	C2 80 cm R
		T+GC 60 cm L	T+GC 30cm L		T+GC 30cm R	T+GC 60 cm R
CH 1 E	21.5	1.287 r	1.357 o	1.399 m	1.354 o	1.300 pq
CH 1 F	20.8	1.369 n	1.510 i	1.581 e	1.514 i	1.362 no
CH 2 E	21.3	1.309 p	1.438 k	1.464 j	1.437 k	1.301 pq
CH 2 F	21.4	1.421 l	1.583 e	1.672 b	1.602 d	1.430 kl
T + GC E	21.5	1.293 qr	1.548 g	1.644 c	1.537 h	1.290 qr
T + GC F	21.0	1.554 fg	1.674 b	1.734 a	1.670 b	1.564 r
Control Treatment	21.6	1.298 pqr	1.298 pqr	1.298 pqr	1.298 pqr	1.298 pq

Different letters present statistical differences. Duncan test $p < 0.05$.

Diferentes letras presentan diferencias estadísticas. Test de Duncan p < 0,05.

References to tables 3 to 10

Center (Center of the footprint); C1 30 cm L (Combine harvester 1 30 cm to the left of the center of the footprint); 30 cm R C1 (Combine harvester 1.30 cm to the right of the center of the footprint); C1 60 cm L (Combine harvester 1.60 cm to the left of the center of the footprint); 60 cm R C1 (Combine harvester 1.60 cm to the right of the center of the footprint); C2 40 cm L (Combine harvester 2.40 cm to the left of the center of the footprint); C2 40 cm R (Combine harvester 2.40 cm to the right of the center of the footprint); C2 80 cm L (Combine harvester 2.80 cm to the left of the center of the footprint); C2 80 cm R (Combine harvester 2.80 cm to the right of the center of the footprint); T plus GC 30 cm L (Tractor plus grain cart 30 cm to the left of the center of the footprint); T plus GC 30 cm R (Tractor plus grain cart 30 cm to the right of the center of the footprint); T plus GC 60 cm L (Tractor plus grain cart 60 cm to the left of the center of the footprint); T plus GC 60 cm R (Tractor plus grain cart 30 cm to the right of the center of the footprint). CH 1 E, combine harvester 1 empty; CH 1 F, combine harvester 1 fully loaded; CH 2 E, combine harvester 2 empty; CH 2 F, combine harvester 2 fully loaded; T plus GC E, Tractor plus grain cart; T plus GC F, Tractor plus fully loaded grain cart.

Referencias para tablas 3 a 10

Centro (centro de huella); C1 30 cm L (Cosechadora 1 30 cm a la izquierda del centro de huella); C1 30 cm R (Cosechadora 1 30 cm a la derecha del centro de huella); C1 60 cm L (Cosechadora 1 60 cm a la izquierda del centro de huella); C1 60 cm R (Cosechadora 1 60 cm a la derecha del centro de huella); C2 40 cm L (Cosechadora 2 40 cm a la izquierda del centro de huella); C2 40 cm R (Cosechadora 2 40 cm a la derecha del centro de huella); C2 80 cm L (Cosechadora 2 80 cm a la izquierda del centro de huella); C2 80 cm R (Cosechadora 2 80 cm a la derecha del centro de huella); T más BC 30 cm L (Tractor más carro granelero 30 cm a la izquierda del centro de huella); T más BC 30 cm R (Tractor más carro granelero 30 cm a la derecha del centro de huella); T más BC 60 cm L (Tractor más carro granelero 60 cm a la izquierda del centro de huella); T más BC 60 cm R (Tractor más carro granelero 30 cm a la derecha del centro de huella). CH 1 E, cosechadora 1 vacía; CH 1 F, cosechadora 1 llena; CH 2 E, cosechadora 2 vacía; CH 2 F, cosechadora 2 llena; T más BC E, tractor más carro granelero vacío; T más BC F, tractor más carro granelero lleno.

Conclusions

The passage of the tractor plus the fully loaded grain cart caused the greatest compaction at all sampling depths, exceeding 60 cm on both sides of the center of the footprint. The values obtained were close to or higher than the those suitable for crop development.

The compaction effect caused by the passage of the two combined harvesters in fully loaded condition is considered critical for crop development up to 30 cm on both sides of the center of the footprint.

The tractor plus the grain cart traffic within crop lots is neither convenient nor advisable according to the obtained results. To design a strategy for harvesting with controlled traffic becomes necessary. Nevertheless, if the detected levels of compaction remain the same in the next crop cycle should be verified.

To verify that tires used in harvesting equipment can withstand the stresses, turns important. Inflation pressure should also be verified since a tire's load carrying capacity is related to this pressure.

References

1. ASAE. 1985. Standard soil cone penetrometer S 313.2.
2. Botta, G. F.; Tolón-Becerra, A.; Lastra-Bravo, X.; Hidalgo R.; Rivero D.; Agnes D. 2015. Alternatives for handling rice (*Oryza sativa* L.) straw to favor its decomposition in direct sowing systems and their incidence on soil compaction. Geoderma Vols. (239-240): 213-222.
3. Botta, G.; Tolon Becerra, A.; Bienvenido, F.; Rivero, E.; Laureda, D.; Contesotto, E.; Fonteros, R.; Agnes, D. 2018. Traffic of harvester combines: effect on maize yields (*Zea Mays* L.) and soil compaction under direct sowing system. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 85-100.
4. Firestone. Catálogo Neumáticos. 2010. Bridgestone Argentina S.A.I.C. 43 p.
5. Firestone. 2018 (a). Catálogo Neumáticos. Avaliable in: https://commercial.firestone.com/en-us/ag-tire-selector?page=1. (Fecha de consulta: 10/5/2019).
6. Firestone. 2018 (b). Tire-pressure-inflation-calculator. Avaliable in: https://commercial.firestone.com/en-us/agriculture/resources/tire-pressure-inflation-calculator. (Fecha de consulta: 10/5/2019).
7. Goodyear. 2014. Catalogo Neumáticos Agrícolas. Avaliable in: http://www.titanlat.com/es/neumaticos-agricolas/index.aspx. (Fecha de consulta: 10/5/2019).
8. Hidalgo, R. 2003. Efectos del tránsito de ruedas especiales y neumáticos de tacos profundos sobre la compactación en suelos arroceros. Caracterización del desempeño de ruedas especiales en suelos inundados. Maestría en Mecanización Agrícola. Tesis, Facultad de Ciencias Agrarias y Forestales de la Universidad Nacional de La Plata. La Plata. Argentina. 65 p.
9. Hidalgo, R.; Botta, G.; Pozzolo O.; Santajuliana H.; Domínguez F., Curro C. 2010. Cosecha de soja. efecto sobre la compactación del suelo. Actas XX Congreso Argentino de Mecánica de Suelos e Ingeniería. Geotécnica 2010 CAMSIG 2010. Mendoza. Argentina.
10. Laureda, D.; Botta, G.; Tolón Becerra, A.; Rosatto, H. 2016. Soil compaction induced by machinery at polo fields in Argentina. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 48(1): 79-99.
11. Ligier, H.; Kurtz D.; Matteio H.; Perucca, R.; Vallejos, O. 2001. Suelos y vegetación de los departamentos Sauce y Curuzú Cuatiá, Provincia de Corrientes. INTA. EEA Corrientes. Recursos Naturales. Provincia de Corrientes-Consejo Federal de Inversiones (CFI). 282 p.

12. McKyes, E. 1985. Soil cutting and tillage. Elsevier Science . Amsterdam. Netherlands. p. 217.
13. Mur, M.; Balbuena, R. 2014. Compactación de un suelo argiudol típico por tráfico en un sistema de producción de forrajes. Rev. Ciencia del suelo. 32(1): 1-12.
14. O'Sullivan, M.; Henshall, J.; Dickson, J. 1998. A simplified method for estimating soil compaction. Soil & Tillage Research. Vol. 49. p. 325-335.
15. Palancar, T.; Terminiello A.; Jorajuría, D. 2009. Determinación expeditiva del área de contacto rueda-suelo en máquinas agrícolas. Congreso Argentino de Ingeniería Rural. 209-212.
16. Pérez, G. R.; Barbieri, P. A.; Hernandez Guijarro, K.; Echeverría. H. E.; Covacevich, F. 2017. Labranza y fertilización como moduladores de la dinámica de comunidades microbianas asociadas a un cultivo de trigo en el sudeste bonaerense (Argentina). Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 49(2): 219-234.
17. Pozzolo, O.; Gange, J; Ferrari, H.; Hegglin, J.; Rivarola, S.; Curró, C.; De Battista, J. 2012. Comportamiento de suelos vertisoles al tráfico en sistemas de siembra directa y convencional. Avaliable in: http://www.cosechaypostcosecha.org/data/articulos/maquinaria/ComportamientoSuelosVertisoles.asp Diciembre 2012. (Fecha de consulta: 10/5/2019).
18. Reinert, D. J.; Albuquerque, J. A.; Reichert, J. M.; Aita, C.; Andrada, M. M. C. 2008. Limites críticos de densidade do solo para o crescimento de raízes de plantas de cobertura em Argissolo Vermelho. R. Bras. Ci. Solo. 32: 1805-1816.
19. Rivero, E. R. D. 2012. La compactación del suelo y la resistencia a la rodadura en relación al diseño del tractor y a la condición mecánica del suelo. Maestría en Mecanización Agrícola. Tesis, Facultad de Ciencias Agrarias y Forestales de la Universidad Nacional de La Plata. La Plata Argentina. 60 p.
20. Sadras, V.; Calviño, P. 2001. Quantification of grain yield response to soil depth in soybean, maize, sunflower and wheat. Agron. J. 93(3): 577-583.
21. Schjønning, P.; Lamande´, M.; Tøgersen, F.; Arvidsson, J.; Keller, T. 2008. Modeling effects of tyre inflation pressure on the stress distribution near the soil-tyre interface. Biosyst. Eng. 99: 119-133
22. Tolon Becerra, A.; Botta, G.; Lastra Bravo, X.; Tourn, M.; Bellora Melcon, F.; Vazquez, J.; Rivero, D.; Linares, P.; Nardon, G. 2009. Soil compaction distribution under tractor traffic in almond (*Prunus amigdalus* L.) orchard in Almería España. Soil & Tillage Research 107(2010): 49- 56.

Monitoring vegetation using remote sensing time series data: a review of the period 1996-2017

Monitoreo de vegetación utilizando datos de series de tiempo de teledetección: una revisión de 1996-2017

José Manuel Zúñiga-Vásquez [1], Carlos Arturo Aguirre-Salado [2], Marín Pompa-García [1*]

Originales: *Recepción:* 17/05/2018 - *Aceptación:* 04/07/2019

Abstract

Analyzing time series data with remote sensing provides a better understanding of vegetation dynamics, since previous conditions and changes that have occurred over a given period are known. The objective of this paper was to analyze the current status and recent advances in the use of time series data obtained from remote sensors for vegetation monitoring. A systematic search of scientific papers was performed and 167 papers were found, published during the period 1996 to 2017. No significant difference in the amount of years analyzed was found between time series analyzed with a single sensor and those analyzed with a combination of several sensors (*i.e.* Landsat and SPOT, Landsat and Sentinel, among others). However, the combination of data from different sensors (fusion of images) can improve the quality of the results. Special attention must also be given to the fusion of optical and radar data, since this offers more unique spectral and structural information for land cover and land use assessments.

Keywords

phenology • land cover • analysis of multi-temporal remote sensing • spatio-temporal analysis • image fusion

1 Universidad Juárez del Estado de Durango. Facultad de Ciencias Forestales. Río Papaloapan y Blvd. Durango s/n. Col. Valle del Sur. 34120, Durango. Durango, México. josema.zuva@gmail.com, * mpgarcia@ujed.mx

2 Universidad Autónoma de San Luis Potosí. Facultad de Ingeniería. Manuel Nava N° 8. Zona Universitaria. 78280. San Luis Potosí. S. L. P. México. carlos.aguirre@uaslp.mx

Resumen

El análisis de datos de series de tiempo con sensores remotos proporciona una mejor comprensión de la dinámica de la vegetación, ya que se conocen las condiciones y cambios previos que se han producido en un período determinado. El objetivo de este trabajo fue analizar el estado actual y los avances recientes en el uso de datos de series de tiempo obtenidos de sensores remotos para el monitoreo de vegetación. Se realizó una búsqueda sistemática de artículos científicos y se encontraron 167 artículos publicados entre 1996 y 2017. No se encontraron diferencias significativas en la cantidad de años analizados entre las series temporales analizadas con un solo sensor y aquellas analizadas con una combinación de varios sensores (es decir, Landsat y SPOT, Landsat y Sentinel, entre otros). El número de años analizados con datos de series de tiempo fue similar, ya sea utilizando un solo sensor o una combinación de diferentes sensores. Sin embargo, la combinación de datos de diferentes sensores (fusión de imágenes) puede mejorar la calidad de los resultados. También se debe prestar especial atención a la fusión de datos ópticos y de radar, ya que ofrecen información espectral y estructural más exclusiva para las evaluaciones de la cubierta terrestre y el uso de la tierra.

Palabras clave

fenología • cobertura de la tierra • análisis multi-temporal de teledetección • análisis espacio-temporal • fusión de imágenes

Introduction

Characterizing plant cover is essential for managing natural resources, modeling environmental variables and understanding habitat distribution (14). Vegetation dynamics have been monitored using a variety of approaches, from observational methods conducted directly in the field (26) to those based on remote sensing (2) from plot level (1), to regional (9, 37) and global scales (7). Different vegetation classes or types can be identified by their unique spectral characteristics *i.e.* reflectance or emissivity (44). Thus, remote sensing offers an advantageous method of monitoring since imagery can cover large geographic areas, and has become an essential method for understanding large-scale environmental changes (22). While a plethora of remote sensors exist, those most commonly applied in vegetation monitoring include Landsat (TM, ETM + and currently Landsat 8 OLI), SPOT, MODIS, NOAA-AVHRR, IKONOS and QuickBird (36, 44).

In recent years, sensor capabilities have considerably improved in terms of spatial, spectral and temporal resolution (44). This technological evolution provides information in greater quantity and with improved precision (5). Increased accessibility to remote sensing data and greater computing capacity have completely changed the way of using these data. At present, the use of more complex analysis with novel algorithms for detecting changes in vegetation cover using time series data is becoming more frequent (46). Although there is current research that reports trends in the use of time series data, these studies only report trends for a single sensor (*e.g.* Landsat) (4, 46), while other remote sensors have not been the subject of extensive review. It is therefore necessary to report trends in the use of time series data for the monitoring of vegetation with respect to more sensors, greater periodicity and involving the study of more ecosystems. For this reason, the main objective of this research was to analyze the current status and recent advances in the use of time series data obtained from remote sensors for vegetation monitoring. Systematization of this collection in a database will provide an overview with which to identify the background, knowledge gaps and trends of the current research.

Materials and Methods

A search of scientific papers exploring the topic of "monitoring of vegetation using time series data" was carried out using the Web of Science website (http://apps.webofknowledge.com). A database of published papers was constructed containing the following information fields: reference, year of publication, paper title, journal, impact factor, source of funding, spatialized (mapped) results, number of authors, number of institutions involved, study objective, area of influence, country, size and location of the study area, vegetation and land use, climate, platform/sensor, number of sensors used, spatial resolution, number of images analyzed, years analyzed, main data, ancillary data, software, main algorithms used and purpose of algorithm and variables of interest. The data were subsequently grouped into ranges and categories of similar data in order to facilitate their representation and statistical description.

The papers were classified into two groups: those using one sensor and those presenting a combination of two or more sensors (*e.g.* Landsat sensors and SPOT sensors combination, Landsat sensors and Sentinel sensors combination, among others). A means test (Kruskal-Wallis test, $p \leq 0.05$) was performed in R Studio (2013) between these groups in order to identify which group featured the most robust time series (more years analyzed). To ensure coherence of the results in this review, opinions were sought from experts.

Results

Temporal trend

Initially, 186 papers were detected by the search, of which 167 were chosen for analysis (see supplementary material). Studies of all types of vegetation and climate were included. The rest of papers did not study vegetation, and some articles even belonged to other branches of science. The 167 papers analyzed were published in the period 1996 to 2017 (August). No papers were found for the year 1998, and the highest number of papers on the study topic was published in 2012. Three periods of research output productivity were identified: the first was observed during the period 1996-2002, with an average of 1 to 3 papers published per year; the second covered the period 2003-2010, with an average of 3 to 10 papers published per year; and the third was identified for the period 2011-2017, with an average of 11 to 18 papers. This revealed a clear and increasing trend in the number of papers published per year from 1996 to 2017 (figure 1, page 178).

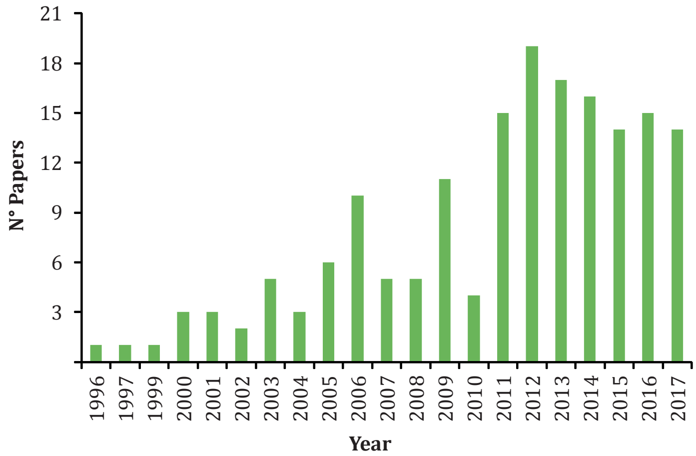


Figure 1. Number of papers/studies grouped by year of publication in vegetation monitoring using time series data (167 papers consulted).

Figura 1. Número de investigaciones/estudios agrupados por año de publicación en el monitoreo de vegetación usando datos de series de tiempo (167 documentos consultados).

Most preferred journal for publication

The published papers were mainly distributed among 70 journals, where the lowest impact factor was 0.2442 for the "Iranian Journal of Science & Technology", and the highest was 8.502 for the journal "Global Change Biology". In addition, there were journals that were prominent in terms of quantity of publications (table 1, page 179).

Papers published per country

While time series data analysis for monitoring vegetation has been conducted practically worldwide, China and Brazil are the prominent countries in terms of the number of studies published (figure 2, page 179).

Most studied ecosystems

The main ecosystems studied, as well as the main types of climate that occur in the data analyzed, are shown in figure 3 (page 180). It should be noted that the main ecosystem and climate studied worldwide are forests and the tropical climate, respectively.

Most used approach/methodology

The main methodologies found are based on classification of plant cover and monitoring of phenological states. Most of these methods use the vegetation index NDVI as the main element. These, in combination with other algorithms, help the extraction of results. An interesting aspect found was that the research mostly utilized climatic information as ancillary data. In table 2 (page 181), vegetation monitoring is summarized. The NDVI is the most reported algorithm in the literature.

Sensors used

The most used satellites were SPOT, followed by Landsat, although it should be noted that a combination of data from several different satellites is used in some studies (table 3, page 181).

Table 1. Journals with highest number of publications in vegetation monitoring using time series data (167 papers consulted).

Tabla 1. Revistas con más publicaciones en el monitoreo de vegetación usando datos de series de tiempo (167 artículos consultados).

Journal	Impact factor	Number of publications	Percentage
Remote Sensing of Environment	6.265	27	16.16
International Journal of Remote Sensing	1.724	27	16.16
Remote Sensing	3.244	11	6.58
International Journal of Applied Earth Observation and Geoinformation	3.93	9	5.38
Forest Ecology and Management	3.064	5	2.99
Journal of Applied Remote Sensing	1.107	5	2.94
Others (5 publications)	-	83	49.70
Total		167	100

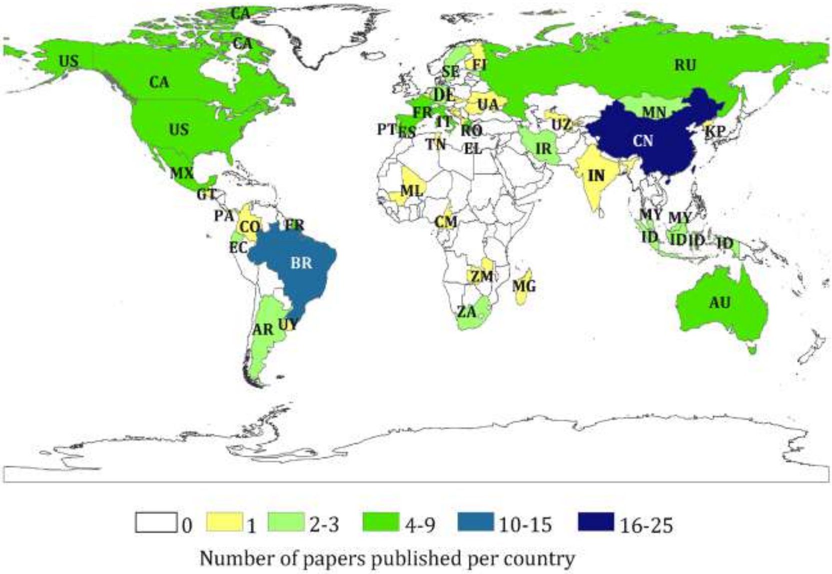


Figure 2. Global studies published in the field of vegetation monitoring using time series data, grouped by country (167 papers consulted).

Figura 2. Estudios publicados a nivel global en el campo del monitoreo de vegetación usando datos de series de tiempo, agrupados por país (167 documentos consultados).

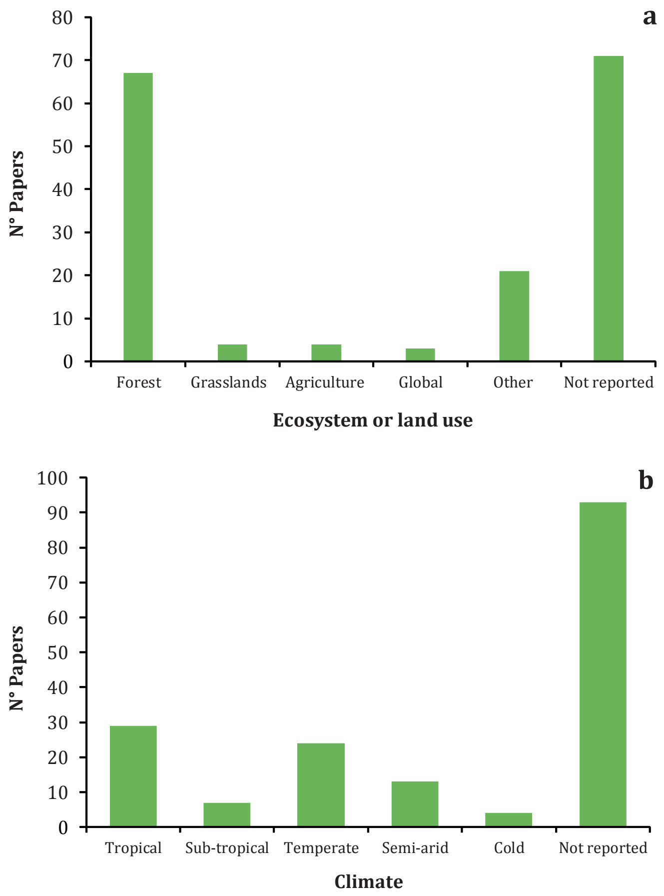


Figure 3. Papers published on vegetation monitoring using time series data, grouped by type of ecosystem (a) and by type of climate (b), from a total of 167 papers analyzed.

Figura 3. Documentos publicados sobre el monitoreo de la vegetación usando datos de series de tiempo, agrupados por tipo de ecosistema (a) y por tipo de clima (b) estudiado, 167 artículos analizados.

Table 2. Main algorithms/indexes and approaches for vegetation monitoring using time series data.

Tabla 2. Principales algoritmos/índices y enfoques para el monitoreo de la vegetación usando datos de series de tiempo.

Algorithm/index	Number of publications in which it appears	Main purpose
Normalized Difference Vegetation Index (NDVI)	72	To determine vegetation status, discrimination of vegetation cover and soil loss.
Supervised Classification	12	Classify types of land use and vegetation.
Leaf Area Index (LAI)	10	Condition of vegetation, vegetation cover.
Difference Normalized Burn Ratio (DNBR)	9	Classification of post-fire vegetation.
Enhanced Vegetation Index (EVI)	6	To determine vegetation phenology.
Others	58	-

Table 3. Most used satellites/sensors in published papers addressing vegetation monitoring using time series data (167 papers consulted).

Tabla 3. Satélites/sensores más utilizados en artículos publicados sobre el monitoreo de la vegetación utilizando datos de series de tiempo (167 documentos consultados).

Satellite	Sensor	Spatial resolution (m)	Number of papers
SPOT 5	Vegetation (VGT-II)	1000	15
	HRG (Xi)	10-20	7
SPOT 4	Vegetation (VGT-I)	1000	77
	HRVIR (Xi)	20	3
SPOT 2, 3	HRV (XS)	20	12
Landsat 8	OLI	30	9
Landsat 7	ETM+	30	24
Landsat 5	TM	30	24
Landsat 1, 2,3	MSS	30	6
Aqua	MODIS (MYD)	250, 500, 1000	4
Terra	MODIS (MOD)	250, 500, 1000	32
	MISR	1100	1
Aqua/Terra	MODIS (MCD)	250, 500, 1000	9
NOAA	AVHRR	1000, 4000, 8000, 64000	28
Others		1.8, 4, 300, 500, 1000	13

It was also found that most of the studies featured analysis of periods from 1 to 10 years, while only one study analyzed a period of more than 40 years (110 years) (figure 4).

Specifically, the most analyzed period was from 2000 to 2010 (figure 5) since it was observed that the time series studies mainly analyzed the period from the year 1980 to date.

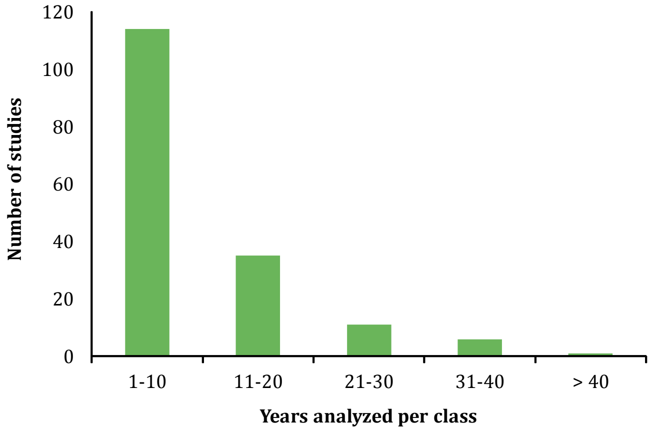


Figure 4. Number of papers grouped by the category of number of years of remotely sensed time series data used for vegetation monitoring.

Figura 4. Número de artículos agrupados por la categoría de años de datos de series temporales de teledetección utilizados para el monitoreo de la vegetación.

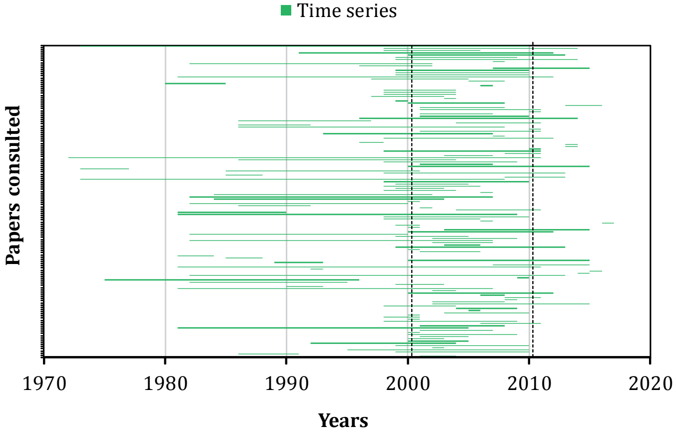


Figure 5. Starting and ending years of the time series data analyzed in vegetation monitoring studies (167 papers consulted, the period between the dotted vertical line denotes the most commonly analyzed period of time).

Figura 5. Año de inicio y final de las series de tiempo analizadas en estudios de monitoreo de la vegetación (167 documentos consultados, el período entre la línea vertical punteada indica el período de tiempo más analizado).

The reviewed papers and the opinions of experts indicate that, in recent years, the combination of data (image fusion) from different sensors (*e.g.* Landsat sensors and SPOT sensors combination, Landsat sensors and Sentinel sensors combination, among others) has served to improve its quality (45) and therefore deserves special attention. Our results indicated that 77 of 167 articles used combinations of several sensors. These papers have mostly been published in the last decade, thus representing a current trend in the analysis of time series data.

No significant difference was found between the average of the number of years analyzed using only one sensor (8.18 years) and that using a combination of several sensors (8.4 years) (significance of 0.05) (figure 6).

Discussion

Our study reveals some important trends found in vegetation monitoring studies using time series data over the period 1996-2017 (August). The number of vegetation monitoring papers published using time series data has shown a considerable increase over the last 7 years of this period. This supports that reported by Zhuang *et al.* (2013), who also found a significant increase in the number of papers published in recent decades in the field of remote sensing. This pattern is due to the fact that remote sensing is a rapidly advancing technology and has in recent years experienced unprecedented growth due to the development of sensors and increased information technology capacities, including processing, storage and data base formation (30).

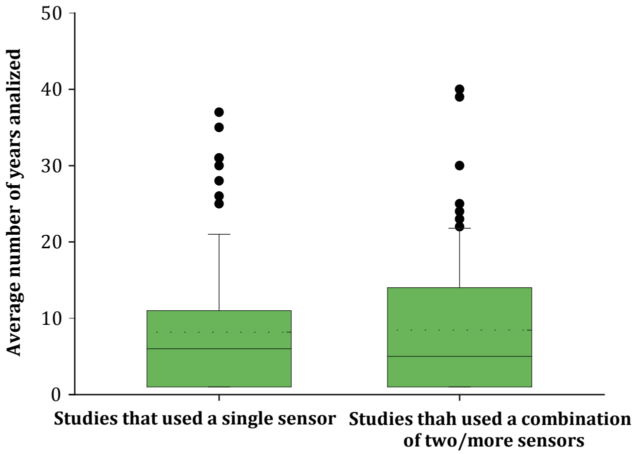


Figure 6. Comparison of the number of years of vegetation monitoring analyzed using time series data, performed with a single sensor or with a combination of several sensors. Total of 167 papers analyzed.

Figura 6. Comparación del número de años de monitoreo de vegetación analizados utilizando datos de series de tiempo, realizados con un solo sensor y con una combinación de varios sensores, 167 artículos analizados.

The journals that published the greatest number of papers in the field of vegetation monitoring using time series data were Remote Sensing of Environment and International Journal of Remote Sensing. Likewise, Zhuang *et al.* (2013) reported that these two journals are among those with the most papers published in the field of remote sensing over recent years. In terms of the geographic location of scientific production, China and Brazil are the leaders in this field (48). These two countries are in the group of 20 countries with the highest scientific production (published papers) in the field of remote sensing. Indeed, China is the second most productive country in the world, after the United States (48).

The most studied ecosystem is forest, followed by grassland and then by general global monitoring of vegetation. This is logical, since forests are the most widely distributed ecosystems on the planet (42). However, it should be noted that the study of other ecosystems is also of great importance.

Of the 167 papers reviewed, the methodologies used for vegetation monitoring most commonly featured the Normalized Difference Vegetation Index (NDVI). The NDVI, developed by Rouse *et al.* (1974), stands out in that, since its appearance, it has quickly become the most dominant satellite observable metric for spatio-temporal changes (18) and has been successfully used to explore vegetation dynamics (20), although in recent years there has also been an attempt to improve and evaluate the performance of algorithms and indices to improve vegetation monitoring (35). Some notable studies were also found, using a considerable number of indices (6, 11, 19, 23).

A further important factor in vegetation monitoring using time series data, is that of the satellites utilized. Landsat satellites are the most commonly used in remote sensing; however, in the analyzed data, SPOT satellites appear as the most used in multi-temporal studies. This could be explained by some limitations of Landsat, (*e.g.* temporal resolution or images contaminated by cloud and shadow) (12). For this reason, the suite of SPOT sensors is the most widely used alternative to Landsat (32) and some authors state that it is particularly suitable for vegetation mapping at global and regional scales (44). With regards to Landsat, it should be noted that most studies that use Landsat data correspond to more recent years, since initiation of the free and open Landsat data policy in 2008 (47). This is therefore a satellite of great potential for the future given its availability (*i.e.* free and open access). In recent decades, the cost of data storage has dramatically decreased, providing a viable basis for time series analysis that demands Landsat data (46).

Most studies have analyzed time series of between 1 and 10 years. However, since 2010, more complex investigations have been carried out. This could also be strongly influenced by the free access policies of Landsat, as mentioned above, as well as the launch of the MODIS sensor onboard the Aqua/ Terra satellites notable for its temporal resolution (28). Remote sensing is a rapidly advancing technology (40) and has now reached a price point where the images acquired by these systems are truly accessible (36).

New trends for the analysis of time series data

Advances in remote sensing technology, in terms of software, processes and information acquisition, mean that it is now possible to conduct research with data from several different sensors (2, 15, 24, 29, 31). While this could in theory produce more robust time series, statistical testing shows that there is no significant difference between time series analyzed with data from a single sensor and those analyzed with a combination of data from several sensors. Nevertheless, the combination of data (image fusion) improves the quality of the results (13) since this technique can integrate different image data and provide more information than the derived from a single sensor (39).

In this sense, K. de Beurs (personal communication, February 16, 2018) indicates that special attention should be given to the use of fusion of optical and radar data, since this offers more unique spectral and structural information for land cover and land use assessments. This coincides with Joshi *et al.* (2016), who indicate that future research should focus on the development of robust optical and radar data fusion techniques, including those that test the frequency with which time series and variable spatial resolution data sets can be combined in a significant manner with a minimum loss of information.

The main difference between these types of data is that, compared to optical satellite images, synthetic aperture radars (SARs) have certain advantages for vegetation monitoring due to the fact that microwave sensors have longer wavelengths, can penetrate vegetation canopies, and are not influenced by the presence of clouds or haze (16, 17). Some SARs have a short revisit time and high spatial resolution, which could be beneficial for vegetation monitoring. Since optical and SAR image data respond to crop characteristics differently, their complementary information can be valuable to support vegetation monitoring (39).

Another aspect of great interest, at present and for the future, is the use of free access data and software. According to Wulder *et al.* (2012), open access to data promotes greater international collaboration to meet the land observation needs of the twenty-first century. In this sense, the Landsat satellite network stands out for both the long history and free access of its data set (25). It is also important to mention the establishment of a Landsat-9 Architecture Study Team that define capabilities and implementation strategy. Landsat-9 has been authorized and is proceeding towards a December 2020 launch. Planning for missions beyond Landsat-9 is also underway, with the USGS defining future Landsat measurement needs (*e.g.*, Landsat-10 and -11) (43).

While the ability to use a multitude of images acquired in a single region has changed the perception on the Landsat value, some properties of this satellite are currently critical (41). One limitation of Landsat is that the satellites can only revisit the same area every 16 days, and the acquired Landsat data for specific areas can be contaminated by cloud and shadow. The temporally sparse time-series Landsat data are, therefore, unsuitable for global monitoring of rapid changes in the vegetation and terrestrial surface (12).

A potential approach for utilization of Landsat data is represented by data fusion techniques that integrate imagery across sensors, effectively leveraging the most desirable characteristics from multiple sensors. The spatial, spectral and temporal resolution of any given sensor can thus potentially be enhanced by merging bands within or across sensors (32).

Special attention should also be paid to the Sentinel-2 satellite since, in terms of spatial and temporal resolution, it provides an alternative to SPOT and Landsat, with narrower bands for better identification of characteristics, additional channels in the red edge spectral domain that allow evaluation of vegetation status and specific bands for improved atmospheric correction (10, 27). There has even been experimentation with the fusion of Landsat and Sentinel-2 satellite data, showing great potential for timely monitoring of rapid changes (38). Currently, the Harmonized Landsat/ Sentinel-2 (HLS) project provides a surface reflectance product that combines observations from USGS/NASA's Landsat 8 and ESA's Sentinel-2 satellites at moderate spatial resolution (30 m). The main goal is to provide a unique dataset based on the data of both satellites in order to reduce the revisit time to 3-5 days, depending on latitude (8).

Conclusions

Most of the articles analyzed (64.7%) were published in the last 7 years of the study period, reflecting the great changes that remote sensing has undergone in terms of data availability. China and Brazil are the countries with the highest number of vegetation monitoring studies using time series data. The main ecosystems studied are the forests, although in recent years it is common to find studies of vegetation monitoring at the global scale.

The SPOT satellites have been the most used for vegetation monitoring using time series data, but Landsat satellites aim to be the most popular in the future due to the availability, periodicity and easy handling of its data. Also important is the Sentinel-2 satellite, which is notable for its greater temporal resolution.

On average, the number of years analyzed with time series data was similar using either a single sensor or a combination of different sensors. However the combination of data from different sensors (image fusion) can improve results quality, while the use of fusion of optical and radar data points towards a new trend in this field.

Our findings in this review of literature on vegetation monitoring using time series data will contribute to future work in this field, improving decision-making about periods for analysis and appropriate data selection from remote sensors.

Supplementary Material

https://drive.google.com/open?id=1bMilH96d1E--7PLI1VhwkzIFX-pT3Z4GL

References

1. Albornoz, L.; Rodríguez Plaza, L.; Navarro, A.; López, M.; Bageta, C.; Mercado, L. 2019. Vineyard zoning of cv Bonarda argentine (*Vitis vinífera* L.), from Sentinel satellite images and three vegetation indexes. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(2): 167-176.
2. Arino, O.; Casadio, S.; Serpe, D. 2012. Global night-time fire season timing and fire count trends using the ATSR instrument series. Remote Sensing of Environment. 116: 226-238.
3. Baeza, S.; Baldassini, P.; Bagnato, C.; Pinto, P.; Paruelo, J. 2014. Caracterización del uso/cobertura del suelo en Uruguay a partir de series temporales de imágenes MODIS. Agrociencia Uruguay. 18(2): 95-105.

4. Banskota, A.; Kayastha, N.; Falkowski, M. J.; Wulder, M. A.; Froese, R. E.; White, J. C. 2014. Forest monitoring using Landsat time series data: A review. Canadian Journal of Remote Sensing. 40(5): 362-384.
5. Camps-Valls, G.; Tuia, D.; Bruzzone, L.; Benediktsson, J. A. 2014. Advances in hyperspectral image classification: Earth monitoring with statistical learning methods. IEEE Signal Processing Magazine. 31(1): 45-54.
6. Chen, B.; Cao, J.; Wang, J.; Wu, Z.; Tao, Z.; Chen, J.; Yang, C.; Xie, G. 2012. Estimation of rubber stand age in typhoon and chilling injury afflicted area with Landsat TM data: a case study in Hainan Island, China. Forest Ecology and Management. 274: 222-230.
7. Chen, J.; Chen, J.; Liao, A.; Cao, X.; Chen, L.; Chen, X.; Chaoying, H.; Gang, H.; Shu, P.; Miao, L.; Weiwei, Z.; Xiaohua, T.; Jon, M. 2015. Global land cover mapping at 30 m resolution: A POK-based operational approach. ISPRS Journal of Photogrammetry and Remote Sensing. 103: 7-27.
8. Claverie, M.; Ju, J.; Masek, J. G.; Dungan, J. L.; Vermote, E. F.; Roger, J.-C.; Skakun, S. V; Justice, C. 2018. The Harmonized Landsat and Sentinel-2 surface reflectance data set. Remote Sens. Environ. 219: 145-161.
9. Di Bella, C. M.; Beget, M. E.; Campos, A. N.; Viglizzo, E.; Jobbágy, E.; García, A. G.; Sycz, A.; Cotroneo, C. 2019. Changes in vegetation seasonality and livestock stocking rate in La Pampa Province (Argentina). Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 79-92.
10. Drusch, M.; Del Bello, U.; Carlier, S.; Colin, O.; Fernandez, V.; Gascon, F.; Hoersch, B.; Isola, C.; Laberinti, P.; Martimort, P.; Meygret, A.; Spoto, F.; Sy, O.; Marchese, F.; Bargellini, P. 2012. Sentinel-2: ESA's optical high-resolution mission for GMES operational services. Remote Sensing of Environment. 120: 25-36.
11. Einzmann, K.; Immitzer, M.; Böck, S.; Bauer, O.; Schmitt, A.; Atzberger, C. 2017. Windthrow detection in European forests with very high-resolution optical data. Forests. 8(1): 21.
12. Gao, F.; Hilker, T.; Zhu, X.; Anderson, M.; Masek, J.; Wang, P.; Yang, Y. 2015. Fusing Landsat and MODIS data for vegetation monitoring. IEEE Geoscience and Remote Sensing Magazine. 3(3): 47-60.
13. Ghassemian, H. 2016. A review of remote sensing image fusion methods. Information Fusion. 32: 75-89.
14. Gómez, C.; White, J. C.; Wulder, M. A. 2016. Optical remotely sensed time series data for land cover classification: A review. ISPRS Journal of Photogrammetry and Remote Sensing. 116: 55-72.
15. Hardtke, L. A.; Blanco, P. D.; del Valle, H. F.; Metternicht, G. I.; Sione, W. F. 2015. Semi-automated mapping of burned areas in semi-arid ecosystems using MODIS time-series imagery. International Journal of Applied Earth Observation and Geoinformation. 38: 25-35.
16. Herold, N. D.; Haack, B. N. 2002. Fusion of radar and optical data for land cover mapping. Geocarto International. 17(2): 21-30.
17. Herold, N. D.; Haack, B. N. 2006. Comparison and integration of radar and optical data for land use/cover mapping. Geocarto International. 21(4): 9-19.
18. Houborg, R.; Fisher, J. B.; Skidmore, A. K. 2015. Advances in remote sensing of vegetation function and traits. International Journal of Applied Earth Observation and Geoinformation.1-6.
19. Huang, H.; Roy, D. P.; Boschetti, L.; Zhang, H. K.; Yan, L.; Kumar, S. S.; Gomez-Dans J.; Li, J. 2016. Separability analysis of Sentinel-2A multi-spectral instrument (MSI) data for burned area discrimination. Remote Sensing. 8(10): 873.
20. Hutchinson, J. M. S.; Jacquin, A.; Hutchinson, S. L.; Verbesselt, J. 2015. Monitoring vegetation change and dynamics on US Army training lands using satellite image time series analysis. Journal of Environmental Management. 150: 355-366.
21. Joshi, N.; Baumann, M.; Ehammer, A.; Fensholt, R.; Grogan, K.; Hostert, P.; Jepsen, M. R.; Kuemmerle, T.; Meyfroidt, P.; Mitchard, E. T. A.; Reiche, J.; Ryan, C.; Waske, B. 2016. A review of the application of optical and radar remote sensing data fusion to land use mapping and monitoring. Remote Sensing. 8(1): 70.

22. Khatami, R.; Mountrakis, G.; Stehman, S. V. 2016. A meta-analysis of remote sensing research on supervised pixel-based land-cover image classification processes: General guidelines for practitioners and future research. Remote Sensing of Environment. 177: 89-100.
23. Kim, J.; Grunwald, S.; Rivero, R. G.; Robbins, R. 2012. Multi-scale modeling of soil series using remote sensing in a wetland ecosystem. Soil Science Society of America Journal. 76(6): 2327-2341.
24. Kim, Y.; Huete, A. R.; Miura, T.; Jiang, Z. 2010. Spectral compatibility of vegetation indices across sensors: band decomposition analysis with Hyperion data. Journal of Applied Remote Sensing. 4(1): 043520.
25. Loveland, T. R.; Dwyer, J. L. 2012. Landsat: Building a strong future. Remote Sensing of Environment. 122: 22-29.
26. Malhi, Y.; Phillips, O. L.; Lloyd, J.; Baker, T.; Wright, J.; Almeida, S.; Arroyo, l.; Frederiksen, T.; Grace, J.; Higuchi, N.; Killeen, T.; Laurance, W. F.; Leaño, C.; Lewis, S.; Meir, P.; Monteagudo, A.; Neill, D.; Núñez-Vargas, P.; Panfil, S. N.; Patiño, S.; Pitman, N.; Quesada, C. A.; Rudas-Ll, A.; Salomão, R.; Saleska, S.; Silva, N.; Silveira, M.; Sombroek, W. G.; Valencia, R.; Vásquez-Martínez, R.; Vieira, I. C.; Vinceti, B. 2002. An international network to monitor the structure, composition and dynamics of Amazonian forests (RAINFOR). Journal of Vegetation Science. 13(3): 439-450.
27. Martimor, P.; Arino, O.; Berger, M.; Biasutti, R.; Carnicero, B.; Del Bello, U.; Fernandez, V.; Gascon, F.; Silvestrin, P.; Sy, O. 2007. Sentinel-2 optical high-resolution mission for GMES operational services. In Geoscience and Remote Sensing Symposium. IGARSS 2007. IEEE International. 2677-2680.
28. National Aeronautics and Space Administration (NASA). (2019). Moderate Resolution Imaging Spectroradiometer (MODIS). Available online: https://modis.gsfc.nasa.gov/about/components.php (Accessed on May 24, 2019).
29. Nyamugama, A.; Kakembo, V. 2015. Estimation and monitoring of aboveground carbon stocks using spatial technology. South African Journal of Science. 111(9-10): 01-07.
30. Pajares, G. 2015. Overview and current status of remote sensing applications based on unmanned aerial vehicles (UAVs). Photogrammetric Engineering & Remote Sensing. 81(4): 281-329.
31. Pickett-Heaps, C. A.; Canadell, J. G.; Briggs, P. R.; Gobron, N.; Haverd, V.; Paget, M. J.; Pinty, B.; Raupach, M. R. 2014. Evaluation of six satellite-derived Fraction of Absorbed Photosynthetic Active Radiation (FAPAR) products across the Australian continent. Remote Sensing of Environment. 140: 241-256.
32. Powell, S. L.; Pflugmacher, D.; Kirschbaum, A. A.; Kim, Y.; Cohen, W. B. 2007. Moderate resolution remote sensing alternatives: a review of Landsat-like sensors and their applications. Journal of Applied Remote Sensing. 1(1): 012506.
33. R Development Core Team. 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
34. Rouse, Jr. J.; Haas, R. H.; Schell, J. A.; Deering, D. W. 1974. Monitoring vegetation systems in the Great Plains with ERTS. In Third Earth Resources Technology Satellite (ERTS) symposium. Washington. DC: NASA. 309-317.
35. Schultz, M.; Clevers, J. G.; Carter, S.; Verbesselt, J.; Avitabile, V.; Quang, H. V.; Herold, M. 2016. Performance of vegetation indices from Landsat time series in deforestation monitoring. International Journal of Applied Earth Observation and Geoinformation. 52: 318-327.
36. Toth, C.; Jóźków, G. 2016. Remote sensing platforms and sensors: A survey. ISPRS Journal of Photogrammetry and Remote Sensing. 115: 22-36.
37. Volante, J.; Mosciaro, J.; Morales, P. M.; Vale, L.; Castrillo, S.; Sawchik, J.; Tiscorna, G.; Fuente, M.; Maldonado, I.; Vega, A.; Trujillo, R.; Cortéz, L.; Paruelo, J. 2015. Expansión agrícola en Argentina, Bolivia, Paraguay, Uruguay y Chile entre 2000-2010. Caracterización espacial mediante series temporales de índices de vegetación. Revista de Investigaciones Agropecuarias. 41(2): 1.

38. Wang, Q.; Blackburn, G. A.; Onojeghuo, A. O.; Dash, J.; Zhou, L.; Zhang, Y.; Atkinson, P. M. 2017. Fusion of Landsat 8 OLI and Sentinel-2 MSI data. IEEE Transactions on Geoscience and Remote Sensing. 55(7): 3885-3899.
39. Wang, X. L.; Chen, C. X. 2016. Image fusion for synthetic aperture radar and multispectral images based on sub-band-modulated non-subsampled contourlet transform and pulse coupled neural network methods. The Imaging Science Journal. 64(2): 87-93.
40. Watts, A. C.; Ambrosia, V. G.; Hinkley, E. A. 2012. Unmanned aircraft systems in remote sensing and scientific research: Classification and considerations of use. Remote Sensing. 4(6): 1671-1692.
41. Wulder, M. A.; Masek, J. G.; Cohen, W. B.; Loveland, T. R.; Woodcock, C. E. 2012. Opening the archive: How free data has enabled the science and monitoring promise of Landsat. Remote Sensing of Environment. 122: 2-10.
42. Wulder, M. A.; Coops, N. C. 2014. Make Earth observations open access. Nature. 7516 (513): 30-31. doi: http://dx.doi.org/10.1038/513030a
43. Wulder, M. A.; Loveland, T. R.; Roy, D. P.; Crawford, C. J.; Masek, J. G.; Woodcock, C. E.; Allen, R. G.; Anderson, M. C.; Belward, A. S.; Cohen, W. B.; Dwyer, J.; Erb, A.; Gao, F.; Griffiths, P.; Helder, D.; Hermosilla, T.; Hipple, J. D.; Hostert, P.; Hughes, M. J.; Huntington, J.; Johnson, D. M.; Kennedy, R.; Kilic, A.; Li, Z.; Lymburner, L.; McCorkel, J.; Pahlevan, N.; Scambos, T. A.; Schaaf, C.; Schott, J. R.; Sheng, Y.; Storey, J.; Vermote, E.; Vogelmann, J.; White, J. C.; Wynne, R. H.; Zhu, Z. 2019. Current status of Landsat program, science, and applications. Remote Sensing of Environment. 225: 127-147.
44. Xie, Y.; Sha, Z.; Yu, M. 2008. Remote sensing imagery in vegetation mapping: a review. Journal of Plant Ecology. 1(1): 9-23.
45. Zhang, J. 2010. Multi-source remote sensing data fusion: status and trends. International Journal of Image and Data Fusion. 1(1): 5-24.
46. Zhu, Z. 2017. Change detection using landsat time series: A review of frequencies, preprocessing, algorithms, and applications. ISPRS Journal of Photogrammetry and Remote Sensing. 130: 370-384.
47. Zhu, Z.; Wulder, M. A.; Roy, D. P.; Woodcock, C. E.; Hansen, M. C.; Radeloff, V. C.; Healey, S.; Schaaf, C.; Hostert, P.; Strobl, P.; Pekel, J. F.; Lymburner, L.; Pahlevan, N.; Scambos, T. A. 2019. Benefits of the free and open Landsat data policy. Remote Sensing of Environment. 224: 382-385.
48. Zhuang, Y.; Liu, X.; Nguyen, T.; He, Q.; Hong, S. 2013. Global remote sensing research trends during 1991-2010: a bibliometric analysis. Scientometrics. 96(1): 203-219.

Use of indicators as a tool to measure sustainability in agroecosystems of arid land, San Juan, Argentina

Uso de indicadores como herramienta para medir la sustentabilidad en agroecosistemas de tierras áridas, San Juan, Argentina

Julieta Carmona Crocco [1, 2*], Silvina Greco [3], Raúl Tapia [1, 2, 4], Mariana Martinelli [2, 5]

Originales: *Recepción:* 21/12/2018 - *Aceptación:* 06/03/2020

Abstract

The main objective of this study was to determine the sustainability status of three agroecosystems in arid areas, and to identify the critical aspects that limit it, through the use of economic, ecological and socio-cultural indicators. Three agroecosystems (AE) were selected from the Department of 25 de Mayo, San Juan. Its main economic activity is goat farming and, to a lesser extent, cultivation of orchards, as well as subsistence economic activities. To determine the state of sustainability, the indicators were selected and subsequently standardized and weighted according to their relative importance with respect to sustainability. The results indicated that none of the dryland AE achieves sustainability simultaneously in its three dimensions. The systemic analysis through indicators showed that the critical variables are food self-sufficiency, water access, livestock survival, and technical assistance, among others. The methodology used is simple, allowing to detect the sustainability status of the three EAs, and to identify the critical variables that jeopardize the permanence of the AE over time.

Keywords
multidimensional • analysis agroecology • dryland • non-irrigated land

1 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
* juli.carmonacrocco@gmail.com
2 Instituto Nacional de Tecnologías Agropecuarias (INTA). Estacion Experimental Agropecuaria San Juan Calle 11 y Vidart 5427. Villa Aberastain. San Juan. Argentina.
3 Universidad Nacional de Cuyo. Facultad de Ciencias Agrarias. Almirante Brown 500. Chacras de Coria. Luján de Cuyo. CPA M5528AHB. Mendoza. Argentina.
4 Universidad Nacional de San Juan (UNSJ). Facultad de Ingenieria. Av. Lib. San Martín (Oeste) 1109. C. P. A. J5400ARL.
5 Universidad Nacional de San Juan (UNSJ). Facultad de Ciencia Exactas, Físicas y Naturales (FCEFyN). Av. Ignacio de la Roza 590 (O). Complejo Universitario "Islas Malvinas". Rivadavia. C. P. A. J5402DCS. San Juan. Argentina.

Resumen

El objetivo principal de este estudio fue determinar el estado de sustentabilidad de tres agroecosistemas en tierras áridas, e identificar los aspectos críticos que la limitan, mediante el uso de indicadores económicos, ecológicos y socioculturales. Se seleccionaron tres agroecosistemas (AE) del departamento de 25 de Mayo, San Juan. Su principal actividad productiva es la producción caprina y en menor medida la producción de huerta, ambas orientadas a una economía de subsistencia. Los indicadores seleccionados se estandarizaron y ponderaron de acuerdo a su importancia relativa con respecto a la sustentabilidad. Los resultados indican que ninguno de los AE alcanza la sustentabilidad en sus tres dimensiones simultáneamente. El análisis sistémico a través de indicadores mostró que las variables críticas son la autosuficiencia alimentaria, el acceso al recurso hídrico, la supervivencia del ganado y la asistencia técnica, entre otras. La metodología utilizada fue fácil de usar, permitió detectar el estado de la sustentabilidad de los tres AE e identificar las variables críticas que ponen en peligro la permanencia de los AE en el tiempo.

Palabras clave
analisis multidimensional • agroecología • tierras secas • tierra no irrigada

Introduction

One of the subjects addressed by agroecology is the evaluation of agroecosystems (AE) in terms of their state of sustainability. This responds to certain concerns expressed by scientists and technicians, given the environmental and social crisis in rural production systems, as a consequence of the "modern" agriculture imposed by the Green Revolution (10, 42, 47). The "sustainable development" concept was officialized in 1987 by the World Commission on Environment and Development, and it was defined as that which "meets the needs of the present without compromising the ability of future generation to fulfill theirs". In that context, the term "sustainable agriculture" was raised in response to the decreasing quality of natural resources, or of the productive base of modern agriculture (7). The concept is linked to the objectives of sustainable development launched by the UN in 2015 aiming to eradicate poverty, ensure food security, stop land degradation, and biodiversity loss, among other aspects (54). Although the sustainability of a production system is a controversial concept under permanent construction, there is broad consensus on the fact that agriculture production has gone from purely technical to a much complex issue, requiring consideration of ecological, economic, and sociocultural aspects for its assessment (19, 42, 58).

Agroecology postulates that, in order to understand the reality of AE, an integrative, holistic and systemic perspective is required, leaving aside the reductionist vision usually held by agrarian sciences when analyzing production systems. The agroecosystem approach has commonly been simplified to one or few of its components, without addressing their interactions or including the man as one of its main components (44, 49). In this sense, the agroecology approach must be put into practice through research that allows understanding the socio-ecological relationships within agroecosystems; accounting for the complex phenomena that result from these interactions; and making a diagnosis of such realities from a systemic approach (40, 52, 58). Also, agroecology acknowledges and values traditional indigenous and rural agriculture knowledge wisdoms (9, 13, 35, 48). This way of understanding nature has reached its objectives of productivity accounting for biodiversity and using the natural resources in a sustainable fashion (1, 22, 56), where techniques are adapted to the local socioeconomic and ecological conditions. Although some of these systems, mainly developed by farmers for food self-sufficiency are considered as sustainable a priori, publications operationalizing the concept by using tools to effectively evaluate the condition of these systems are scarce (18), or only consider one dimension (21, 25). Since the evaluation of AE sustainability involves the analysis of multiple dimensions, the use of indicators as tools for such end is proposed. This methodology has been tested by several authors that evaluated the sustainability of traditional production systems (1, 11, 19, 35, 39, 57). For example, commonly used indicators are food self-sufficiency (46), soil cover (10), biodiversity (45), water quality (5, 43), and basic needs satisfaction (24). An indicator is defined as a variable selected according to an adopted criterion, which responds to social, ecological and economic local characteristics, and is in agreement with the selected scale of analysis. In consequence, a group of indicators measured in a given AE inform about its state of sustainability in a given moment, and allow detecting the critical variables that jeopardize the permanence of AE in time.

On the other hand, experiences evaluating sustainability of AE in drylands, more specifically in non-irrigated lands, are scarce compared to those in AE of oases or irrigated lands (3, 39, 58). In general, non-irrigated AE are characterized by being of difficult access and isolated from the main urban cores, not presenting well-defined property boundaries. Also, their access to water comes from different sources (20). San Juan province is located in the arid strip of center-west Argentina. Here, territory is sectorized in oases, occupying 3% of the area, and where most urban and rural-urban centers merge with majorly intensive production systems. The rest of the territory is comprised by mountain chains and drylands. In these areas, the rural communities and indigenous descendants are strategically and sparsely distributed within extensive production systems based on a subsistence economy. Their main economic activity is goat farming, and to a lesser extent, orchard cultivation for self-sufficiency. In this context, we ask: 1- which is the state of these AE in the economic, ecological and sociocultural fields? 2- Are these systems sustainable?

To answer these questions, sustainability was considered as an historical, dynamic and situated concept, subjected to constant reconceptualization and change (18). An AE is considered sustainable if it meets the following sustainability criteria (46): economically viability, sociocultural acceptance, and productive and ecological suitability. Each of these objectives are measured by a group of indicators.

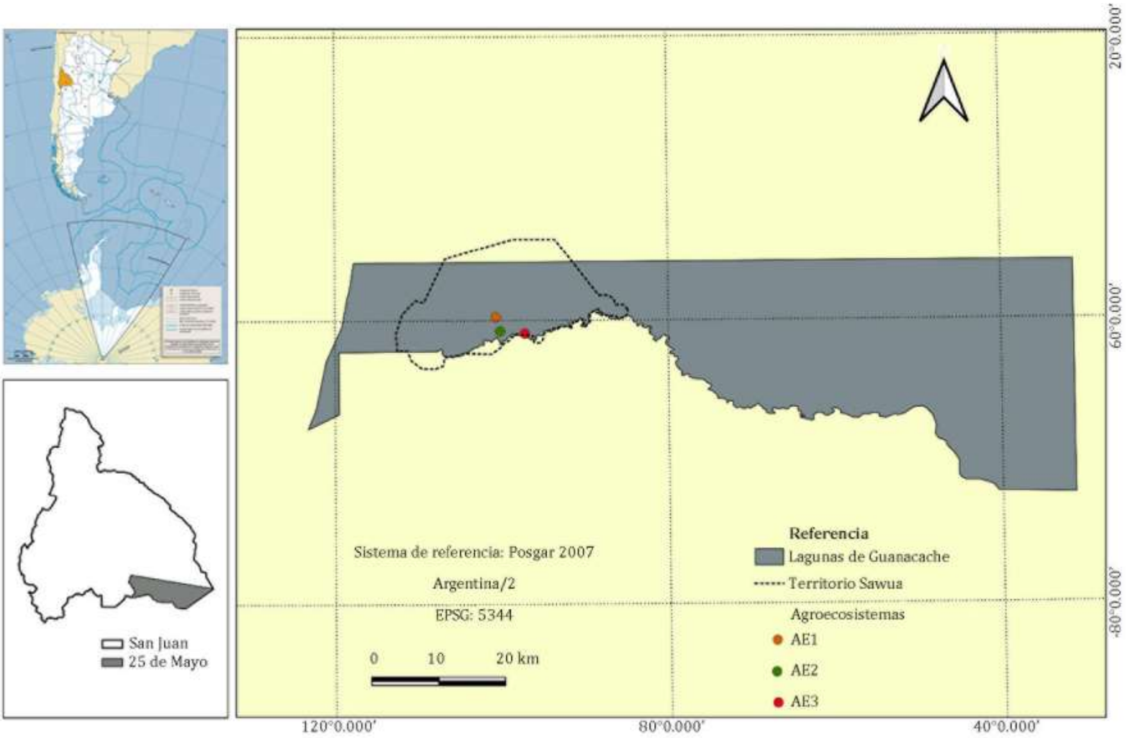
Our hypothesis was that local economic, ecological and sociocultural indicators determine critical points of sustainability in the production systems of the arid communities established in the

Central Mountain. The objective of this study was to determine the state of sustainability of three agroecosystems of arid lands, and to identify the critical aspects that limit this sustainability, using economic, ecological and sociocultural indicators.

Materials and methods

Study area

The study area is located towards the South of San Juan province, in 25 de Mayo department, which corresponds to the former wetland Lagunas de Guanacache, declared as a Ramsar Site in 1999 (figure 1).



The polygon towards the the right corresponds to the Wuarpe Sawa Community area, within the Ramsar Lagunas de Guanacache Site (gray polygon). The color points correspond to the studied agroecosystems.

El polígono de puntos ubicado a la derecha corresponde con el área de la Comunidad Wuarpe Sawa, dentro del Sitio Ramsar Lagunas de Guanacache (polígono gris). Los puntos de color corresponden con los agroecosistemas en estudio.

Figure 1. Study area, in 25 de Mayo, San Juan, Argentina.

Figura 1. Área de estudio, departamento de 25 de Mayo, San Juan, Argentina.

It is the seventh wetland of Argentina and the first in extension (980000 hectares including the Guanacache, Desaguadero and Bebedero lacoons (www.ramsar.org.ar) (33, 50, 51). The Ramsar Site is located in the center-west of Argentina, in the Cuyo Region, and it belongs to the Desaguadero - Salado basin. These wetlands form a system of lagoons and chained marshes, fed by the Mendoza and San Juan rivers, which discharge into the Desaguadero river. At the present, this system behaves like an endorheic basin, due to the great use of its waters from the main tributaries in the upper and middle parts of its routes. It is a continental wetland system of natural origin composed of rivers, lagoons and marshes, and of historical, archaeological and cultural value.

Regarding relief, Guanacache is a plain called "Gran llanura de la travesía". It is a deep sedimentary basin formed by a great depression slightly tilted towards the east, filled by sandy, silty and clayey sediments of lacustrine, fluvial, and wind origin (2, 4, 26).

From a biogeographic point of view, the area corresponds to the Monte province, which is characterized by a dry, warm climate, with summer, torrential rainfall that varies between 80 and 200 annual mm, and wide thermal amplitude (17). Physiognomically, the Monte is a mosaic of three types of vegetation: a) shrub steppes, dominated by species of the family Zigophyllaceae; b) edaphic steppes of halophyte shrubs, such as *Suaeda divaricata*, Atriplex spp., *Allenrolfea vaginata*; and c) forests, mostly dominated by species of the genus Prosopis.

Description of the production systems

Three AE located on the south section of the San Juan river were selected. The 3 AE belong to the Wuarpe Sawa community, acknowledged as descendants of indigenous peoples by the Instituto Nacional de Asuntos Indígenas (INAI) in the year 2000 (figure 1, page 193). The AE were selected using the snowball method (15). The average extension of these production systems is 560 hectares, but they do not present defined boundaries, and the parcels are delimited by the foraging resources of the herds of each AE (20). The most common productive activities are cattle breeding (with an average of 80 heads per AE), and, to a lesser extent, orchard production (vegetables and seasonal fruits) for self-consumption. Goats are one of the few resources that provide income to the families, and their breeding takes place traditionally, with animals foraging extensively in the open field. In the recent past, the productive landscape was much different, and the farmers called themselves "dwellers", due to their tight relationship with activities related to the presence of lagoons (29). Other activities that provide income to the AE families are temporary pruning and harvest in the nearby vine and pistachio settlements, as well as craft making with wool, leather and ceramic.

Water access is a key point for the development of productive activities, and the quantity and quality of water varies according to the four available sources: the San Juan river, water wells, perforations, or water transported through a municipal tank truck (the only way of access to drinking water). It should be highlighted that the water well and perforation technologies (present in AE1 and AE2, and absent in AE3) do not provide quality water for animal or people consumption, or to be used in the orchard (55); thus, not used by the farmers. For that reason, such sources were not considered when performing the sustainability analyses, and only sources with permanent use were addressed.

The marketing routes of young and adult goats take place through a "cabritera", which buys the animals from the community to resell them in the nearby urban center. Other marketing routes include the landowners of the region and particular customers who go to the farms. Certain farm products are directly sold to the individual customers by personal delivery, and crafts are made on request and sold to individual customers or craft centers in Mendoza.

Data collection

To collect agroecological information regarding ecological, economic and sociocultural aspects of the production systems, as well as information about the perception and knowledge of the AE, the following methods were used: participant observation (14), semi-structured interviews to each familiar unit (AE1: a couple, a son, a daughter and a granddaughter, AE2: a couple and two sons, AE3: two people), and visits around the AEs with a family member.

To address water quality (whether it is suitable for different uses; *e. g.* human and animal consumption, orchard irrigation, other uses) water samples were taken from each surveyed source of each AE according to the protocol proposed by INTA (27). Samples were placed in 1-liter aseptic plastic containers for physical-chemical determinations, and 250 ml were used for bacteriological measures. The samples were analyzed by the INA-CRAS (National Institute of Water-Regional Center for Groundwater), and by the Institute of Technological Research.

To address the offer of forage resources, 3 physio-structural transects within the farm area of each AE were established during the forage supply period, using the Point Quadrat method adapted for the Monte area. At each transect, the frequency of forage species, their specific contribution, and specific contribution by contact (considered as a relative expression of biomass) were determined (23, 34, 36).

Sustainability indicators

Sustainability indicators were built, standardized and weighted according to the methodology proposed by Sarandón (44). The threshold value of sustainability (TVS), defined as the mean value of the adopted scale of values (0 to 4, in this case) corresponded to 2. Weighting was performed by multiplying the value from the scale by a coefficient according to the relative importance of each of the sustainability variables. The economic, ecological and sociocultural indicators were calculated as the algebraic sum of their components, multiplied by their weight, to finally estimate the General Sustainability Index (GSI).

Description and weight of the indicators

The methodology applied to build the indicators allowed obtaining a series of standardized and weighted indicators and sub indicators for each analyzed sustainability dimension (economic, ecological and sociocultural, table 1, page 196). Below, the way in which each indicator and sub indicator were measured, the categories adopted by each indicator within the standardized scale, and their subsequent weighting are shown.

Table 1. Summary of the indicators, sub-indicators, their estimation methodology, and weighing adopted in the estimated indexes (IE, IEC, ISC).

Tabla 1. Resumen de los indicadores, subindicadores, metodología utilizada para calcular cada uno y la ponderación que adoptó cada uno en el cálculo de los indices (IE, IEC, ISC).

Economic Indicators	**Economic subindicators**	**Method**	**Weighing**
Food Self-sufficiency (FS)	Number of products for self-consumption (NPSC)	Interview, participant observation	2
	Area destined to Self-Consumption (ASC)	*In situ* measurement	2
Economic Risk (ER)	Number of Marketing Channels (NMC)	Interview	
	Diversification of Sale Products (DSP)	Interview, participant observation	
	Dependence on external inputs (DI)	Interview	
	Productive Activities (PA)	Interview, participant observation	2
Extraproperty Work (EPW)		Interview	2
Ecological Indicators	**Ecological Subindicators**		
Access to Water (AW)	Water - Quantity (QUAN)	Interview	2
	Water - Quality (QUAL)	Water analysis	4
Livestock Survival (LS)		Interview	2
Foraging Resources (FR)	Quality of the Foraging Resources (QUALFR)	Transects	2
	Quantity of the Foraging Resource (QUANFR)	Interview, Transects	
Social Indicators	**Social Subindicators**		
Basic Needs Met (BNM)	Household (H)	Interview, participant observation	
	Health (HE)	Interview	
	Education (ED)	Interview	
Technical Assistance (TA)		Interview	2
Satisfaction of the Production System (SPS)		Interview	2

The economic dimension was measured through three indicators:

1-Food Self-sufficiency (FS). A self-sufficient AE, in terms of the food produced within its limits, is considered sustainable. This indicator is composed by two sub indicators:

-Number of products for self-consumption (NPSC). Measured as the N° of products aimed at family consumption, including animal and plant products.

0: 1 product is produced.
1: 2-3 products are produced.
2: 4-5 products are produced.
3: 5-6 products are produced.
4: More than 7 products are produced.

-Area destined to Self-Consumption (ASC). Measured as the area destined for the cultivation of vegetables and fruits for family consumption.

0: less than 15 m^2.

1: between 15-30 m^2.

2: between 30-40 m^2.

3: between 40 - 50 m^2.

4: Higher than 50 m^2.

2-Economic Risk (ER). An AE that reduces the economic risk and insures the productive-economic capital for the future generations is considered sustainable. This indicator is composed by four sub indicators:

-Number of Marketing Channels (NMC). Measured as the N° of channels through which each AE commercializes goats and/or other products.

0: no marketing channel.

1: 1-2 marketing channels.

2: 3 marketing channels.

3: 4 marketing channels.

4: more than 5 marketing channels.

- Diversification of Sale Products (DSP).

Measured as the N° of products destined to market, either from the orchard, livestock, craft, fabric, and/or processed products.

0: 1 product for sale.

1: 2 products for sale.

2: 3 products for sale.

3: 4 products for sale.

4: More than 5 products for sale.

-Dependence on external inputs (DI).

Measured as the % of inputs that an AE must destine in order to guarantee livestock and/or orchard production.

0: 80 - 100% of dependence.

1: 60 -80% of dependence.

2: 40 -60% of dependence.

3: 20 al 40% of dependence.

4: 0 al 20% of dependence.

-Productive Activities (PA). Measured as the N° of productive activities carried out within the limits of the AE.

0: No productive activities.

1: 1-2 productive activities are carried out.

2: 3 productive activities are carried out.

3: 4 productive activities are carried out.

4: More than 5 productive activities are carried out.

3-Extraproperty Work (EPW). An AE in which its members use labor to enhance or maintain the intra property production is considered sustainable. This indicator was measured as the participation of the members of the family within each AE in extraproperty work throughout the year.

0: extraproperty work during the whole year.

1: extraproperty work during three seasons.

2: extraproperty work during half of the year.

3: occasional extraproperty work.

4: no need of extraproperty work.

Since AEs respond to a self-sufficiency economic model, the indicators of food self-sufficiency and extraproperty work; along with the sub indicator of number of productive activities were assigned twice the weight of the rest of the indicators for the economic dimension. For the calculation of the economic dimension index, the following formula was used:

$$IE = \frac{(2*NPSC + 2*ASC)/4 + (NMC + DSP + DI + 2*AP)/5 + (2*EPW)/2}{3}$$

The ecological dimension was measured through 3 indicators:

1-Access to Water (AW). An AE with free access to quality water quality resources, for all the activities that depend on this resource, is considered sustainable. This indicator is composed by two sub indicators:

-Water - Quantity (QUAN). Measured as the % of activities (that depend on water resources) covered within the AE, as a function of the quantity of water within that AE.

0: Covers 0-20% of its needs.

1: Covers 20-40%.

2: Covers 40-60%.

3: Covers 60-80%.

4: Covers 80-100%.

-Water - Quality (QUAL). Measured through the analysis of water samples from the sources, as the quality of the resource for different uses (water for human and animal consumption, for irrigation, and other uses).

0: Unsuitable.

1: Suitable for two uses.

2: Suitable for three uses, including human consumption.

3: Suitable for four uses.

4: Suitable for multiple uses.

2-Livestock Survival (LS). An AE that minimizes livestock losses through time is considered sustainable. This indicator is measured as goat losses in % of mortality/ year, and acknowledges the multiple causes that can derive in livestock death.

0: More than 20% of mortality.

1: Between 15-20% of mortality.

2: Between 10-15% of mortality.

3: Between 5-10% of mortality.

4: Between 0-5% of mortality.

3-Foraging Resources (FR). An AE with enough foraging resources to meet livestock demands, is considered sustainable. This indicator is composed by two sub indicators:

-Quality of the Foraging Resources (QUALFR). Measured as a function of the specific quality of the species with higher contribution of forage biomass within the AE.

0: Bad.

1: Regular.

2: Good.

3: Very Good.

4: Excellent.

- Quantity of the Foraging Resource (QUANFR). Measured as the percentage of food needs that the foraging resource of the AE is able to fulfill.

0: Covers up to 20% of the diet.

1: Covers between 20-40% of the diet.

2: Covers between 40- 60% of the diet.

3: Covers between 60 - 80% of the diet.

4: Covers between 80 - 100% of the diet.

The calculation of the ecological dimension index assigned double weight to the water access indicators, especially water quality, as well as to livestock survival, since it is the main productive activity within the AE. Also, double weight was assigned to forage quality, given its importance in the nutritional intake of the herd. The following formula was used:

$$IEC = \frac{2*((W-QUAN+2*W-QUAL)/3)+2*LS+(2*QUALFR+QUANRF)/3}{5}$$

The sociocultural dimension was measured through 3 indicators:

1-Basic Needs Met (BNM): An AE in which the farmers have insured housing with services, permanent access to health service and to the different educational levels is considered sustainable. This indicator is composed by three sub indicators:

-Household (H). Measured as the state of the household, including water and electricity services.

0: Without household and services.

1: Incomplete household/ no service.

2: Incomplete household/one service.

3: Complete household/two services.

4: Complete household/all the services.

-Health (HE). Measured as the access to a health center (the possibility of arriving or having access to the health center without inconveniences) with medical staff and adequate equipment/ infrastructure for medical assistance.

0: Without access to health center.

1: Access to health center/ no equipment/ temporary medical staff.

2: Access to health center/ scarce equipment/ temporary medical staff.

3: Access to health center/ moderately equipped/ temporary medical staff.

4: Access to health center/ adequate equipment/ permanent medical staff.

-Education (ED). Measured as the access to the different education levels (the possibility of arriving or having access to the educational center).

0: No access to education.

1: Access to primary level.

2: Access to primary and secondary levels/ with restrictions.

3: Access to primary and secondary levels/ without restrictions.

4: Access to primary, secondary and higher level.

2-Technical Assistance (TA). A system is sustainable if it is reachable to technicians that can improve the production status. It is measured as the presence of technical assistances in the field, and the feasibility of carrying out technical measures that improve the production status.

0: No technical assistance.

1: Eventual technical assistance/ no proposals.

2: Eventual technical assistance/ undone proposals.

3: Frequent technical assistance/ developing proposals.

4: Frequent technical asssistance/ completed proposals.

3-Satisfaction of the Production System (SPS). An AE is considered sustainable and able to support itself in time if its farmers are satisfied with the productive activities, measured as the degree of satisfaction in relation to the productive activities carried out.

0: Discontent, would not do it anymore.

1: Not at all satisfied, would live somewhere else.

2: Moderately satisfied, would keep producing.

3: Satisfied, did better before.

4: Satisfied, would not do anything else.

The sociocultural dimension index was calculated giving double weight to the satisfaction of the production system, since a discontent farmer has higher probabilities of abandoning the activity. Also, the technical assistance was weighted with the following formula, since AE have few resources and are isolated from urban centers and roads:

$$ISC = \frac{(H + HE + ED)/3 + 2*SPS + 2*TA}{5}$$

Finally, the General Sustainability Index was calculated as the algebraic sum of all the indexes that compose the sustainability dimensions:

$$ISG = (IE + IEC + ISC)/3$$

Results

Sustainability analysis

The use of indicators allowed detecting the state of sustainability of the three analyzed AE. Broadly, the only AE with a general sustainability index (GSI) higher than the threshold value was AE 1 (GSI= 2.3), while AE2 and AE3 were below that threshold (table 2 and figure 2, page 200). Figure 3 (page 201) and table 1 (page 196) show the individual analysis of the economic (EI), ecological (ECI) and sociocultural indexes (SCI) for each AE. The results indicate that the three studied AE varied according to the addressed dimensions, and that neither of them reached the TSV in the three dimensions.

Table 2. Values of the set of indicators with their respective economic (IE), ecological (IEC), and sociocultural (ISC) indexes; and the general sustainability index (ISG) for the three agroecosystems in drylands of San Juan, Argentina.

Tabla 2. Valores del conjunto de los indicadores con sus respectivos índices económico (IE), ecológico (IEC), sociocultural (ISC) y el índice de sustentabilidad general (ISG) para los tres agroecosistemas de zonas áridas, San Juan, Argentina.

	FS	ER	EPW	IE	AW	FR	LS	IEC	BNM	SPS	TA	ISC	ISG
AE1	4	3.2	3	3.44	1.33	2.00	1	1.07	1. 67	3	2	2.33	2.3
AE2	0.5	1.4	1	0.88	1.33	2.00	1	1.07	1. 67	3	2	2.33	1.4
AE3	0.5	1.2	4	2.04	1	2.00	1	1.00	1.33	3	0	1.47	1.5

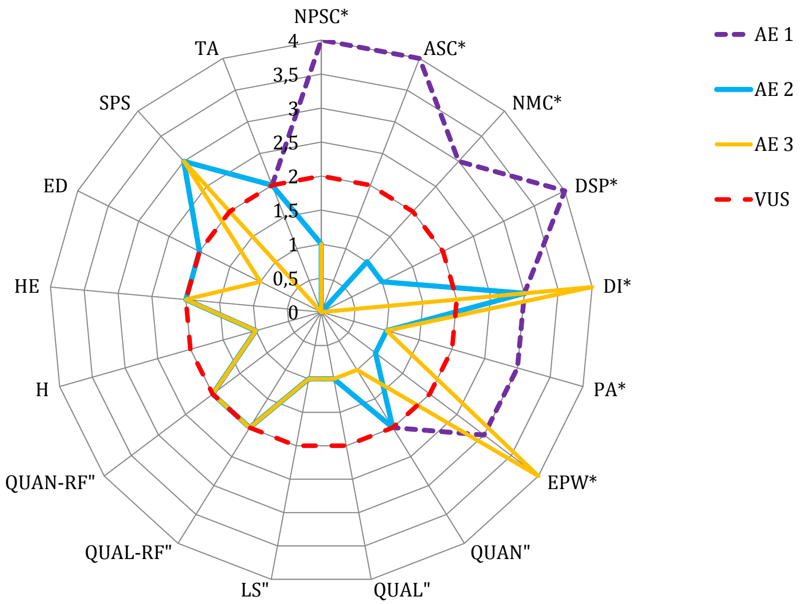


Figure 2. Spider web chart of the sustainability indicators in the three agroecosystems in drylands of San Juan, Argentina. The outer limits represent the ideal value of sustainability (4), and the intermediate limit the threshold value (2). In asterisk: economic (E), ecological (EC) and Sociocultural (SC) indicators are indicated with asterisks, quotes, and normal text, respectively.

Figura 2. Representación gráfica en un diseño en tela de araña, de los indicadores de sustentabilidad en tres agroecosistemas de tierras áridas, San Juan, Argentina. Los límites exteriores representan el valor ideal de sustentabilidad (4) y el intermedio el valor umbral (2). Con asterisco: económicos (E); con comillas: ecológicos (EC) y los que no tienen marca socioculturales (SC).

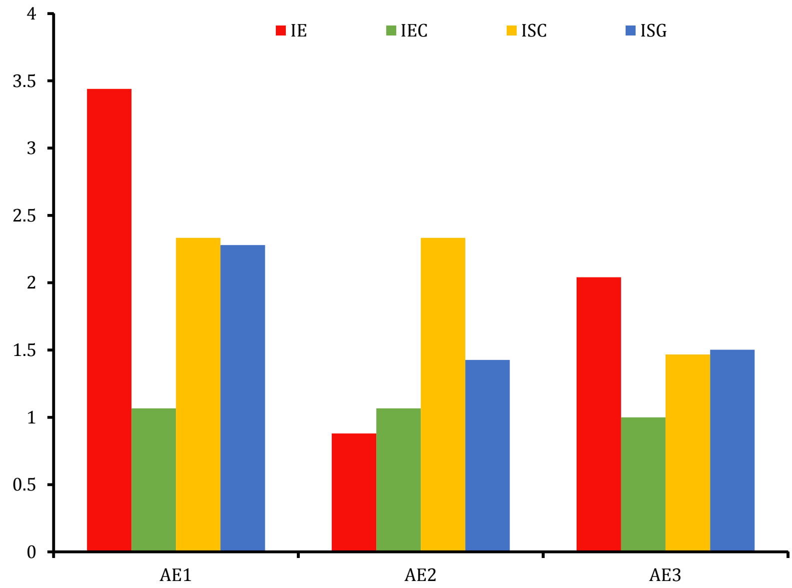


Figure 3. Values of the three dimensions of sustainability indexes: economic (EI), ecological (ECI) and sociocultural (SCI) dimensions. The bar on the far right indicates the value of the general sustainability index (GSI).

Figura 3. Valores de los índices de las tres dimensiones de la sustentabilidad: índice de la dimensión económica (IE), de la dimensión ecológica (IEC) y de la dimensión sociocultural (ISC). La última barra a la derecha indica el valor del índice de sustentabilidad general (ISG).

In the case of EI, AE1 widely exceeded the other AE within the established scale (EI= 3.44), while AE2 did not reach sustainability in this dimension (EI= 0.88) and AE3 reached the TSV.

However, for the ECI the situation was similar in the three agroecosystems, with none reaching the TSV; while for SCI, AE1 and AE2 obtained the same value (SCI= 2.33), reaching the threshold sustainability value. On the other hand, AE3 reached a lower to the TSV value (SCI= 1.47).

When analyzing the indicators for each dimension, it was observed that AE3 reached food self-sufficiency with a high number of foods produced in the area, enough to cover the whole family needs (figure 2, page 200). Also, AE3 reduced the economic risk by diversifying production and increasing the number of products for sale and the marketing channels. In the other AE, food self-sufficiency was not reached (both with FS= 0.5, table 2, page 200), nor did they reduce the economic risk (ER= 1.4 and 1.2 in AE2 and AE3, respectively). These two AE did not exhibit diversification of productive activities and had none to 1 or 2 marketing channels. The only indicator within economic risk that was similar among the three AE was the low dependence of external inputs.

On the other hand, the indicator extra-property work exceeded the TSV in AE1 and AE3 (figure 2, page 200), but not in AE2, since this system is mainly sustained by such incomes (EPW= 1).

For the ecological dimension, a critical situation was found in the three analyzed systems. For the indicator access to quality water, the resource was partially available in AE1 and AE2 (for example, human consumption was only partially satisfied), while AE3 showed an even worse situation (AW= 1), since it only counts with one water source. The San Juan river is shared by the three AE, but its flow varies throughout the year, and its quality is only suitable for animal consumption and orchard. Regarding goat survival, the three AE showed 15-20% losses due to mortality (LS= 1 in the three AE, table 2; figure 2, page 200), with the following causes mentioned in the interviews: illness, attacks by wild dogs and malnutrition. The latter was related with foraging resource availability in the area, which did not cover 60% of the diet, although its quality (*i.e.*, the nutritional status of the available foraging species) was good in the three systems. The species that provide the higher foraging biomass for livestock are *Prosopis alpataco* and Lycium spp. (figure 4).

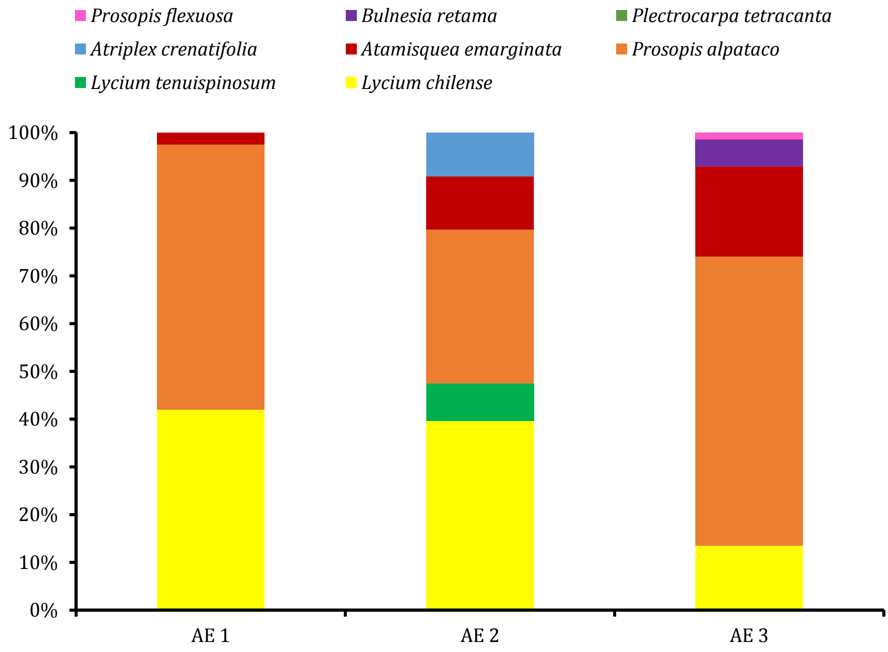


Figure 4. Specific contribution by contact (CSC), expressed as percentage of the foraging species to the forage supply season.

Figura 4. Contribución específica por contacto (CSC) expresada en porcentaje de las especies forrajeras para la época de oferta forrajera.

Regarding the social dimension indicators, they showed that the basic needs were not satisfied in neither of the AE, with AE3 showing the lowest values (BNS= 1.33, table 2, page 200).

Despite this, farmers were satisfied with the productive activities, and did not express they would rather perform other activities. Regarding the technical assistance indicator, AE3 did not present any type of assistance, while in the other two systems technicians reached the area and made suggestions, yet, not carried out.

Discussion

Our results confirm the former hypothesis of this research: the indicators for the three dimensions (economic, ecological and sociocultural) used at the local scale, determine the critical points that jeopardizes the sustainability of the AEs over time. Sustainability is a multidimensional concept, that should thus be understood and evaluated in a systemic and holistic fashion. The obtained results suggest that the analyzed AE do not reach sustainability, since it is not achieved in its three dimensions. Furthermore, considering the objectives that sustainable agriculture should meet (46), and that were proposed as a framework for this study, none of the analyzed systems meets them all simultaneously. Instead, differences in the values of the economic, ecological and sociocultural dimension indices were found. This situation agrees with that found in other studies that have evaluated sustainability at the farm scale, although in those studies the systems did reach sustainability (3, 46). The dimensions were composed by indicators that reflected their state. The economic dimension was found to be the most variable for the three systems, while the ecological dimension was very similar, and the sociocultural dimension resulted similar between AE1 and AE3, but differed for AE3.

The variability in the economic dimension indicators of the three AE might be due to the fact that each AE adopts diverse strategies that define the economy of each system. Although AE1 and AE3 are economically sustainable, substantial differences were found between these systems. AE1 is held due to the diversification of its productive activities, products for self-consumption and sale, and the relation established with the diverse consumers. Also, this AE is sustained through family labor, which occurs exclusively within its limits, and which might explain why the members of this AE do not participate in extraproperty jobs. On the other hand, AE3 is not sustained by a diversification of productive activities, therefore it does not present a diversity of products for consumption, while livestock production is lower and occurs only to sustain domestic demand. This might be due to the fact that this AE is composed by elderly members that have retired from market-oriented production and that cannot carry out extraproperty work as another possible strategy for the economic growth of the system. Their main objective is to keep the productive capital needed to subsist. On the other hand, AE2 did not reach sustainability in the economic dimension, and a different life strategy than that of AE1 and AE3 was detected. In this system, there is no diversity of productive activities, since it dedicates exclusively to goat breeding, such as AE3. However, AE2 commercializes livestock, ensuring an influx of money for family needs or for buying livestock forage. In contrast to the other AE, this system is largely sustained by extraproperty work, for which the family invests time, leaving aside diversification within the farm.

As in this study, a study performed in Salinas Grandes, Catamarca addressing economic diversification in multiple-use systems (28) found a diversity of life strategies within the community members, with some people carrying out diversified productive activities while others dedicated almost exclusively to a certain activity (cattle ranching in that case) and others had extraproperty jobs. Several agroecology publications promote the diversification strategy, and it is usually referred to as that one of the bases to reach sustainable agroecosystems. Furthermore, it is recognized that polycultures, agroforestry and other diversification methods imitate natural ecological processes (8, 12, 16, 30, 35, 45, 53). On the other hand, extraproperty jobs reduce the use of natural resources within the farm, and gives independence of the climatic and economic uncertainties to the people who adopt this strategy (28).

The analysis of the ecological dimension was similar for the three AE, but certain differences regarding the components of this dimension were detected in AE3, mainly due to the lack of water resources in this system, compared to the other two. This could be explained by the fact that this AE is more isolated, (figure 1, page 193) impeding the municipal tanker truck to and provide drinking water. "Not even the technicians with technical proposals can reach the farm" (information from one of the interviews). In AE1 and AE2, the water extracted through the wells and perforations has very high salt content, and thus cannot be used for productive activities. These sources are virtually voided, despite being functional (55). The San Juan river is the common water source for all the AE, through which the herd water needs of the three AE, the orchard water needs of AE1, and the consumption needs through filtration in AE3 are provided. To this respect, a study by Tapia *et al.* (2017) about water source quality in the study area, found that the water provided by the San Juan river is suitable for the different activities. It is worth mentioning that this source does not have a permanent water course, and water is scarce in the seasons where it is most needed, due to its utilization in the upstream oasis.

In response to this situation, the members of the AE develop different strategies to provide water to the herds, such as the fabrication of pastures inside the river, where they extract water through excavation. These kind of subsistence strategies, together with solid the strong roots, might explain that the AE still remain in the area, even under critical conditions.

In the Lavalle desert, towards the south of the study area, the situation regarding water resources is similar to that from our study area, where the surface flows that supply water to the area are scarce and discontinuous, and subterranean waters have high salinity levels, even exhibiting high levels of hydroarsenism (59).

Regarding foraging resources, the indicators reflect that its quality is good for the three AE, while its quantity is not enough to meet goat demands. The latter explains why these systems have to buy extra foraging inputs for the herds, although they are still insufficient and usually destined to cover feeding needs of horses (mean of transport). On the other hand, goat survival is critical in the three systems. The most common identified causes of goat death are starvation, attacks by dogs, and diseases due to lack of sanitary control, among others. Although this is the main productive activity and farmers would not dedicate to anything else, it is deteriorating. To this respect, a study case with goat farmers in the Lavalle desert (59), next to the study area, showed that goat production is strongly compromised because ranching is held in degraded areas with low foraging availability, due to a lack of calving planification, a high percentage of animals with brucellosis (chronic infectious contagious disease produced by *Brucella melitensis*), and a lack of adequate management practices, among other factors. The analysis of the ecological dimension could deepen, through the consideration of variables related to orchard development, such as soil management or crop rotation, among others. They were left aside in this study due to orchard being a secondary activity.

Sustainability of the socioeconomic dimension was reached in AE1 and AE2, while AE3 did not exceed the STV. Although livelihood and health access conditions are similar among the three systems, access to education and technical assistance is not. The members of AE3 claimed not having the opportunity to go to school, thus they dedicated to farming activities since childhood. On the other hand, technical assistance for this AE does not have continuity in time, and technical proposals are only partially achieved, for which resources (such as vaccines) are only guaranteed at certain seasons or times of the year. The situation is more critical in AE3, where technical assistance is null. Once more, this could be due to this farm being isolated. Finally, farmers are satisfied with the activity, to the point that they do not do anything else. Such satisfaction, their roots, and the "hope that the lagoons will appear again", justify why these systems remain in the site despite the critical sustainability conditions identified in this study.

The results of this study confirm the utility of indicators for multidimensional evaluations, through which an analysis of the state of the productive systems can be held at different levels. Addressing both general issues, such as the GSI, to more specific situations, allow detecting economic, ecological and sociocultural-factors under critical states, that might jeopardize sustainability at the farm level. In turn, this methodology allows detecting how the different values interrelate among each other, and explains the functioning of each system. Also, the weight of the indicators came out through consensus within the working group, and has been used in other studies (46). Undoubtedly, results can vary according to the criteria used to weight the indicators, including the participation of the farmers in this decision, as suggested by Roming *et al.* (1996), and Lefroy *et al.* (2000). Since this study is a synchronic evaluation, it has certain limitations that can be overcome through an evaluation in time (diachronic), addressing the trends of the system, analyzing whether the compromising reality of these AE might change if, for example, all the AE had unrestricted access to quality water.

Although sustainability is a situated concept, the local indicators used in this study can be applied in other rainfed agroecosystems with similar characteristics. The analysis performed can be deepened by measuring other variables. For example, in the ecological dimension, goat production can be evaluated through technical-productive parameters, such as flock structure, or pregnancy and birth rates (37, 38). In the social dimension, community interaction and land tenure could be measured (32); while in the economic sphere, other indicators can be addressed, such as family income composition (6).

Ultimately, the decision about the variables chosen to assess sustainability is determined by the objective of the study, the scale of analysis (farm, land, region), the type of establishment, productive activity, farmer characteristics, and the possibilities of carrying out the effective measurement of the mentioned variables.

Conclusions

The indicators used to measure sustainability in dryland agroecosystems allow identifying critical points that might jeopardize the sustainability of the different dimensions analyzed. In our study, no agroecosystem exceeds the threshold sustainability value simultaneously in all three dimensions, even when the general sustainability index does reach this value. In general, the indicators with a critical state were food self-sufficiency in the economic dimension; access to water resources and livestock survival in the ecological dimension; and technical assistance in the field, and satisfaction of basic needs in the socio-cultural dimension. In this sense, the multidimensional analysis of sustainability showed that these indicators should be taken into account when seeking intervention alternatives in the agroecosystems, and when making decisions regarding their sustainable development.

The development and use of indicators, even with their limitations, is an adequate and flexible tool to evaluate trends, establish differences among productive systems, and detect critical variables that hinder the achievement of sustainable agriculture. In turn, the detection of these variables can derive in the implementation of management and self-governance measures by the technicians and local actors, aiming at improving sustainability in the AE. The indicators used are easy to measure, inexpensive, and reflect the reality in the different dimensions addressed.

References

1. Abbona, E. A.; Sarandón, S. J.; Marasas, M. E.; Astier, M. 2007. Ecological sustainability evaluation of traditional management in different vineyard systems in Berisso, Argentina. Agriculture, Ecosystems & Environment. 119(3-4): 335-345.
2. Abraham, E.; Prieto, R. 1999. Guanacache, la travesía de los profundos cambios. Guanacache, Fidel Roig Matons, pintor del desierto. 107-125.
3. Abraham, L.; Alturria, L.; Fonzar, A.; Ceresa, A.; Arnés, E. 2014a. Propuesta de indicadores de sustentabilidad para la producción de vid en Mendoza, Argentina. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 46(1): 11-180.
4. Abraham, R.; Vitarelli, M. 2014b. Las lagunas de Guanacache como espacio de recuperación. Un caso de estudio para la enseñanza del manejo integral de cuencas.
5. Achkar, M.; del Territorio, G. A. 2005. Indicadores de sustentabilidad. Ordenamiento ambiental del territorio. Montevideo: DIRAC Facultad deficiencias.
6. Acuña, N. R. F.; Marchant, C. 2016. ¿Contribuyen las prácticas agroecológicas a la sustentabilidad de la agricultura familiar de montaña? El caso de Curarrehue, región de la Araucanía, Chile. Cuadernos de Desarrollo Rural. 13(78): 35-66.
7. Altieri, M. A. 1995. Agroecology: the science of sustainable agriculture (No. Ed. 2). Intermediate Technology Publications Ltd (ITP).

8. Altieri, M. A. 2002. Agroecología: principios y estrategias para diseñar sistemas agrarios sustentables. Sarandón, S. J. Agroecología: el camino hacia una agricultura sustentable. Buenos Aires-La Plata. 49-56.
9. Altieri, M. A. 2016. Breve reseña sobre los orígenes y evolución de la Agroecología en América Latina. Agroecología. 10(2): 7-8.
10. Altieri, M. A.; Nicholls, C. I. 2000. Teoría y práctica para una agricultura sustentable. Serie Textos Básicos para la Formación Ambiental. 1.
11. Altieri, M. A.; Nicholls, C. I. 2002. Un método agroecológico rápido para la evaluación de la sostenibilidad de cafetales. Manejo integrado de plagas y Agroecología. 64(1): 7-2.
12. Altieri, M. A.; Nicholls, C. I. 2007. Conversión agroecológica de sistemas convencionales de producción: teoría, estrategias y evaluación. Revista Ecosistemas. 16(1).
13. Alzate, C.; Mertens, F.; Fillion, M.; Rozin, A. 2019. The study and use of traditional knowledge in agroecological contexts. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 337-350.
14. Bernard, H. R. 1988. Research methods in cultural anthropology. Newbury Park, CA: Sage Publications.
15. Blanco, M. C. M. C.; Castro, A. B. S. 2007. El muestreo en la investigación cualitativa. 27.
16. Bonaudo, T.; Bendahan, A. B.; Sabatier, R.; Ryschawy, J.; Bellon, S.; Leger, F.; Tichit, M. 2014. Agroecological principles for the redesign of integrated crop–livestock systems. European Journal of Agronomy. 57: 43-51.
17. Cabrera, A. L. 1994. Enciclopedia Argentina de agricultura y jardinería, Tomo II, Fascículo 1: regiones fitogeográficas Argentinas. ACME. Buenos Aires.
18. Cáceres, D. M. 2006. Sustentabilidad como concepto situado. Un marco conceptual para la construcción de indicadores. Des. Rural Coop. Agrario. 8: 165-178.
19. Cáceres, D. M. 2009. La sostenibilidad de explotaciones campesinas situadas en una reserva natural de Argentina central. Agrociencia. 43(5): 539-550.
20. Carmona C. J.; Tapia R.; Martinelli, M. 2018. Definición del área predial de agroecosistemas de secano a través del mapeo participativo. San Juan. Argentina. Periurbano hacia el consenso: ciudad, ambiente y producción de alimentos: propuestas para ordenar el territorio. Córdoba. Available in: https://inta.gob.ar/sites/default/files/inta_periurbanos_hacia_el_consenso_libro_1_resumenes_ampliados.pdf.
21. Chiappe, M. 2002. Dimensiones sociales de la agricultura sustentable. Agroecología: El camino para una agricultura sustentable. 83-98.
22. Convention on biological diversity (CBD). 1996. In: Conference of the Parties 3. Consideration of Agricultural Biological Diversity under the Convention on Biological Diversity. UNEP/CBD/COP/3/14. Buenos Aires.
23. Dalmasso, A. D.; Márquez, J.; Navas, A. L; Herrera Moratta, M.; Gordillo, L.; Salomón, E. 2018. Lasprincipales pasturas del Secano Cuyano. Ed. Universidad Nacional de San Juan.
24. Delgado, P.; Salcedo, T. 2008. Aspectos conceptuales sobre los indicadores de calidad de vida. La sociología en sus escenarios. p. 17.
25. Foladori, G. 2007. Paradojas de la sustentabilidad: ecológica versus social. Trayectorias. 9(24): 20-30.
26. García Llorca, J.; Cahiza, P. A. 2007. Aprovechamiento de recursos faunísticos en las Lagunas de Guanacache (Mendoza, Argentina): Análisis zooarqueológico de La Empozada y Altos de Melién II. Chungará (Arica). 39(1): 117-133.
27. INTA. 2011. Protocolo de Muestreo, Transporte y Conservación de Muestras de Agua con Fines Múltiples (consumo humano, abrevado animal y riego).
28. Karlin, M.; Ruiz Posse, E.; Contreras, A.; Coirini, R. 2014. Diversificación económica y diversidad ecológica en sistemas de uso múltiple de salinas grandes, Catamarca (Argentina). Multequina. 23(1): 5-15.
29. Katzer, L. 2010. Tierras indígenas, demarcaciones territoriales y gubernamentalización: El caso Huarpe. Provincia de Mendoza. Avá. (16): 1-1.
30. Kremen, C.; Iles, A.; Bacon, C. 2012. Diversified farming systems: an agroecological, systems based alternative to modern industrial agriculture. Ecology and Society. 17: (4).

31. Lefroy, R. D.; Bechstedt, H. D.; Rais, M. 2000. Indicators of sustainable land management based on farmer surveys in Vietnam, Indonesia and Thailand. Agriculture, Ecosystems & Environment. 81: 137-146.
32. Loewy, T. 2008. Indicadores sociales de las unidades productivas para el desarrollo rural en Argentina. Revibec: revista iberoamericana de economía ecológica. 9: 75-85.
33. Manual "Lagunas del Desierto: el valor de la naturaleza oculto en la identidad de su gente". 2012. Ramsar. Editorial APN.
34. Martinelli, M.; Martínez Carretero, E. 2014. Matorrales forrajeros en zonas áridas: indicadores de estado. Multequina. 23(1): 29-40.
35. Noguera-Talavera, A.; Salmerón, F.; Reyes-Sánchez, N. 2019. Bases teórico-metodológicas para el diseño de sistemas agroecológicos. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 273-293.
36. Passera, C. B.; Dalmasso, A. D.; Borsetto, O. 1983. Método de point quadrat modificado. Taller de arbustos forrajeros para zonas áridas y semiáridas. 71-79.
37. Paz, R.; Álvarez, R.; Castaño, L. 2000. Parámetros técnico-productivos y tipologías en los sistemas caprinos tradicionales en áreas de secano. Archivos latinoamericanos de producción animal. 8(2): 59-68.
38. Paz, R.; Castaño, L.; Álvarez, R. 2008. Diversidad en los sistemas cabriteros tradicionales y estrategias tecnológico-productivas. Archivos de zootecnia. 57(218): 207-218.
39. Pérez, L. B.; Esquivel, C. G.; Hernández, L. G. 2005. Evaluación de la sustentabilidad de dos agroecosistemas campesinos de producción de maíz y leche, utilizando indicadores. Livestock Res. Rural Dev. 17: (7).
40. Polanco-Echeverry, D. N.; Rios-Ososrio, L. A. 2015. Proposed methodology for research into the socioecological resilience of agroecosystems. Tropical and Subtropical Agroecosystems. 18: (2).
41. Roming, D. E.; Jason Garlynd, M.; Harris, R. F. 1996. Farmer- based assessment of soil quality: a soil health scorecard. In Methods for Assessing Soil Quality (Doran JW, Jones AJ, eds.). SSSA Special Publication. 49: 127-158.
42. Rosset, P. 1998. La crisis de la agricultura convencional, la sustitución de insumos y el enfoque agroecológico. Food First. Institute for Food and Development Policy.
43. Rubio, C.; Rubio, M. C.; Abraham, E. 2018. Poverty Assessment in Degraded Rural Drylands in the Monte Desert, Argentina. An Evaluation Using GIS and Multi-criteria Decision Analysis. Social Indicators Research. 137(2): 579-603.
44. Sarandón, S. J. 2002. El desarrollo y uso de indicadores para evaluar la sustentabilidad de los agroecosistemas. Agroecología: El camino para una agricultura sustentable. 393-414.
45. Sarandón, S. J. 2010. Biodiversidad, agrobiodiversidad y agricultura sustentable. Análisis del Convenio sobre Diversidad Biológica. León Sicard, T. E.; Altieri, M. Vertientes del pensamiento agroecológico: fundamentos y aplicaciones, edit. Instituto de Estudios Ambientales. Universidad Nacional de Colombia Sede Bogotá. Sociedad Científica Latinoamericana de Agroecología (SOCLA), Medellín. Colombia. 105-129.
46. Sarandón, S. J.; Zuluaga, M. S.; Cieza, R.; Janjetic, L.; Negrete, E. 2008. Evaluación de la sustentabilidad de sistemas agrícolas de fincas en Misiones, Argentina, mediante el uso de indicadores. Agroecología. 1: 19-28.
47. Sarandón, S. J.; Marasas, M. E. 2015. Breve historia de la agroecología en la Argentina: orígenes, evolución y perspectivas futuras. Agroecología. 10(2): 93-102.
48. Sarandón S. J.; Bonicatto M. M.; Gargoloff, N. A. 2016. Rol de la agrobiodiversidad para un manejo sustentable y resiliente de los agrosistemas: importancia del componente cultural. Cuadernos de la Biored, Numero 1: 21-33. Biored Iberoamericana, CYTED.
49. Sarandón, S. J. 2019. Potencialidades, desafíos y limitaciones de la investigación agroecológica como un nuevo paradigma en las ciencias agrarias. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 383-394.
50. Sosa, H. J. 2012. Restauración y conservación del Sitio Ramsar Lagunas de Guanacache, Desaguadero y del Bebedero.

51. Sosa, H.; Vallve, S. 1999. Lagunas de Guanacache (centro-oeste de Argentina). Procedimiento de inclusión a la convención sobre los humedales (Ramsar, 71). Multequina. 8: 71-85.
52. Stockle, C. O.; Papendick, R. I.; Saxton, K. E.; Campbell, G. S.; Van Evert, F. K. 1994. A framework for evaluating the sustainability of agricultural production systems. American Journal of Alternative Agriculture. 9(1-2): 45-50.
53. Stupino, S.; Iermanó, M. J.; Gargoloff, N. A.; Bonicatto, M. M. 2014. La biodiversidad en los agroecosistemas. Agroecología: bases teóricas para el diseño y manejo de agroecosistemas sustentables. Colección libros de cátedra. Editorial de la Universidad Nacional de La Plata. Capítulo 5. 131-158.
54. Sustainable Development Goals. 2015. Available in: https://sustainabledevelopment.un.org/?menu=1300
55. Tapia, R.; Scaglia, J.; Andrieu, J.; Martinelli, M. 2017. Acceso y calidad del agua para su uso en múltiples actividades por parte de pequeños productores caprinos situados en el sureste del secano de San Juan (Argentina). Multequina 0327-9375.
56. Toledo, V. M. 2001. Indigenous peoples and biodiversity. Encyclopedia of biodiversity. 3: 451-463.
57. Tonolli. A. J. 2019. Propuesta metodológica para la obtención de indicadores de sustentabilidad de agroecosistemas desde un enfoque multidimensional y sistémico. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(2): 381-399.
58. Tonolli, A.; Sarandón, S.; Greco, S. 2019. Algunos aspectos emergentes y de importancia para la construcción del enfoque agroecológico. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 205-212.
59. Torres, L. 2010. Claroscuros del desarrollo sustentable y la lucha contra la desertificación: las racionalidades económicas en el ojo de la tormenta: Estudio de caso con productores caprinos de tierras secas (Mendoza, Argentina). Mundo agrario. 11(21): 00-00.

Agro-economic viability from two croppings of broadleaf vegetables intercropped with beet fertilized with roostertree in different population densities

Viabilidad agroeconómica de dos cultivos de hortalizas de hoja ancha intercalados con remolacha y fertilizados con roostertree en diferentes densidades de población

Francisco Cicupira de Andrade Filho [1], Eliane Queiroga de Oliveira [1], Jailma Suerda Silva de Lima [2], Joserlan Nonato Moreira [1], Ítalo Nunes Silva [2], Hamurábi Anizio Lins [2], Arthur Bernardes Cecílio Filho [3], Aurélio Paes Barros Júnior [2], Francisco Bezerra Neto [2]

Originales: *Recepción:* 23/08/2018 - *Aceptación:* 12/03/2019

Abstract

The objective of this study was to evaluate the sustainability and agro-economic viability from two croppings of coriander (C) and two of arugula (A) intercropped with beet (B) as a function of roostertree additions to the soil in different population densities. The experimental design was a randomized complete block, with treatments arranged in a 4 x 4 factorial scheme with four replications. The treatments resulted from the combination of four amounts of roostertree biomass (6, 19, 32 and 45 t ha^{-1} on dry basis) with four population densities of coriander, beet and arugula (20_C-50_B-20_A%, 30_C-50_B-30_A%, 40_C-50_B-40_A% and 50_C-50_B-50_A% of the recommended densities in their single crops). The maximum agronomic efficiency of the polyculture of coriander, beet and arugula was obtained with the density of 40_C-50_B-40_A and the amount of 19 t ha^{-1} roostertree biomass incorporated into the soil. The highest profitability of the polyculture was obtained with the density of 20_C-50_B-20_A (%) and the amount of 45 t ha^{-1} of this green manure. High agro-economic efficiency can be obtained by cultivating the polyculture of coriander, beet and arugula when well-manageding the factors of production, fertilization with roostertree and population densities.

Keywords

Coriandrum sativum • *Beta vulgaris* • *Eruca sativa* • *Calotropis procera* • Polyculture • Agronomic and economic viability • arugula

1 Instituto Federal de Educação. Ciência e Tecnologia. Sousa. Paraiba. Brazil.

2 Centro de Ciências Agrárias. Departamento de Ciências Agronômicas e Florestais. Universidade Federal Rural do Semi-Árido. Mossoró. Rio Grande do Norte. Brazil. Av. Francisco Mota 572. Costa e Silva. Caixa-postal: 137. CEP: 59625-900. Mossoró. RN. Brazil. aurelio.barros@ufersa.edu.br

3 Departamento de Produção Vegetal. Universidade Estadual Paulista Júlio de Mesquita Filho. Jaboticabal. São Paulo. Brazil.

Resumen

El objetivo de este estudio fue evaluar la sostenibilidad y la viabilidad agroeconómica de dos cultivos de cilantro (C) y dos de rúcula (A) intercalados con remolacha (B) en función de las cantidades de diferentes densidades de población de roostertree añadidas al suelo. El diseño experimental fue un bloque completo aleatorizado, con tratamientos dispuestos en un esquema factorial de 4 x 4 con cuatro repeticiones. Los tratamientos fueron el resultado de la combinación de cuatro cantidades de biomasa de roostertree (6, 19, 32 y 45 t ha^{-1} en base seca) con cuatro densidades de población de cilantro, remolacha y rúcula (20_C-50_B-20_A%, 30_C-50_B- 30_A%, 40_C-50_B-40_A% y 50_C-50_B-50_A% de las densidades recomendadas en sus cultivos individuales). La máxima eficiencia agronómica del policultivo de cilantro, remolacha y rúcula se obtuvo con la densidad de 40_C-50_B-40_A en la cantidad de 19 t ha^{-1} de biomasa de roostertree incorporada al suelo. La mayor rentabilidad del policultivo se alcanzó a la densidad de 20_C-50_B-20_A (%) y la cantidad de 45 t ha^{-1} de este abono verde. Se puede obtener una alta eficiencia agro-económica cultivando el policultivo de cilantro, remolacha y rúcula cuando se manejan bien los factores de producción, la fertilización con roostertree y las densidades.

Palabras clave

Coriandrum sativum • *Beta vulgaris* • *Eruca sativa* • *Calotropis procera* • Policultivo • Viabilidad agronómica y económica • rúcula

Introduction

The demand for healthy and quality products is increasing every day. Thus, the concerns cannot only be limited to the crop productivity and quality of production sought, but also to how it will be achieved. Therefore, there is a need for adaptation of agronomic practices and their economic evaluations, which will guarantee such requirements (5).

The intercropping systems are presented as viable alternatives, although they already are traditional practices present in Brazilian agriculture and applied mainly to in family-based rural properties. Characterized by the planting of two or more crops, in the same space and time, these technologies use little external inputs and strongly influence productivity, since they provide a better use of the environmental resources and the interaction between the system's component crops (1). Proper management of production factors such as fertilization and population densities of the component crops, can reduce competition for environmental resources and increase crop and system productivity (19).

As cultivation of vegetables requires is notable for its intense management, leading to considerable environmental impacts, it can be said that this agricultural segment can benefit from the use of these practices, with the possibility of locating the olericulture within the context of more sustainable agriculture and for small producers that act with extreme difficulties in the sector (18, 24).

In Brazil, these systems have received increasing attention from researchers. Increasing use and efficiency in the production of broadleaf, tuberous vegetables has provided positive with regards to the agricultural sustainability indicators (4).

However, polycultures from the broadleaf crops coriander and arugula intercropped with beet have been practiced in the Brazilian semi-arid region given that they are economically valuable crop that offer healthy products for northeastern consumers. The obtention of efficiency and productivity with the best combination of these crops in polyculture system, should meet the demand in the region. It is known, however, that the efficiency of these cropping systems is conditioned by a series of important factors of production, with emphasis on crop types, population densities, spatial arrangements, spacing, and fertilization, among others.

Efficiency of these intercropping systems can be evaluated through different methods. One of the most used is the amount of food produced per unit area, considered of greatest interest for the small farmers, the main users of the system. Another evaluation considers the generated profit, through economic analysis. Researchers usually use the land equivalent ratio (LER) and more recently, the productive efficiency index of intercropping systems through Data Envelopment Analysis (DEA), incorporating biological and economic advantages of the intercropped systems (11).

Studying the multiple cultivation of arugula (A), carrot (C) and coriander (Co) fertilized with different amounts of hairy woodrose and population densities, Oliveira *et al.* (2017) observed that the most productive agroeconomic performance of the system was obtained with a quantity of biomass of 18.21 t ha^{-1} of hairy woodrose incorporated in the soil and in the population density of 50_A-50_C-50_{Co} (%) of the RDSC.

On the other hand, Oliveira *et al.* (2015) investigating the viability of the multiple cropping of arugula, carrot and lettuce fertilized with different amounts of roostertree in different proportions, observed that the best agronomic performance of this multiple cropping was recorded with the amount of 55 t ha^{-1} of roostertree incorporated into the soil. The proportion of population density of these three vegetables of 50_A-50_C-50_L (%) of RDSC was that that provided the greatest agronomic viability of the multiple cropping systems.

Aiming to contribute with information on viable and sustainable technologies for the production of coriander, beet and arugula vegetable crops in multiple crop system, this work had the objective of evaluating the agro-economic performance of coriander and arugula intercropped with beet as a function of fertilization with roostertree biomass at different population densities in semi-arid environment.

Materials and Methods

The experiment was conducted at the Experimental Farm 'Rafael Fernandes', belonging to the Universidade Federal Rural do Semi-Árido (UFERSA), located in the Alagoinha district, a rural area of Mossoró-RN (5° 03' 37" S and 37° 23' 50" W Gr) June to November 2011. According to Thornthwaite, the local climate is BShw, *i.e.* semi-arid (2). During the experimental period, the values of average temperature was 27 °C; minimum of 25 °C; maximum of 31°C; relative humidity of 66 %; radiation of 918 kJ m^{-2}; rainfall of 0 mm; atmospheric pressure of 1011 h Pa and temperature of dew point of 19 °C.

The soil of the experimental area was classified as Oxisol dystrophic argisolic (7). In this area, soil samples were collected at a depth of 0-20 cm and then sent to the Water Analysis Laboratory, Soils and Plants of the EMBRAPA-PE for analysis, yielding the following results: pH = 7.7;

organic matter = 4.34 g kg^{-1}; E.C. = 2.81 dS m^{-1}; P = 3.0 mg dm^{-3}; K^+ = 0.016 $cmol_c$ dm^{-3}; Ca^{+2} = 3.54 $cmol_c$ dm^{-3}; Mg^{+2} = 1.05 $cmol_c$ dm^{-3} and Na = 0.029 $cmol_c$ dm^{-3}.

The experimental design was a randomized complete block, with treatments arranged in a 4 x 4 factorial scheme with four replications. The treatments resulted from the combination of four amounts of roostertree biomass (6, 19, 32 and 45 t ha^{-1} on dry basis) added to the soil with four population densities of coriander (*Coriandrum sativum*), beet (*Beta vulgaris*), and arugula (*Eruca sativa*) of 20_C-50_B-20_A%, 30_C-50_B-30_A%, 40_C-50_B-40_A% and 50_C-50_B-50_A% of the recommended densities in their single crops – RDSC.

The preparation of the soil consisted of manual cleaning of the area with the aid of a hoe, followed by harrowing and rising of beds. Pre-planting solarization with transparent plastic, type Vulcabrilho Bril Fles 30 microns for 45 days was done in order to reduce soil phytopathogens and the possible appearance of damping off, which would affect crop productivity.

Roostertree (*Calotropis procera)* was collected from native vegetation of areas of the rural zone of the municipality of Sousa-Paraiba, Brazil. They were crushed into pieces of two to three centimeters and left to dry at room temperature until they reached the point of hay, being stored with a moisture content of 10%. Nutient content was quantified with randomly chosen samples. The obtained chemical composition was: N = 29.58 g kg^{-1}; P = 4.08 g kg^{-1}; K = 50.09 g kg^{-1}; Ca = 16.55 g kg^{-1}; Mg = 9.50 g kg^{-1}, S = 4.39 g kg^{-1}; Fe = 700 mg kg^{-1}; Zn = 44 mg kg^{-1}; Cu = 13 mg kg^{-1}; Mn = 220 mg kg^{-1}, B = 56.49 mg kg^{-1}; Na = 995.13 mg kg^{-1} and a C:N ratio = 16: 1.

Intercropping was established in alternating strips of coriander and arugula between the beet strips in the of 50% area occupied by beet, 25% by coriander and 25% by arugula. Each plot consisted of four strips of four rows each: a strip of broadleaf, a strip of beet, a strip of other broadleaf and a strip of beet, flanked in the side of the first strip with a strip of beet and in the other side flanked by an arugula strip, used as borders. The total area of the plot was 4.80 m^2, with a harvest area of 3.20 m^2. The spacing and number of broadleaf plants in the plots varied according to the population densities studied. The population densities used in single crop in the region are 500,000 plants per hectare for beet (27) and 1,000,000 plants per hectare for coriander and arugula (10, 14).

The experimental plots were fertilized with the respective quantities of roostertree studied, and 50% of the quantities for each plot were incorporated 16 days before planting the crops in the intercropping (15). The remaining 50% were incorporated 40 days after sowing (26). After the incorporation of roostertree to the soil, daily irrigations were carried out in two shifts with the purpose of favoring the microbial activity of the soil in the decomposition process.

The vegetable cultivars were Verdão, Early Wonder and Cultivada. The sowing of the component crop was performed on August 25 and 26, 2011, in holes of approximately three centimeters deep, placing three to four seeds per hole. Roughing of arugula and beet was made 11 days after the planting, leaving only two plants per hole for arugula and one for beet. Coriander thinning was done 14 days after sowing, leaving two plants per hole.

During the conduction of the experiment, weed control was manually. Harvesting of arugula and coriander occurred on September 26 and 28, 2011, respectively.

The second cropping of coriander and arugula was performed on October 18, 2011, with the same procedures of the first cultivation as marking, sowing, adding coconut substrate on the rows and providing first irrigation. In the following week, some seeds were replanted and 12 to 14 days after sowing, arugula and coriander were thinned, leaving two plants per hole. Beet was harvested on November 08, 2011.

In addition to the green mass yields of coriander (Y_c) and arugula (Y_a) and the commercial productivity of beet roots (Y_b), the following agronomic and economic indices were evaluated in the polycultures:

a) Score of the canonical variable (Z) - Obtained from the multivariate analysis of the green mass yields of coriander and arugula and commercial productivity of beet roots.

b) Productive efficiency index (PEI) – It was obtained from the Data Envelopment Analysis (DEA) model (22) with constant returns to the scale (29), since there was no significant difference in the scales. This model has a mathematical formulation X_{ik}: is the input i value (i = 1, ..., s) for treatment k (k = 1, ..., n); Y_{jk}: is the output j value (j = 1, ..., r), for treatment k; $_{vi}$ and $_{uj}$: are weights assigned to inputs and outputs, respectively; and O: is the treatment being analyzed.

$$Max \sum_{i=1}^{v} v_i x_{io}$$

$$\sum_{j=1}^{s} u_j y_{jo} = 1$$

$$\sum_{j=1}^{s} u_j y_{jk} - \sum_{i=1}^{r} v_i x_{ik} \leq 0, k = 1, \ldots, n$$

$$u_j, v_i \geq o, i = 1, \ldots, s, j = 1, \ldots, r$$

The evaluation units were each treatment, from a total of sixteen resuting from the combination of four roostertree biomass amounts incorporated to the soil and four populations densities of the component crops. The outputs were the green mass yields of coriander and arugula (sum of the first and the second harvest), and the commercial productivity of beet roots. To evaluate yield of each plot, it was assumed that each plot utilized a single resource with a unitary level, following a similar approach to that used by Soares de Melo and Gomes (2004), since the outputs incorporated the possible inputs.

In the modeling of this study, the profit margin (index described in the following item) was used as input.

c) Gross Income (GI) - It was obtained through the value of the production per hectare, based by the price paid to producers in the region in December 2011. For coriander, the paidprice was R$ 6.25 kg-1, R$ 1.50 kg-1 for beet and R$ 4.60 kg-1 for arugula.

d) Total costs (TC) production – Calculated after Silva et al. (2015). The production total costs were calculated at the end of the productive process in December 2011, based on total expenditure per hectare of cultivated area, covering the services provided by stable capital, i.e., the contribution of current capital and the value of alternative costs or opportunities.

e) Net income (NI) - Obtained from the difference between gross income (GI) and total costs (TC).

f) Return Rate (RR) - Calculated from the ratio between gross income and total costs. It reveals how many reals are obtained in return for every real invested in the evaluated intercropping system.

g) Profit margin (PM) - It was obtained by the ratio between net income (NI) and gross income (GI), expressed as a percentage.

Univariate analyses of variance were performed on agronomic and economic indices of polycultures using the statistical package SISVAR (9) for the randomized complete block design with treatments arranged in factorial scheme. Tukey's test at 5% probability was used to compare the averages between the population densities of vegetable crops. A fitting procedure of response curves was performed in each index as a function of roostertree amounts incorporated into the soil through Table Curve package (6).

Results

Agronomic indices

Significant interactions between the amounts of roostertree biomass added to soil and population densities of the component crops were observed in the vectors of coriander and arugula yields and beet root productivity, by the Wilks criterion (table 1, page 216).

By examining the eigenvalues and vectors associated with the significant effects of the interaction (A x D), it was observed that 87.83% of the total variance was explained by the linear combination of X_1 given the first eigenvalue. This result was different from those obtained by Porto *et al.* (2011) in a polyculture of lettuce, carrot and arugula conducted in the same region. The linear combination was dominated entirely by X_1 (Y_a = arugula yield), where in terms of relative importance to the linear combination of variable X_1 was about 2.18 times more efficient than variable X_2 and 500 times more efficient than variable X_3 (table 1, page 216).

The discriminant function or canonical variable obtained from crop yields was $Z = 0.458\ Y_c + 0.012\ Y_b + 0.751\ Y_a$ (table 1, page 216). This equation obtained the scores of each treatment, which subsequently, were then submitted to the univariate analysis of variance. From this analysis, it was found that, by partitioning the amounts of roostertree biomass within each population density, the scores of the canonical variable of the densities of 40_C-50_B-40_A and 50_C-50_B-50_A, increased with increasing amounts of roostertree biomass until maximum values of 7.11 and 5.49 in the green manure amounts of 13.02 and 20.09 t ha^{-1}, decreasing up to the highest amount of manure (45 t ha^{-1}) incorporated into the soil. There was no adjustment of response function for the scores of the canonical variable in the population densities of 20_C-50_B-20_A and 30_C-50_B-30_A (figure 1, page 217).

On the other hand, by partitioning the population density interaction within each amount of the green manure, it was observed that the scores of the canonical variable Z in the population densities of 40_C-50_B-40_A and 50_C-50_B-50_A stood out from scores of the densities of 20_C-50_B-20_A and 30_C-50_B-30_A with 6 t ha^{-1} of added roostertree biomass. The population density score of 40_C-50_B-40_A overcame the other scores of the densities of 20_C-50_B-20_A, 30_C-50_B-30_A and 50_C-50_B-50_A in the amounts of 19 and 45 t ha^{-1} of roostertree biomass and the population density score of 50_C-50_B-50_A outperformed the scores of the densities of 20_C-50_B-20_A, 30_C-50_B-30_A and 40_C-50_B-40_A with 32 t ha^{-1} of the incorporated green manure (figure 1, page 217).

Table 1. Multivariate variance analysis of the coriander and arugula yields and commercial productivity of beet roots, eigenvalues, and vectors associated with significant effect of the interaction between roostertree biomass amounts incorporated into the soil and the population densities, and discriminant function of the Z canonical variable.

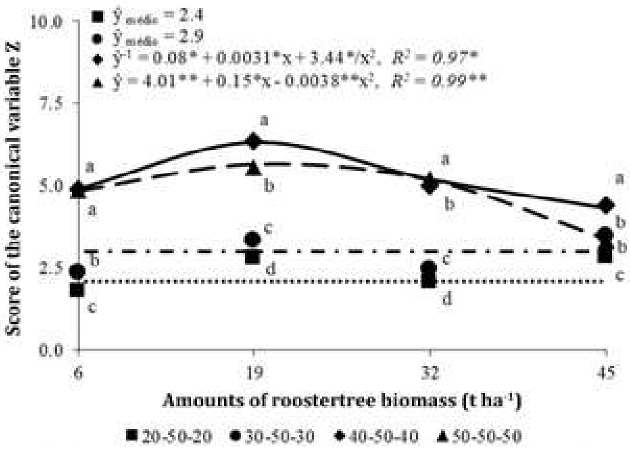
Tabla 1. Análisis de varianza multivariado de los rendimientos de cilantro y rúcula y productividad comercial de raíces de remolacha, valores propios y vectores asociados con el efecto significativo de la interacción entre las cantidades de biomasa de roostertree incorporadas en el suelo, las densidades de población, y la función discriminante de la variable canónica Z.

Source of variation		**Degrees of Freedom for F**	**λ (Wilks)**	**F**	**Prob> F**
Blocks		(9;104.8)	0.002	137.13	0.0001
Amounts (A)		(9;104.8)	0.079	21.28	0.0001
Combinations of population densities (D)		(9;104.8)	0.002	137.13	0.0001
A x D		(9;104.8)	0.024	12.06	0.0001
Significant effect of A x D					
Variate	Latent root	% Variance	Coefficient	Standard deviation	Relative Importance
*$Y_c - X_1$	12.63	87.83	0.416	1.009	0.458
$Y_b - X_3$			0.002	13.844	0.002
$Y_a - X_2$			0.909	1.241	1.000
$Y_c - X_1$			0.528	1.009	2.721
$Y_b - X_3$			0.019	13.844	0.098
$Y_a - X_2$	1.71	11.82	-0.194	1.241	1.000
$Y_c - X_1$			-0.930	1.009	8.455
$Y_b - X_3$	0.12	0.85	0.042	13.844	0.382
$Y_a - X_2$			1.109	1.241	1.000
	Canonical variable (Z)				
	86.65	90.28	$Z = 0.458\ Y_c + 0.012\ Y_b + 0.751\ Y_a$		

* Yc - Green mass yield of coriander; Yb - Commercial Productivity of beet roots; Ya - Green mass yield of arugula.

* Yc - rendimiento de masa verde de cilantro; Yb - Productividad comercial de raíces de remolacha; Ya - rendimiento de masa verde de rúcula.

There was a significant interaction between the different population densities of the component crops and the amounts of roostertree biomass added to the soil in the productive efficiency index (PEI). Partitioning the amount of roostertree, within each population density, it was observed that in the population density of 40_C-50_B-40_A there was an increase of the PEI with the increasing amounts of green manure incorporated up to the maximum value of 1.00 with 12.87 t ha^{-1}. No response function adjustment for PEI was observed for densities of 20_C-50_B-20_A, 30_C-50_B-30_A and 50_C-50_B-50_A (figure 2, page 217).

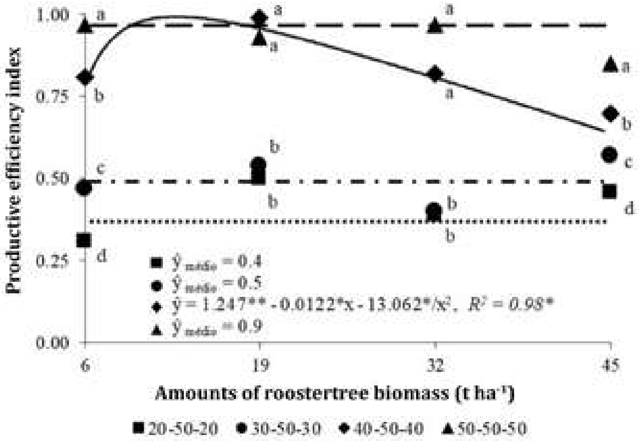


Means followed by different lowercase letter in the Y axis differ statistically by Tukey test at 5% probability.

Las medias seguidas por diferentes letras minúsculas en el eje Y difieren estadísticamente según la prueba de Tukey con un 5% de probabilidad.

Figure 1. Score of the canonical variable Z as a function of the amounts of roostertree biomass for the population densities.

Figura 1. Puntuación de la variable canónica Z en función de las cantidades de biomasa de roostertree para las densidades de población.



Means followed by different lowercase letter in the Y axis differ statistically by Tukey test at 5% probability.

Las medias seguidas por diferentes letras minúsculas en el eje Y difieren estadísticamente según la prueba de Tukey con un 5% de probabilidad.

Figure 2. Productive efficiency index as a function of the amounts of roostertree biomass for the population densities.

Figura 2. Índice de eficiencia productiva en función de las cantidades de biomasa de roostertree para las densidades de población.

Partitioning the population densities within each amount, it was recorded that the PEI in the density of 50_C-50_B-50_A stood out significantly from the others within the amounts of 6 and 45 t ha^{-1} roostertree biomass, while this index in the densities of 40_C-50_B-40_A and 50_C-50_B-50_A stood out from the densities of 20_C-50_B-20_A and 30_C-50_B-30_A in the amounts of 19 and 32 t ha^{-1} of the green manure added to the soil. The highest productive efficiency index (1.00) was obtained in the density of 50_C-50_B-50_A with 19 t ha^{-1} of roostertree biomass (figure 2, page 217).

Economic indicators

A significant interaction was also found between the different combinations of population densities of the component crops and the amounts of roostertree added to the soil in the gross income (GI), net income (NI), rate of return (RR) and profit margin (PM) (figure 3).

By partitioning the roostertree biomass amount within each population density, increases in the GI, NI, RR and PM values were observed in the densities of 20_C-50_B-20_A and 50_C-50_B-50_A. It was observed, with the increasing amounts of

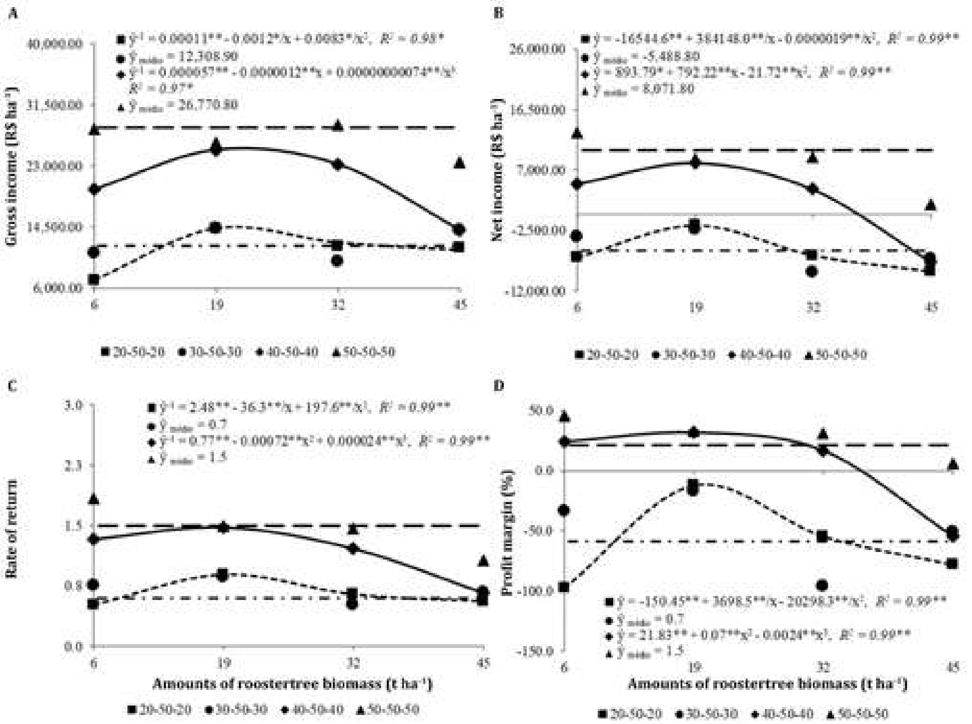


Figure 3. Gross income (A), net income (B), rate of return (C) and profit margin (D) of the coriander, beet and arugula polyculture as a function of the amounts of roostertree biomass and population densities.

Figura 3. Ingreso bruto (A), ingreso neto (B), tasa de rendimiento (C) y margen de ganancia (D) del policultivo de cilantro, remolacha y rúcula en función de las cantidades de biomasa de roostertree y densidades poblacionales.

roostertree until the maximum values of R$ 28,962.60 and R$ 23,448.98 ha^{-1}; R$ 13,482.67 and R$ 975.40 ha^{-1}; 1.89 and 1.05, and 45.40 and 3.36%, in the amounts of 13.60 and 23.39 t ha^{-1}; 10.16 and 18.23 t ha^{-1}; 10.88 and 19.91 t ha^{-1}, and 10.97 and 19.91 t ha^{-1}, respectively. Then decreased up to the greatest amount of green manure added to the soil. For the above mentioned indices, no adjustments of response functions were observed in the population densities of 30_C-50_B-30_A and 40_C-50_B-40_A (figure 3, page 218).

On the other hand, after partitioning the population densities of the component crops within each amount of roostertree biomass incorporated into the soil, it was recorded that the gross income in the densities of 30_C-50_B-30_A, 40_C-50_B-40_A and 50_C-50_B-50_A stood out from that density of 20_C-50_B-20_A in the roostertree amounts of 6 and 19 t ha^{-1}. Whit the amount of 32 t ha^{-1} of roostertree, GI in the densities of 30_C-50_B-30_A and 40_C-50_B-40_A stood out from that of the densities of 20_C-50_B-20_A and 50_C-50_B-50_A. With the amount of 45 t ha^{-1} of the green manure, there were no significant differences in the gross income between population densities within each amount of roostertree added to the soil (table 2, page 220).

The net income in the density of 30_C-50_B-30_A stood out significantly from the densities of 20_C-50_B-20_A, 40_C-50B-40_A and 50_C-50_B-50_A in the amounts of 6, 19 and 32 t ha^{-1} of roostertree biomass, even when expressing negatively in the amounts of 6 and 19 t ha^{-1} of roostertree added to the soil. Whith 45 t ha^{-1} of the green manure, NI in the density of 20_C-50_B-20_A outperformed with respect to the population densities of 30_C-50_B-30_A, 40_C-50_B-40_A and 50_C-50_B-50_A (table 2, page 220).

The rate of return of the density of 30_C-50_B-30_A stood out from the densities of 20_C-50_B-20_A, 40_C-50_B-40_A and 50_C-50_B-50_A in the amounts 6, 19 and 32 t ha^{-1} of roostertree biomass. For 45 t ha^{-1}, this rate of return in the density of 20_C-50_B-20_A outperformed that of the densities of 30_C-50_B-30_A, 40_C-50_B-40_A and 50_C-50_B-50_A (table 2, page 220).

There was no profit margin between the population densities studied in the amounts of roostertree of 6 and 19 t ha^{-1} incorporated into the soil. With 32 t ha^{-1} of the green manure, this profit margin was expressed in the population densities of 20_C-50_B-20_A, 30_C-50_B-30_A and 40_C-50_B-40_A outperforming the density of 50_C-50_B-50_A. Finally, the highest profitability was achieved in the population density of 20_C-50_B-20_A with 45 t ha^{-1} roostertree biomass added to the soil (table 2, page 220).

Discussion

Agronomic/biological efficiency

Efficiency of an intercropping system depends directly on the management and crops involved (23). Thus, the appropriate management of factors of production, such as fertilization, population density, planting spatial arrangement, among others, can reduce the competition between the component crops by environmental resources and increase the efficiency of the system in agronomic and economic terms. This increase in efficiency is due to the ecological and economic benefits of the complementarity of the species involved, increasing the production of the intercropping when compared to monocultures, or to the chemical, physical and biological enrichment of the soil that improve root exploration (3, 12).

Table 2. Mean values of gross income (GI), net income (NI), rate of return (RR) and profit margin (PM) of the coriander, beet and arugula polyculture as a function of different amounts of roostertree biomass added to the soil and population densities.

Tabla 2. Valores medios del ingreso bruto (IG), el ingreso neto (NI), la tasa de rendimiento (RR) y margen de ganancia (PM) del policultivo de cilantro, remolacha y rúcula en función de las diferentes cantidades de biomasa de roostertree añadido al suelo y las densidades de población.

Combinations of population densities	Amounts of roostertree (t ha^{-1})			
	6	19	32	45
GI (R$ ha^{-1})				
20_C-50_B-20_A	7,200.14 b	9,760.56 b	19,785.99 b	28,257.81 a
30_C-50_B-30_A	14,403.97 a	14,373.52 a	25,288.71 a	26,351.43 a
40_C-50_B-40_A	11,864.37 a	10,911.26 a	23,264.97 a	28,827.51 a
50_C-50_B-50_A	11,630.03 a	14,190.22 a	14,166.45 c	23,646.23 a
NI (R$ ha^{-1})				
20_C-50_B-20_A	-6,734.31 b	-3,574.84 a	4,849.22 ab	12,870.38 a
30_C-50_B-30_A	-1,732.28 a	-2,314.38 a	8,150.14 a	8,762.20 b
40_C-50_B-40_A	-6,448.42 b	-9,103.90 b	3,949.85 b	9,061.73 b
50_C-50_B-50_A	-8,970.42 b	-6,961.88 b	-7,436.32 b	1,592.80 c
RR				
20_C-50_B-20_A	0.52 b	0.75 ab	1.33 ab	1.84 a
30_C-50_B-30_A	0.89 a	0.86 a	1.47 a	1.50 b
40_C-50_B-40_A	0.65 b	0.52 c	1.20 b	1.46 b
50_C-50_B-50_A	0.56 b	0.67 bc	0.65 c	1.07 b
PM (%)				
20_C-50_B-20_A	-97.87 d	-33.72 b	23.97 a	45.40 a
30_C-50_B-30_A	-12.07 a	-16.88 a	31.59 a	32.81 b
40_C-50_B-40_A	-54.54 b	-96.47 d	16.42 a	31.25 b
50_C-50_B-50_A	-78.38 c	-50.95 c	-54.49 b	5.88 c

* Means followed by different lowercase letters in the column differ statistically by Tukey test at 5% probability.

* Medias seguidas de diferentes letras minúsculas en la columna difieren estadísticamente mediante la prueba de Tukey al 5% de probabilidad.

The results of the significant interaction recorded in this research between the amounts of roostertree added to the soil and the population densities of the component crops in the canonical variable score and in the productive efficiency index show that the levels of one factor behaved differently within each level of the other factor, thus revealing that there are more productive and agronomically efficient intercropping systems with high quality products. These results for the polyculture of coriander, beet and arugula in terms of agronomic efficiency are explained by better use of environmental resources in the density of 40_C-50_B-40_A with the amount of 19 t ha^{-1} roostertree biomass, not observing the negative influence of competition for water and nutrients to the plants. This means that when we combine production factors such as population density of the component crops and fertilizer doses in polyculture, it is possible to obtain satisfactory and viable results in terms of production and agronomic efficiency, making it easier for farmers to practice this type of cultivation.

Given this, it is up to us to select the agronomically most productive systems. Working with the polyculture of arugula, carrot and lettuce, Oliveira *et al.* (2015) did not obtain significant interaction in the agronomic variables evaluated but recorded that the intercropped system of better agronomic efficiency of the polyculture was that obtained with the amount of 55 t ha^{-1} of roostertree added to the soil and in the population density of 50_A-50_C-50_L (%) of the RDSC. This result differed from that obtained in this research, where the best productive efficiency index of the polyculture was reached with 14.23 t ha^{-1} of roostertree incorporated into the soil in the population density of 40_C-50_B-40_A (%) of the RDSC. This difference in results is due to the types of polycultures used, being stem vegetables more efficient than broadleaf vegetables and beet, where the benefits of complementarity between the species occur more efficiently (19).

The organic fertilizer to be decomposed, besides providing nutrients, stimulating root growth and increasing absorption after humi-fication, becomes a main source of negative loads in the soils increasing cation retention and allowing greater absorption of nutrients by plants (16).

In addition, it also has high soil buffering power, *i.e.*, the higher the content of organic matter humidified in the soil, the greater its resistance to the sudden change in pH of the medium. One of the main characteristics related to the quality of an organic fertilizer for soil is its C/N ratio, controlling the availability of nutrients to plants (13).

When used in an adequate quantity, the organic matter immediately reduces the apparent density of the fertilized layer and promotes the aggregation of particles, giving the soil favorable conditions of aeration and friability, increasing its water retention capacity (17). The increase in water retention may be related to the decrease in density and increase in total porosity and change in aggregate size distribution, which may change the pore size distribution (8).

It is known that population densities have been used in intercropped systems of cultivated species, and their use in vegetable crops has been increasing. With the proper management of this production factor, it is possible to increase the efficiency in the use of fertilizer and available resources (water, light and nutrients), consequently increasing crop productivity and agronomic efficiency in the association of crops.

The overall density of the intercrops and the relative proportions of the component crops are important in determining yield and production efficiency of these systems. When components are present in approximately equal numbers, productivity and efficiency appear to be determined by the most aggressive culture in the intercropping (30).

IIn the case of this research the most aggressive crop was beet. In agronomic terms, the best productive performance of the polyculture of cori- ander, beet and arugula was reached at the density of 40C-50B-40A of the RDSC with 19 t ha-1 roostertree biomass incorporated into the soil.

Economic efficiency

One of the questions that arise when working with vegetable crop polycultures is whether the productive or agronomic performance of the intercropping systems evaluated is translated in terms of economic efficiency. This is not an easy question to answer, since polyculture systems with vegetables are complex and depend on a number of factors, such as the crops involved, type and quality of products produced, prices of products, indicators and indices used in economic evaluation, among others.

Based on the partitioning of the interaction between the amounts of roostertree biomass added to the soil and the population densities evaluated in the polyculture of coriander, beet and arugula, it can be verified that the best agronomic/ biological efficiency of the polyculture was reached with the density of 40_C-50_B-40_A of the RDSC and the incorporation of 19 t ha^{-1} of roostertree biomass into the soil, while the best economic efficiency of the polyculture was obtained with the density of 20_C-50_B-20_A and 45 t ha^{-1} of this green manure. These results lead producer choose whether to use a high population with intercropped systems fertilized with a low roostertree amount or a low population with a high roostertree amount.

The indicators chosen to express economic efficiency of the polyculture of coriander, beet, and arugula in this research were the net income and profit margin, which are strongly dependent on the total cost of production of each treatment and on the product prices coming from each treatment.

The net income is one of the indicators that best expresses the economic value of an intercropping system when compared to gross income, because the total costs of production are deducted. The expenses that most affect these costs of production are those with inputs, labor, maintenance and conservation of facilities and equipment. If these intercropping systems are run by family farmers, where the workforce in the production of crops is carried out by the family itself, this means that the expenditure of this labor force would become an extra profit for the farmer and the intercropped production systems would increase their economic efficiency. When production factors like fertilization and population density in polyculture of broadleaf and tuberous crops are well managed, family farmers can benefit from efficient production systems with high quality products for the market. These indexes allow the producer to visualize the best technology for the coriander, beet and arugula production process, in terms of agro- economic efficiency.

Cultivating a polyculture of arugula, carrot and lettuce in semi-arid region, Oliveira et al. (2017) obtained agroeconomic efficiency using a high population density of 50A-50C-50L (%) of the RDSC and a low roostertree amount of 25 t ha-1 incorporated into the soil. This result is close to the obtained agronomic efficiency of the polyculture of coriander, beet and arugula of this research. In view of this, one can observe agroeconomic advantage in cultivating polycultures among broadleaf and tuberous crops in semi-arid environment.

Conclusions

The maximum agronomic efficiency of the polyculture of coriander, beet and arugula was obtained with the density of 40_C-50_B-40_A with of 19 t ha^{-1} roostertree biomass incorporated into the soil. The maximum economic efficiency of the polyculture of coriander, beet and arugula was reached with the density of 20_C-50_B-20_A with the amount of 45 t ha^{-1} roostertree biomass added to the soil. High agro-economic efficiency can be obtained by cultivating the polyculture of coriander, beet and arugula by well managing production, fertilization with roostertree and population densities among the component crops. This cropping system should be recom- mended to family farmers who produce leafy and tuberous vegetable crops in a sustainable way in semi-arid environment.

References

1. Almeida, A. E. S.; Bezerra Neto, F.; Costa, L. R.; Silva, M. L.; Lima, J. S. S.; Barros Júnior, A. P. 2015. Eficiência agronômica do consórcio alface-rúcula fertilizado com flor-de-seda. Revista Caatinga. 28(3): 79-85.
2. Alvares, C. A.; Stape, J. L.; Sentelhas, P. C.; Gonçalves, J. L. M.; Sparovek, G. 2014. Koppen´s climate classification map for Brazil. Metereologische Zeitschrift. 22(6):711-728.
3. Batista, M. A. V.; Bezerra Neto, F.; Silva, M. L.; Ambrósio, M. M. Q.; Cunha, J. L. X. L. 2016. Atributos de solo-planta e de produção de beterraba influenciados pela adubação com espécies da Caatinga. Horticultura Brasileira. 34(1):12-19.
4. Bezerra Neto, F.; Porto, V. C. N.; Gomes, E. G.; Cecílio Filho, A. B.; Moreira, J. N. 2012. Assessment of agroeconomic indices in polycultures of lettuce, rocket and carrot through uni-and multivariate approaches in semi-arid Brazil. Ecological Indicators. 14(1): 11-17.
5. Blandi, M. L.; Rigotto, R. M.; Sarandón, S. J. 2018. Influencia de factores contextuales en la adopción de modelos de agricultura insustentables. La incorporación del invernáculo en agricultores platenses. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 203-216.
6. Cody, R. P.; Smith, J. K. 2004. 5th ed. Applied statistics and the SAS programming language. New Jersey: Prentice Hall.
7. EMBRAPA. (Empresa Brasileira de Pesquisa Agropecuária). 2006. 2ª ed. Sistema brasileiro de classificação de solos. Rio de Janeiro: Embrapa.
8. Fageria, N. K. 1998. Otimização da eficiência nutricional na produção das culturas. Revista Brasileira de Engenharia Agrícola e Ambiental. 2(1): 6-16.
9. Ferreira, D. F. 2011. Sisvar: A computer statistical analysis system. Ciência e Agrotecnologia. 35(6): 1039-1042.
10. Freitas, K. K. C.; Bezerra Neto, F.; Grangeiro, L. C.; Lima, J. S. S.; Moura, K. H. S.; Barros Júnior, A. P. 2009. Desempenho agronômico de rúcula sob diferentes espaçamentos e épocas de plantio. Revista Ciência Agronômica. 40(3):449–454.
11. Gomes, E. G.; Souza, G. S. 2005. Avaliação de ensaios experimentais com o uso da análise de envoltoria de dados: uma aplicação a consórcios. In: Simpósio de Estatística Aplicada à Experimentação Agronômica (SEAGRO). 11. Londrina. Resumos. Londrina. IBS.
12. Grisa, C.; Schneider, S. 2008. "Plantar pro gosto": a importância do autoconsumo entre famílias de agricultores do Rio Grande do sul. Revista de Economia e Sociologia Rural. 46(2): 481-515.
13. Kiehl, E. J. 1985. Fertilizantes orgânicos. Piracicaba: Agronômica Ceres.
14. Lima, J. S. S.; Bezerra Neto, F.; Negreiros, M. Z.; Freitas, K. K. C.; Barros Júnior, A. P. 2007. Desempenho agroeconômico de coentro em função de espaçamentos e em dois cultivos. Revista Ciência Agronômica. 38(4): 407-413.

15. Linhares, P. C. F. 2009. Vegetação espontânea como adubo verde no desempenho agroeconômico de hortaliças folhosas. Mossoró: UFERSA. (Tese de Doutorado).
16. Mendonça, E. S.; Loures, E. G. 1995. Matéria orgânica do solo. Brasília: ABEAS. (Curso de Fertilidade e Manejo de solo, Módulo 5).
17. Myasaka, S.; Camargo, A. O.; Cavaleri, P. A.; Godov, I. J.; Werner, J. C.; Curi, S. M.; Lombardi Neto, F.; Medina, J. C.; Cervelline, G. S.; Bulsinane, E. A. 1984. 2ª ed. Adubação orgânica, adubação verde e rotação de cultura no Estado de São Paulo. Campinas: Fundação Cargill.
18. Oliveira, E. Q.; Souza, R. J.; Cruz, M. C. M.; Marques, V. B.; França, A. C. 2010. Produtividade de alface e rúcula, em sistema consorciado, sob adubação orgânica e mineral. Horticultura Brasileira. 28(1): 36-40.
19. Oliveira, L. A. A.; Bezerra Neto, F.; Silva, M. L.; Oliveira, O. F. N.; Lima, J. S. S.; Barros Júnior, A. P. 2015. Viabilidade agronômica de policultivos de rúcula/cenoura/alface sob quantidades de flor-de-seda e densidades populacionais. Revista Caatinga. 28(4): 116-126.
20. Oliveira, L. A. A.; Bezerra Neto, F.; Barros Júnior, A. P.; Silva, M. L.; Oliveira, O. F. N.; Lima, J. S. S.; Barros Júnior, A. P. 2017. Agro-economic efficiency of polycultures of arugula-carrot-lettuce fertilized with roostertree at different population density proportions. Revista Brasileira de Engenharia Agrícola e Ambiental. 21(11): 791-797.
21. Oliveira, L. J.; Bezerra Neto, F.; Lima, J. S. S.; Oliveira, E. Q.; Moreira, J. N.; Silva, I. N. 2017. Viability of polycultures of arugula-carrot-coriander fertilized with hairy woodrose under different population densities. Revista Brasileira de Engenharia Agrícola e Ambiental. 21(9): 611-617.
22. Olmedo Vázquez, V. M.; Minjares Lugo, J. L.; Camacho Poyato, E.; Hernández Hernández, M. L.; Rodríguez Díaz, J. A. 2017. Uso del Análisis Envolvente de Datos (DEA) para evaluar la eficiencia de riego en los Módulos del Distrito de Riego No. 041, Río Yaqui (Sonora, México). Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 49(2): 127.
23. Porto, V. C. N.; Bezerra Neto, F.; Lima, J. S. S.; Barros Júnior, A. P.; Moreira, J. N. 2011. Combination of lettuce and rocket cultivars in two cultures intercropped with carrots. Horticultura Brasileira. 29(3): 404-411.
24. Resende, B. L. A.; Cecílio Filho, A. B.; Martins, M. I. E. G.; Costa, C. C.; Feltrim, A. L. 2005. Viabilidade econômica das culturas de pimentão, repolho, alface, rabanete e rúcula em cultivo consorciado na primavera-verão. Jaboticabal Estado de São Paulo. Informações econômicas. 35(3): 22-37.
25. Silva, A. F. A.; Souza, E. G. F.; Santos, M. G.; Barros Júnior, A. P.; Bezerra Neto, F.; Silveira, L. M. 2015. Rentabilidade do rabanete adubado com flor-de-seda em duas épocas de cultivo no semiárido de Pernambuco. Revista de Ciências Agrárias/Amazonian Journal of Agricultural and Environmental Sciences. 58(2): 198- 207.
26. Silva, M. L. 2012. Viabilidade agronômica de hortaliças fertilizadas com flor-de-seda (*Calotropis procera*). Mossoró: UFERSA. (Tese de Doutorado).
27. Silva, M. L. S.; Bezerra Neto, F.; Linhares, P. C. F.; Sá, J. R.; Lima, J. S. S.; Barros Júnior, A. P. 2011. Produção de beterraba fertilizada com jitirana em diferentes doses e tempos de incorporação ao solo. Revista Brasileira de Engenharia Agrícola e Ambiental. 15(8): 801-809.
28. Soares de Melo, J. C. C. B.; Gomes, E. G. 2004. Eficiências aeroportuárias: uma abordagem comparativa com análise de envoltória de dados. Revista de Economia e Administração. 3(1): 15-23.
29. Soares de Melo, J. C. C. B.; Gomes, E. G.; Abreu, U. G. P.; Carvalho, T. B.; Zen, S. 2013. Análise de desempenho de sistemas de produção modais de pecuária de cria no Brasil. Produção. 23(4): 877-886.
30. Willey, R. W.; Osiru, D. S. 1972. Studies on mixtures of maize and beans (*Phaseolus vulgaris*) with particular reference to plant population**.** Journal of Agricultural Science. 70(2): 517-529.

Acknowledgements

Special thanks are due to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) by the financial support to this research and to the research group that develops technologies for growing vegetables on family farms.

Determination of the price in the fresh fruit market: case of pears

Determinación del precio en el mercado de frutas frescas: caso de peras

Miguel Ángel Giacinti Battistuzzi [1*], José Ramos Pires Manso [2],
Jaime de Pablo Valenciano [3]

Originales: *Recepción:* 10/11/2018 - *Aceptación:* 19/06/2019 *Nota científica*

Abstract

This document aims to evaluate the determinants of the price of pears in the international fresh fruit market, from an innovative vision in a complex world. The panel data methodology was applied. The variables considered were the different prices (CIF/kg) of pear, apple and stone fruits, their per capita consumptions, real per capita income, consumer price indexes and real exchange rates. Pear consumption responds especially to apple consumption, but also to prices of apples and peaches, real per capita income, consumer price indexes and countries' exchange rates. This might imply improving commercial efficiency in international trade, effective budgets in price formation, and giving new impetus to studies on the price of fruits and foods with a new vision.

Keywords
pears • apples • peaches • CIF import price

1 Gabinete MAG: acrónimo de Miguel Angel Giacinti. Argentina. Centenario. Q8309. Argentina. * miguel.giacinti@gabinetemag.com
2 University of Beira Interior (Portugal). University Beira Interior (UBI). Est. Sineiro s/n 6200209 Covilha. Portugal. NECE: Research Center in Business Sciences, is a Department of Management and Economics the University of Beira Interior (UBI) pmanso@ubi.pt
3 University of Almeria. Cañada de San Urbano. 04120 Almería. Spain. jdepablo@ual.es

Resumen

Este documento tiene como objetivo evaluar los determinantes del precio de la pera en el mercado internacional, en la demanda en fresco; desde una visión innovadora en un mundo global y complejo. Se aplica la metodología de panel de datos. Las variables consideradas son los diferentes precios de importación (CIF/kg) de pera, manzana y duraznos; sus consumos per cápita, ingresos reales per cápita, índices de precios al consumidor y tasas de cambio de los países. Esto implica mejorar la eficiencia comercial en el comercio internacional, presupuestos efectivos en la formación de precios, además de dar un nuevo impulso a los estudios sobre el precio de las frutas y los alimentos con una nueva visión.

Keywords
peras • manzanas • duraznos • precio importación CIF

Introduction

The historical paradigm in the fruit market is that the main determinant of the sale price is the volume of supply of the same product. It responds to a simple model, with direct relationship, offer and price. Numerous studies provide information on price elasticity in fruits (2, 7, 12, 18, 19, 20, 30).

Also many studies indicate that demographic factors and economic growth also influence the consumption of fruits (1, 3, 5, 8, 11, 15).

Literature on the topic shows publications on the demand of apples and pears, at the level of individual countries and not globally (Vosloo and Groenewald 1969, Tunstal and Quilkey 1990 and Kavitha *et al.* 2016). Vosloo and Groenewald (1969) focused on the apple demand analysis in South Africa, where the availability of pears and oranges is considered as a factor explaining the price of apples.

On the other hand, Tunstal and Quilkey (1990), used the disappearance of storage pears to explain the average monthly price of apples in the Victorian wholesale market. Other investigations explain the link between prices of pear and apple, as is the case of (Wani *et al.* 2015). A significant change in the fresh fruit market can be driven by the emergence of new consumption preferences (4, 9, 17, 26, 27, 29).

This contrasts with the recent opinion of some commercial operators of the international fruit business, at least partially. They point out that the definition of price for pears is not based on their volume of supply, but on the price of late peaches at the beginning of the pears harvest, and then on the supply of apples for the rest of the season.

The discussion with people linked to the international trade of fruits (mainly pears and apples), highlight the importance of evaluating this new vision in the formation of the sale price in a globalized environment, thinking that perhaps changes in trade are evident and that currently they are not considered in a commercial planning. For an improvement in the efficiency of the value chain. Kevin Moffitt, Pear Bureau Northwest President (22), comments on a favourable opinion to relaunch the research on price behaviour.

This situation encouraged the project of a structural and comprehensive analysis on the determination of the main factors in the price of pears worldwide, combining the offer of both hemispheres -north and south. The novelty is the analysis based on world trade, in relation to other fruits -analysing peaches and apples- and economic variables of the main importing countries - per capita real income, price indexes and actual annual country exchange rates.

Materials and methods

Our analysis used yearly data from the period 1990-2015. The sample panel was composed of 18 countries, the main world importers in the international demand for fresh pears: Brazil, Canada, Denmark, France, Germany, India, Indonesia, Italy, Malaysia, Mexico, Portugal, Russian Federation, Saudi Arabia, Singapore, Spain, Sweden, UK and USA. China and Argentina are the main exporters, but have low relevance as importers worldwide. This research focused on demand factors from the main importing countries.

The variables considered for the analysis were pear prices (cif/kg), per capita consumptions of pears, apples and stone fruits, per capita real income, consumption price indexes and real annual country exchange rates (local currency per USD). The data source was the World Development Indicators (28). As usual, all the values were converted in their natural logarithms, to reduce variability, and were codified to facilitate data handling and computer processing. Logistics of perishable foods in the domestic market affects prices and consumer availability (23), for this reason choosing of the import price (cif: Cost Insurance and Freight) allowed avoiding asymmetries of wholesale and retail prices.

As stated by Greene (2012), Maddala, G. S. (2001), Hsiao *et al*. (1999), in statistics and econometrics, panel data (or longitudinal data) refers to multi-dimensional data frequently involving measurements over time, containing observations of multiple phenomena obtained over multiple time periods for the same firms, regions, countries or individuals. Time series and cross-sectional data are special cases of panel data that are in one dimension with only one panel member or individual for the former, one time point for the latter. Panel data analysis is a statistical method, generally used in social sciences, epidemiology, energy and econometrics, which deals with two and "n"-dimensional (in and by the cross sectional/times series time) panel data. The data are usually collected over time and over the same "individuals". Then a regression is run over these two dimensions. Multidimensional analysis is an econometric method in which data are collected over more than two dimensions (typically, time, individuals, and some third dimensions). A simplified common panel data regression model looks like

$$\mathbf{y_{it}=a+bx_{it}+\varepsilon_{it}}$$

where:
y = the dependent variable
x = the independent variable
a and b = the coefficients
i and t = indices for individuals and time.

The error ε_{it} is subject for hypothesis. Assumptions about this error term determine whether we speak of fixed effects or random effects. In a fixed effects' model, ε_{it}, is assumed to vary non-stochastically over i or t making the fixed effects model similar to a dummy variable model in one dimension. In a random effects' model, ε_{it} is assumed to vary stochastically over i or t requiring special treatment of the error variance matrix.

Panel data analysis has three more-or-less independent approaches: independently pooled panels, used as benchmark, random effects models and fixed effects models (or first differenced models). The selection between these methods depends upon the objective of the analysis, and the problems concerning the exogeneity of the explanatory variables.

The main assumption of the independently pooled panels is that there are no unique attributes of individuals within the measurement set, and no universal effects across time. On his turn, the key assumption of the fixed effect models (FEM), also known as Least Squares Dummy Variable Model (LSDVM), is that there are unique attributes of individuals that are not the results of random variation and that do not vary across time. To draw inferences only about the examined individuals, is adequate. The main statement of the random effect models (REM) is that there are unique, time constant attributes of individuals that are the results of random variation and do not correlate with the individual regressors. This model is adequate if we want to draw inferences about the whole population and not only the examined sample. The Durbin-Wu-Hausman or simply the Hausman specification test, is a statistical hypothesis test in econometrics named after James Durbin, De-Min Wu, and Jerry A. Hausman that evaluates the consistency of an estimator when compared to a less efficient alter- native estimator which is already known to be consistent. It helps to evaluate if a statistical model corresponds to the data.

Let $y=bX+e$ be a linear model

where:
y = the dependent variable
X = a vector of regressors
b = a vector of coefficients
e = the error term.

We have two estimators for b, b_0 and b_1. Under the null hypothesis, both estimators are consistent, but b_1 is efficient (has the smallest asymptotic variance), at least in the class of estimators containing b_0. Under the alternative hypothesis, b_0 is consistent, whereas b_1 is not. The Wu-Hausman statistic is defined as:

$$\mathbf{H = (b_1-b_0)^1(Var(b_0)-Var(b_1))^{\dagger}\,(b_1-b_0),} \tag{1}$$

where:
† = denotes the Moore-Penrose pseudo-inverse. Under the null hypothesis, this statistic has asymptotically the chi-squared distribution with the number of degrees of freedom equal to the rank of matrix Var(b_0) - Var(b_1). If we reject the null hypothesis, it means that b_1 is inconsistent. This test can be used to check for the endogeneity of a variable (by comparing instrumental variable (IV) estimates to ordinary least squares (OLS) estimates). It can also be used to check the validity of extra instruments by comparing IV estimates using a full set of instruments Z to IV estimates that use a proper subset of Z. Note that for the test to work in the latter case, we must be certain of the validity of the subset of Z and that subset must have enough instruments to identify the parameters of the equation. Hausman, also showed that the covariance between an efficient estimator and the difference of an efficient and inefficient estimator is zero.

The advantages and disadvantages of panel data models can also be seen in the referred literature (6, 10, 13, 16).

Results

The explanatory power of the estimated models, were highly and statistically significant. The Hausman test showed that, among the three cited models (table 1), the random effects model (EGLS Panel), was the best for studying this complicated international food market.

However, the type of effects and the statistical significance of the coefficients were concordant in the three considered models. Using the results of the estimation based on the EGLS Panel estimation method, the Hausman test proved to be the best solution in the current situation (table 2, page 230).

Table 1. Results of the three panel data model estimates.

Tabla 1. Resultados de las estimaciones de los tres modelos de panel de datos.

Variable/model	Fixed	Random	Pooled
Method of estimation	Panel GLS	Panel (EGLS)	Panel LS
lpearpr_cifkg	-0.346024 *	-0.348696 *	-0.520346 *
lappleprcif_kg	0.177930 **	0.176639 **	0.069696
lapplepc_cons	0.968417 *	0.969268 *	1.008693 *
lc_pr_index	0.053387 *	0.053628 *	0.097675 *
lexch_rate	-0.122623 **	-0.119824 ***	0.244522 ***
lreal_pc_income	0.076901***	0.076756 **	0.070982 *
lst_fr_prcifkg	0.126818 **	0.131625 **	0.796797 *
lsw_fr_prcif_kg	-0.128052	-0.126397	-0.305942 *
C	-1.651984 *	-1.678863 *	-3.739812 *
R-squared	0.974204	0.727621	0.805164
Adjusted R-squared	0.972705	0.722746	0.801677
S.E. of regression	0.202179	0.201273	0.544976
Sum squared resid	1.757.678	1.492.618	1.327.586
Log likelihood	9.531.239		-3.656.929
F-statistic	649.5805 *	149.2618 *	230.905 *
Prob (F-statistic)	0.0000	0.0000	0.0000
Durbin-Watson stat	1.155.552	1.123.151	0.2616
LR test – Redundant fixed effects			
Cross-section F df: (17,430)	165.753954 *		
Cross-section Chi-squared (17)	922.010666 *		
Correlated Random Effects - Hausman Test			
Chi-Sq. Statistic (df=8)		3.874.579	
Prob (Ch-sq)		0.8683	

Notes: *, **, ***, statistically significant. at 1%,5%,10%, respectively. Our own estimation using Eviews (v.9).

Notas: *, **, ***, estadísticamente significativo al 1%,5%,10%, respectivamente. Nuestra propia estimación utilizando Eviews (v.9).

Table 2. Hausman test comparisons (Fixed/Random).
Tabla 2. Comparación del test de Hausman (Fijo/Aleatorio).

Cross-section random effects test comparisons				
Variable	**Fixed**	**Random**	**Var (Diff.)**	**Prob.**
lpearpr_cifkg	-0.346024	-0.348696	0.000086	0.7739
lappleprcif_kg	0.177930	0.176639	0.000023	0.7861
lapplepc_cons	0.968417	0.969268	0.000056	0.9098
lc_pr_index	0.053387	0.053628	0.000004	0.8986
lexch_rate	-0.122623	-0.119824	0.000030	0.6084
lreal_pc_income	0.076901	0.076756	0.000221	0.9922
lst_fr_prcifkg	0.126818	0.131625	0.000015	0.2215
lsw_fr_prcif_kg	-0.128052	-0.126397	0.000188	0.9039

Notes: Our own estimation using Eviews (v.9). / Notas: Nuestra propia estimación utilizando Eviews (v.9).

All the coefficients or elasticities were statistically significant, four of them at the 1% level of significance, three at 5% and one at 10%. The only coefficient or elasticity that was not significant in statistical terms at the usual levels of significance is the price (cif) of stone fruits. Possibly explained, by the influence of late variety stone fruit that impacts the beginning of the pear harvest.

The overall regression (REM) was highly significant in statistical terms (Prob (F-stat) = 0.0000). The explicative power of this model (random effects model) was 0.72 and highly significant since F-stat=149.26 and Prof (F-stat=0.0000), while in the fixed effects the R-squared is very high (97.4%), and significant (F-Stst=649.58 and Prob (F-statistic) = 0.0000. We had to be cautious about these interpretations since some possibilities of having autoregressive errors taking in account the Durbin-Watson test (Durbin-Watson, d=1.156) were present. However, we did not suspect of heterocedasticity, nor of multicolinearity among the explicative factors considered using the classic test and the correlation's test.

We did not reject the endogeneity problem since we could not reject the null hypothesis (chi-squared stat=3.87, d.f. = 8, Prob (ch-sq) = 0.87) stating that the regressors were correlated with the model errors. As referred before, the Hausman test (table 2) suggested that the best model to identify and measure the explicative factors of the demand for pears was the random effects' one. Furthermore, the Likelihood Ratio (LR) test applied to the results of the fixed effects model suggested the rejection of the redundancy of the different sections.

With these results, we got empirical evidence that pear consumption is positively associated with the prices of apples (elasticity=0.178**), with per capita apple’s consumption (elasticity=0.968*), with the average index price (elasticity=0.053*), with real per capita income (elasticity=0.077***) and with the price of stone fruits (elasticity=0.127**).

On the other hand, pear consumption was negatively correlated both with pear price (cif) (elasticity = -0.346 *) and exchange rate (elasticity = -0.123 **). The demand elasticities of the factors for pears could be classified as rigid since all of them have absolute values less than 1, suggesting that there was no much scope to intervene in the market. Besides pear consumption, price-response to its own price and to the price of fruits like apple and stone fruits should be expected. Pear consumption is especially and moderately responsive to apple consumption (0.968) (the highest elasticity consumption).

The explanatory power of the estimated models, were highly and statistically significant. The Hausman test showed that, among the three cited models, the random effects model, was the most indicated to study this complicated international food market. However, the type of effects and the statistical significance of the coefficients were concordant in the three considered models.

Tunstal and Quilkey (1990) reached the same conclusion established in this work when studying the relationship between pears and apples.

Conclusions

The analysis adds empirical evidence to the fact that the consumption of pear responds especially to the consumption and the price of apple. Other determinants of the demand for pears are the set of selling prices for apples and stone fruits (for example, nectarines and peaches at the beginning of the pear harvest), and the real per capita income. The synergy between pears and apples in the fresh produce market is an important conclusion and a feature that confirms that pear sellers generally also sell apples and vice versa.

The result of the research is relevant to the pear exporting producers and companies (Argentina, the world's leading exporter), as well as to academics linked to the international trade of fresh fruits; providing knowledge that may increase efficiency in the value chain.

Surely, this research will change the paradigm in the fresh pear business in general (confirming the opinion of those linked to foreign trade. It will also boost research on the price elasticity with a new global approach and complex vision in the fruit and other foods market.

References

1. Abler, D. 2010. Demand growth in developing countries. OECD Food, Agriculture and Fisheries Papers. No. 29. OECD Publishing, Paris. https://doi.org/10.1787/5km91p2xcsd4-en.
2. Arnade, C. Pick, D. 1998. Seasonality and unit roots: the demand for fruits. Agricultural Economics. 18(1): 53-62.
3. Burell, A.; Henningsen, A. 2001. An empirical investigation of the demand for bananas in Germany. Agrarwirtschaft. 50(4): 242-248.
4. Carrillo-Rodriguez, L.; Gallardo, K.; Yue, C.; McCracken, V.; Luby, J.; Mcferson, J. R. 2013. Consumer preferences for apple quality traits. In 2013 Annual Meeting.
5. Cramer, J. S. 1973. Interaction of income and price in consumer demand. International Economic Review. p. 351-363.
6. Dawe, D. C.; Morales-Opazo, C.; Balie, J.; Pierre, G. 2015. How much have domestic food prices increased in the new era of higher food prices. Global Food Sec. 5: 1-10.

7. Durham, C.; Eales, J. 2010. Demand elasticities for fresh fruit at the retail level. Applied Economics. 42(11): 1345-1354.
8. Epstein, L. L.; Handley, E.; Dearing, K.; Cho, D.; Roemmich, J.; Paluch, R.; Raja, S.; Pak, Y.; Spring, B. 2006. Purchases of food in youth: influence of price and income. Psychological Science. vol. 17(1): 82-89.
9. Gallardo, R.; Hong, Y.; Jaimes, M.; Flores, J. 2018. Investigating consumer food choice behavior: An application combining sensory evaluation and experimental auctions. Ciencia e Investigacion Agraria. 45(1): 1-10.
10. Greene, W. 2012. Econometric Analysis (7th ed.). Pearson. p. 234-237.
11. Gwynne, Robert N. 1999. Globalisation, commodity chains and fruit exporting regions in Chile. Tijdschrift voor economische en sociale geografie. 90(2): 211-225.
12. Harker, F. R.; Gunson, F. A.; Jaeger, S. R. 2003. The case for fruit quality: an interpretive review of consumer attitudes, and preferences for apples. Postharvest Biol Technol. 28: 333-347.
13. Hsiao, C.; Lahiri, K.; Lee, L.; Pesaran, M. H. 1999. Analysis of panels and limited dependent variable models. Cambridge: Cambridge University Press.
14. Kavitha, V.; Umanath, M.; Paramasivam, R.; Chandran, K. 2016. Determinants of consumption probability and demand for fruits in India. Agricultural Economics Research Review. 29: 161-170.
15. Kyriakidi, A.; Drichoutis, A. 2007. Determinants of demand for fruit variety. 3rd HO PhD Symposium on Contemporary Greece. London. UK.
16. Maddala, G. S. 2001. Introduction to Econometrics (3rd. ed.). New York: Wiley.
17. Millichamp, A.; Gallegos, D. 2013. Comparing the availability, price, variety and quality of fruits and vegetables across retail outlets and by area-level socio-economic position. Public health nutrition. 16(1): 171-178.
18. Prais, S. J. 1962. Econometric research in international trade: a review. Kyklos. 15(3): 560-574.
19. Price, D. W.; Mittelhammer, Ronald, C. A. 1979. Matrix of Demand Elas: ticities for Fresh Fruits. Western Journal of Agricultural Economics. p. 69-86.
20. Tozer, P.; Marsh, T.; Jíang, M. 2014. Perennial Supply-Substitution in Bearing Acreage decisions. En 2014 Annual Meeting. Agricultural and Applied Economics Association, Minneapolis, Minnesota.
21. Tunstall, A. W.; Quilkey, J. J. 1990. Storage and pricing of apples: Some empirical evidence on the structure of the Victorian wholesale market. Australian Journal of Agricultural Economics. 34(3): 280-291.
22. US PEARS 2017. The Pear Bureau Northwest of United States Available in: (https://usapears.org/)
23. Valdes Salazar, R. 2018. Measuring market integration and pricing efficiency along regional maize-tortilla chains of Mexico. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 279-292.
24. Vosloo, J. J.; Groenewald, J. A. 1969. The demand for apples in South Africa-a statistical analysis. Agricultural Economics Research. Policy and Practice in Southern Africa. 8(4): 21-25
25. Wani, M. H.; Sehar, H.; Paul, R. K.; Kuruvila, A.; Hussain, I. 2015. Supply response of horticultural crops: the case of apple and pear in Jammu & Kashmir. Agricultural Economics Research Review. 28(1): 83-89. DOI: 10.5958/0974-0279.2015.00006.3
26. Watson, J.; Wysocki, A.; Gunderson, M.; Brecht, T.; Sims, C. 2012. Fresh hammock-packed bartlett pears: implications for marketing based on consumers' willingness to pay for sensory attributes and return on invesment potential. Annual Meeting. Agricultural and Applied Economics Association. Seattle. Washington.
27. Wismer, W. V. 2014. Consumer eating habits and perceptions of fresh produce quality. Postharvest Handling. 31-52.
28. WORLD BANK 2016. World Economic Indicators. World bank databases.
29. Yaseen, A.; Mehdi, M.; Somogyi, S.; Ahmad, B. 2016. Consumer preferences to pay a price premium for quality attributes in Pakistani grown mangoes. Pakistan Journal of Commerce and Social Sciences. 10(3): 615-637.
30. You, Z.; Epperson, J.; Huang, C. 1996. A composite system demand analysis for fresh fruits and vegetables in the United States. Journal of Food Distribution Research. 27: 11-22.

Olive oil and the millennial generation in Chile. What do these consumers consider when buying this product?

Aceite de oliva y la generación del milenio en Chile. ¿Qué consideran estos consumidores cuando compran este producto?

Marcos Mora [1*], Berta Schnettler [2], Germán Lobos [3], Cristian Geldes [4], Sofía Boza [1], María del Carmen Lapo [5], Ruth Paz [6]

Originales: *Recepción:* 03/05/2019 - *Aceptación:* 25/10/2019

Abstract

This study aimed to identify the attributes influencing the purchase decision process of the olive oil consumer belonging to "Millennials" in the Metropolitan Region, Chile. The method includes data collection from 408 people and a combination of factorial and cluster analysis. The results identified two segments in the millennial consumers. In the purchasing, one of the consumer groups, with 150 people, attached importance to product information and origin, as well as oil category, colour, and acidity. The other group with 258 people rejected a large part of the attributes, except for that related to electronic commerce. These results suggest that the search for attractive attributes in a broad sense is relevant and should be considered in the development of commercial strategies for the segment that positively values all attributes. However, for that segment rejecting intrinsic attributes, it would be advisable to deepen on aspects related to technology and social network.

Keywords
oil olive • consumer • segmentation • millennials • Chile

1 Universidad de Chile. Santa Rosa 11.315. Faculty of Agricultural Sciences. Department of Management and Rural Innovation. La Pintana Santiago. Chile. C. P. 8820808. * mmorag@uchile.cl
2 Universidad de la Frontera. Faculty of Agricultural and Forestry Sciences. Avda. Francisco Salazar 01145. Temuco. Chile. C. P. 4780000.
3 Universidad de Talca. Faculty of Business and Economics. C. P. 3465548 Talca. Chile. Visiting Professor. Universidad Católica de Santiago de Guayaquil (UCSG) in Ecuador.
4 Universidad Alberto Hurtado. Faculty of Economics and Business. Department of Management and Business. Erasmo Escala 1835. office 204. Santiago. Chile Postal Code 8340539.
5 Universidad Católica de Santiago de Guayaquil. Associate Professor. Ecuador. Address: Av Carlos Julio Arosemena Km 1.5. C. P. 090615.
6 Universidad de Chile. Program of Doctorate in Agricultural Sciences. Santa Rosa 11.315. La Pintana Santiago. Chile. C. P. 8820808.

Resumen

Este estudio tiene como objetivo identificar los atributos que influyen en el proceso de decisión de compra del consumidor de aceite de oliva perteneciente a "Millennials" en la Región Metropolitana, Chile. El método incluye la recolección de datos de 408 personas y una combinación de análisis factorial y de conglomerados. Los resultados identifican dos segmentos en los consumidores milenarios. En la compra, uno de los grupos de consumidores, con 150 personas, da importancia a la información y al origen del producto, así como a la categoría de aceite, al color y a la acidez. El otro grupo con 258 personas rechaza gran parte de los atributos, excepto el relacionado con el comercio electrónico. Estos resultados sugieren que la búsqueda de atributos atractivos en sentido amplio es relevante y debe ser considerada en el desarrollo de estrategias comerciales para el segmento que valoren positivamente todos los atributos. Sin embargo, para ese segmento que rechaza los atributos intrínsecos, sería aconsejable profundizar en aspectos relacionados con la tecnología y las redes sociales.

Palabras clave
aceite de oliva • consumidor • segmentación • millenials • Chile

Introduction

The "Millennials" or "Generation Y" is an increasing market that prefers healthy products, such as olive oil. In Chile, this generation is about 5,5 million people (31% of the population). Reasons to understand this segment of the market in order to develop commercial strategies for companies, as well as designing public policies related to the promotion of healthy food like olive oil are strategic. Thus, the objective of this study is to know what the Chilean Millennial consumers consider when buying olive oil.

Meanwhile, since the year 2000, the olive oil industry has been extraordinarily developed. Olive oil is a recognized product for its health benefits (29), in line with the preferences of "generation Y", including health fact like fat content, and animal or plant origin (26). Moreover, consumer preferences vary among countries. For instance, in Southern Europe, olive oil is widely known and mainly consumed for its health benefits (48, 50, 59). However, there is a relevant gap for market development in emerging countries like Chile, where the use of this product has been increasing progressively among the population, showing special concern about healthy diets (7). Between 2005 and 2015, olive oil consumption in Chile almost doubled, going from 204 grams to over 390 grams (8). Meanwhile production has been significantly growing from 2005 to 2014, going from 2,000 t to 18,500 t (9). Specifically, in the case of the Chilean market, preferences are driven to extra virgin oil in glass bottles, in less-than-one litre formats and fruity flavoured. About the usage given to the oil in this market, it is mostly chosen for salads, whereas the European market uses it in all types of consumption, including cooking and frying. In addition, in Chile, olive oil has a known value in relation to health benefits (48, 50, 59). There is a relevant gap of market development in emerging countries like Chile, where the use of this product has been increasing progres- sively among the population, especially showing more concern about healthy diets (7, 21). Between 2005 and 2015, olive oil consumption in Chile almost doubled, going from 204 grams to over 390 grams (8). While production has been significantly growing from 2005 to 2014, going from 2,000 t to 18,500 t (9). Specifically, in the case of the Chilean market, preferences are driven to extra virgin oil in glass bottles, in less-than-one litre formats and fruity flavoured. About the usage given to the oil in this market, it is mostly chosen for salads, whereas the European one uses it with all types of consumption, including cooking and frying. In Chile, olive oil shows a higher income elasticity of demand than other oils (54). Therefore, the increase in demand in this country is somewhat conditioned by people's purchasing power. On the other hand, the demand between 2006 and 2015 has shown a trend toward decreasing imported oil consumption due to a preference for national oils (9). In Chile, 80% is locally produced, whereas 20% is imported mainly from Argentina and Spain (8).

Given the aforementioned, and since by the year 2020, Millenial will constitute a worldwide market of 200 billion dollars annually it might be relevant to learn these specific consumers (61). In that sense, some researchers consider that "generation Y" is made up of people born between 1980 and 2000 (17). One generation corresponds to a cohort of people born over 20 years, sharing beliefs, attitudes and behaviours, while feeling members of the same generation, and having experienced significant historical events and trends at the same stage of life (63). According to Taylor and Cosenza (2002), most of the millennial generation is already inserted into the workforce and has extensive technological knowledge, which makes them important consumers (62). This point suggests that the "online" market may be thriving in addressing these types of customers (9).

Moreover, to understand the millennials it is necessary to keep in mind that they have dealt with big, exciting, and dynamic changes during their childhood and youth, such as virtual business opportunities and gender equality. They also consider the opinion of non-governmental organizations and groups for their decision-making process (53). In the Chilean case, the Millennial generation is characterized by their appreciation of economic aspects as well as social aspects like social network and pleasant workplaces, among others. Pincheira and Arenas (2016), report that their main characteristics are dependent on social network and access to technology (51).

The industry of olive oil in Chile

The Chilean olive oil industry has had sustained growth, from the nineties to the present. Domestic and export demand-supply have increased (8). This is partly explained because olive oil is considered an essential food in the Mediterranean Diet, and it has been associated with a significant contribution to people's longevity as well as the prevention of heart diseases, diabetes, obesity, and cancer (44, 48).

Attributes olive oil considered by consumers

The description of attributes considered in the consumption of olive oil is based on the "multi-attribute" classification, which conceives the quality of a product as a set of attributes that are separated into "intrinsic" and "extrinsic" signals (25, 35, 41, 49, 65, 66). Intrinsic signals allow the objective measurement of quality. These qualities impregnate the product with its functionality and are related to its physical appearance, are specific to each product, disappear when they are consumed (49), and cannot be altered without changing the nature of the product itself (3). On the other hand, extrinsic attributes have a more or less close relationship with the product, but remain, by definition, outside their essence (49). Cheng *et al.* (2008) consider, totally or partially, as extrinsic attributes the price, the brand, and the advertising (28). Other extrinsic attributes are the date of manufacture and consumption, place of origin of the product and production techniques (6, 28). Specifically, in the case of olive oil, Del Giudice *et al.* (2012), refer to both types of attributes. The extrinsic attributes are, for example, certification of origin of olive oil, safety of the associated product to the production method (organic system and traceability), commercial brand and price (16). On the other hand, intrinsic attributes are mainly the flavour and colour. In Chile, Romo *et al.* (2015) suggested a positive relationship between acidity (intrinsic attribute) and the oil quality as well as between the latter and the origin. They also raised an inverse relationship between payment arrangement and olive oil in plastic bottle (55). Regarding the presented background, this investigation had the following hypothesis (H1): It is possible to identify two or more segments of Chilean consumers in the millennial generation who consider different attributes in the purchase of olive oil

Materials and methods

Data description

The study site was the "Commune of La Florida" (local and administrative area) in the Metropolitan Region of Chile. It has a total population of 397,456 habitants, with 198,706 males and 198,750 females (30). This area is constituted of four main sectors: "San José de la Estrella", "Santa Raquel", "La Florida", and "Lo Cañas". These sectors show different socioeconomic groups allowing to characterize this area as multiclass commune with a heterogeneous population (52).

The sample was selected by "convenience", and it drives to a population segment including 468 Millennials resident in the "Commune of La Florida". From this sample, 408 (87.2%) people declared usual or sporadic consumption of olive oil. The remaining 60 people (12.8%) do not consume olive oil. Consequently, the valid sample number of consumers for the analysis was 408 persons. The survey was conducted face-to-face by stopping people in different places of the Commune of La Florida, between March and June 2018. Convenience sampling has been widely used for market research of different products (18, 26, 37).

Methodological approach and empirical model

In the first instance, descriptive statistics were obtained, including absolute and relative frequency analysis of sociodemographics, purchasing habits, consumption of olive oil, use, and preferences according to the olive oil category. Then a Factor Analysis with maximum likelihood as method of extraction was applied to the statements related to purchasing attributes of olive oil consumers. This is a multivariate analysis technique used to study and interpret the correlations between a group of variables, in which the correlation is due to common factors whose objective is to identify those common factors. At the same time, it seeks to reduce data provided by a correlation matrix making it easier to explain without excessive data loss (48). To validate the former, the dimensionality reduction model required the following conditions: factorial loads (observed component/ variables) over 0.5 (64), constructs made by at least 3 observed variables (31, 32), Cronbach´s Alpha over 0.7 (23), total variance explained over 60% (27) and KMO index over 0.6 (19). Subsequently, those variables obtained by Factor Analysis were analyzed by Cluster Analysis obatining the market segmentation variable, similarly to that used in Irish wine consumers (22), Tunisian and French olive oil consumers, and Chinese wine consumers (11).

This technique allows analysing a set of variables classifying those of maximum homogeneity within the group and maximum heterogeneity among the groups (48).

RESULTS

From the total, 51.7% corresponded to females, whereas 48.3% corresponded to males, a similar result to that reported by National Institute of Statistics - INE (30) about the sociodemographic distribution of the Metropolitan Region. Most respondents were between 31 and 36 years old, corresponding to 41.4% followed by those between 25 and 30 years old with 40.2% and then those between 18 and 24 years old with 18.4% (table 1).

Descriptive background of olive oil consumption in Chilean Millenials

From all the respondents, 87.2% declared that they consumed olive oil, whereas 12.8% claimed they did not. This result is similar to that reported by Matsatsinis *et al.* (2007) for Greek consumers. The difference lies in the frequency of consumption and the consumed quantity, which are higher among the Greeks than the Chileans (36).

From all surveyed consumers of olive oil, 51.1% claimed to do it daily, and 28.2% claimed to do it weekly, which is in accordance with the data obtained in prior studies in Chile (40, 42). Regarding the frequency of olive oil purchase, this is mostly done monthly, with 69.8% (40).

The quantity of olive oil oftenly purchased per occasion by respondents is 500 cubic centimetres (cc) (46.8%). This is similar to that reported by Mora and Magner (2008). Additionally, 13.5% of people buy 1,000 cc formats while 3.2% of person buy-in formats over 1,000 cc. This result is different from that reported by Metta and Guinard (2010) about American consumers with a higher consumption frequency of 1,000 cc (1 Litre) (39).

Table 1. Age and gender of the respondents.

Tabla 1. Características de género y edad de los encuestados.

Gender	Frequency	Percentage
Female	211	51.7%
Male	197	48.3%
Age		
19-24	75	18.4%
25-30	164	40.2%
31-36	169	41.4%

The category of olive oil most frequently consumed by all the respondents was that of extra virgin olive oil with 71.8%, similar to that reported for Greek consumers (36).

Attributes of olive oil purchasing.

A Factorial Analysis was applied to identify the attributes in olive oil purchasing. Initially, the analysis considers 12 observed variables as attributes related to the purchasing process, but only 8 observed variables are maintained. The discarded attributes corresponded to the "trademark", the "system of organic production", "olive variety", and "price". All of them presented correlations with the factor under 0.3. Price, was isolated only by one factor, and consequently excluded from the factor model. The KMO index was 0.76, which is considered acceptable (19). Table 2 shows how the attributes considered when purchasing olive oil can be explained by two groups or "factors" of attributes. The former - called "Extrinsic Attributes" factor included "information at the point of sale", "electronic advertising and offers", "internet sales", and "product origin", being those related to "electronic commerce", the ones that showed negative correlations with the component.

These attributes related to electronic commerce have been considered an important factor by olive growing companies, since the image of the product on the internet is considered essential to boost sales (38). This factor, with 29.9% of variance, was explained by 4 observed variables, with a Conbrach's Alpha of 0.73. The other factor, called "Intrinsic Attributes" included "olive oil category", "acidity", "flavour", and "colour". This factor, representing 27.7% of the variance was explained by 4 observed variables and had a Conbrach's Alpha of 0,74, considered acceptable by literature (2, 23, 43).

Considering the results of Factor Analysis in table 2, a hierarchical Cluster Analysis was applied to obtain two consumer segments (table 3, page 239).

Table 2. Oil olive: Attributes/Dimensions related to purchase decision process.

Tabla 2. Aceite de oliva: atributos / dimensiones relacionadas con el proceso de decisión de compra.

Statement	Extrinsical Attributes	Intrinsical Attributes
Information at the point of sale	**0.80**	0.11
Electronic advertising & offers	**-0.75**	0.02
Internet sales	**-0.70**	0.24
Product origin	**0.68**	0.09
Category of extra-virgin olive oil	0.09	**0.78**
Acidity	0.37	**0.74**
Flavour	0,32	**0,72**
Colour	0.05	**0.69**
Variance explained by factor (%)	33.93%	27.74%
Accumulated variance (%)	33.93%	61.67%
Cronbach's Alpha	0.73	0.74

KMO: 0.76. Varimax Rotation. * Values in columns show factorial loads (factor correlation).

0,76. Rotación varimax. * Los valores en las columnas muestran las cargas factoriales (correlación de factores).

Table 3. Segment characterization based on factors influencing olive oil purchase.

Tabla 3. Caracterización de segmentos basada en factores que influyen en la compra de aceite de oliva.

	Group 1 (n=258)	Group 2 (n=150)	F	Sig.
Extrinsic Attributes (Information at the point of sale, Electronic advertising & offers, Internet sales and Product origin)	-0.340	0.585	86.316	0.000
Intrinsic Attributes (Category of extra-virgin olive oil, Acidity Flavour and Colour)	-0.470	0.808	213.205	0.000

Group 1: "Non- Traditional or Millennials Consumers" (n=258). This result is the biggest of both groups, mainly formed by people between 30 and 35 years old. This segment was characterized by having a negative attitude towards the olive oil origin.

This is similar to that reported for French and Tunisian consumers, where one of the three segments detected showed an unfavourable tendency upon the origin attribute (11). Additionally, it showed negative attitudes upon information at the point of sale and upon intrinsic attributes, such as acidity and flavour, which could be associated with ignorance of the product. On the contrary, it showed positive attitudes upon those attributes related to electronic commerce. It is important to mention that a significant number of consumers in this segment have a daily consumption frequency, mainly used to dress salads and usually purchased monthly at supermarkets (over 96% of the segment) (table 4, page 240).

Group 2: "Traditional Consumers" (N=150). This group was mainly formed by people between 24 and 35 years old, representing 36.7% of the sample, declaring that they consumed olive oil. The main characteristic of this segment was their favourable attitude to consider intrinsic attributes in their purchase, such as category, acidity, flavour and colour. Along with a positive attitude towards advertising at points of sale, which is in line with that reported by Adasme *et al.* in 2013 concerning vegetable consumption (1). Just as the previous group, this segment mainly showed daily consumption and monthly purchases at supermarkets (table 4, page 240).

There were no significant differences in any segment in relation to daily consumption and considering olive oil as an expensive product. More than 90% of surveyed consumers considered olive oil to be expensive and declared to have a daily consumption.

Table 4. Segment characterization based descriptive aspects of olive oil consumption.
Tabla 4. Caracterización de segmentos basados en aspectos descriptivos de consumo de aceite de oliva.

	Frequency Group 1(n=258)	%	Frequency Group 2 (n=150)	%
How often do you buy olive oil? p= 0.029				
Weekly	6	2.3%	1	0.7%
Monthly	163	63.2%	115	76.7%
Occasionally	89	34.5%	34	22.7%
	258	100.0%	150	100.0%
How do you use olive oil? p= 0.050				
Just salads	180	69.8%	85	56.7%
Salads & fried foods	40	15.5%	25	16.7%
Cooking in general	38	14.7%	40	26.7%
	258	100.0%	150	100.0%
Where do you usually buy olive oil? p= 0.003				
Supermarket	248	96.1%	130	86.7%
Specialized Shop	5	1.9%	6	4.0%
Internet	5	1.9%	3	2.0%
Grocery store/Olive mill	0	0.0%	11	7.3%
	258	100.0%	150	100.0%

Significant differences, p<=0.05, with Pearson's Chi-squared Test.
Diferencias significativas, p<=0,05, mediante Test de Chi-cuadrado.

Discussion

What do "Millennials" consumers of oil olive consider when purchasing in the Metropolitan Region, Chile? That was the central question of this survey. The smallest group of olive oil consumers (n=150) declared to consider oil category in the purchase. This preference is in line with that reported by Mtimet *et al.* (2013), who determined that the Tunisian consumers' favourite type of oil is extra virgin followed by virgin olive oil (46). Bernabéu *et al.* (2009) obtained similar results in Spanish consumers.

About the preferred purchase format of the consumers of this survey, our results agree on those found by Santosa and Guinard (2011), who determined that consumers of extra virgin olive oil from the State of California, USA, preferred purchase formats of 750 cc and 500 cc (58). According to the authors, this result could be related to the fact that extra virgin olive oil market in the USA is an emerging market, which is relatively similar to the case of the Chilean market. Additionally, In Chile olive oil is considered as a valuable good because there is a direct relationship between consumers' income and quantities of olive oil demanded (54). The most usual purchase place is the supermarket, which is like that reported for Greek consumers (34).

Regarding the Cluster Analysis, two groups of consumers were found. Group 1 called "Non- Traditional or Millennials Consumers", included the most significant number of consumers with 258 respondents. Group 2 called "Traditional Consumers", included 150 people. The main differences between both groups were centered upon those attributes considering intrinsic characteristics, such as acidity, colour and flavour, and those extrinsic ones, such as online advertising and offers and internet sales.

When purchasing olive oil, Group 1 considered attributes associated with electronic commerce instead of intrinsic ones. About flavour, literature points out a new generation of extra virgin olive oil consumers that seem to prefer a product with a quite simple and neutral flavour, thus generating an important market division (12, 13, 16, 45, 46, 57). For this group of consumers, since they value the intrinsic attributes of olive oil, the attitude towards the product could be improved if information about it was provided, as suggested by Salazar-Ordonez *et al.* (2018), for Spanish consumers.

Group 2 evaluated intrinsic attributes at the moment of purchasing, especially those of flavour and colour, which is similar to that reported by Del Giudice *et al.* (2012). These attributes had been used in the past (16), possibly influenced by the previous generation. This group also considered product origin and information provided at the point of sale. Besides, consumers from Group 1 did not appreciate attributes like origin and information at the point of sale, which could be associated to inadequate knowledge or appreciation of the product. In relation to similarities, both groups considered olive oil to be expensive. In this context, the research made by Dekhili and d'Hauteville (2009) reported that price is the most critical free -choice attribute when choosing olive oil in Tunisianand France (10). Moreover, Delgado and Guinard (2011) determined that price, available information, and the prestige of AOEV (Extra-Virgin Origin Appeal) were crucial factors encouraging purchases of the American survey respondents (12).

Regarding origin, both the studies in Spanish consumers (4), and in northern California consumers of extra-virgin olive oil (58), determined that origin was an important attribute, preferring those olive oils of national origin in Spain as much as in the USA. This result agrees on that found in Segment 2, although this attribute is not considered in Segment 1. Origin of olive oil is positively considered by the consumers of Segment 2 and could work as a driving force to choose this product (11, 60).

Trademark is an important attribute when purchasing olive oil in traditionally producing countries, such as Italy (14, 15) and Spain (20, 23). However, the results obtained in Chilean consumers concerning trademark was not associated with purchasing. This situation is related to the relatively recent development of the olive oil market in Chile.

Finally, it would be interesting to include, in a future research other attributes, such as "packaging design". This attribute should be addressed for millennial consumers, since it is considered as an important factor in non-technological innovation in the Agri-food industry (24).

Conclusions

The results obtained suggest that the decision to purchase olive oil in the surveyed consumers can be explained by intrinsic ("category of oil", "flavour", and "colour") and extrinsic attributes ("information at the point of sale", "electronic advertising & offers", "internet sales", and "product origin").

The results allow establishing two groups or segments of the consumer in Generation Y. There is a group of consumers of a traditional profile, with a similar behaviour to generation X or older ones. This group considers attributes, such as colour, flavour, acidity, oil category, information at the point of sale, and oil origin at the moment of purchase. The other group is a more typical Millennials with a high appreciation of online communication and a rejection of attributes considered by more traditional consumers.

The results indicate the need to develop specific marketing strategies for this generation, emphasizing the creation of product knowledge, related to information on the characteristics of the olive varieties used in oil production, olive oil tastings, and, above all, distribution and communication channels linked to social networks and the Internet. Emphasis should be made in tuning with the requirements of each market segment.

References

1. Adasme-Berríos, C.; Jara-Rojas, R.; Ramos-Cabello, B.; Rodríguez, M.; Mora, M. 2013. Consumer responses to agricultural produce advertising in the O'Higgins Region of Chile. Cienc. Inv. Agr. 40 (1). Available in: http://dx.doi.org/10.4067/S0718-16202013000100003
2. Adasme-Berríos, C.; Sánchez, M.; Mora, M.; Díaz, J.; Schnettler, B.; Lobos, G. 2019. Effects of food-related health concerns and risk perception on the consumption frequency of fresh vegetables. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(2): 289-30.
3. Ardeshiri, A.; Rose, J. 2018. How Australian consumers value intrinsic and extrinsic attributes of beef products. Food Quality and Preference. 65: 146-163.
4. Bernabéu, R.; Olmeda, M.; Díaz, M.; Olivas, R. 2009. Oportunidades comerciales para el aceite de oliva de Castilla-La Mancha. [en línea]. Universidad de Castilla La Mancha. p. 10.
5. Cardona, J.; Paredes, M. 2014. Segmentación de mercados. Revista Académica Facultad de Ciencias Económicas y Empresariales. N° 10.
6. Cavallo, C.; Piqueras-Fiszman, B. 2017. Visual elements of packaging shaping healthiness evaluations of consumers: The case of olive oil. J Sens Stud. 32: e12246. Available in: https://doi.org/10.1111/joss.12246
7. Cheng, J. M. S.; Wang, E.; Lin, J. Y. C.; Chen, L. S.; Huang, W. H. 2008. Do extrinsic cues affect purchase risk at international e-tailers: The mediating effect of perceived e-tailer service quality. Journal of Retailing and Consumer Services. 15: 420-428.
8. CHILEOLIVA. 2016. Informe anual mercado nacional de aceite de oliva. Available in: https://www.chileoliva.cl/wp-content/uploads/2017/04/BOLMERC1SEM2016.pdf. (Date of consultation: 17/10/17).
9. Dawn, V.; Thomas, P. 2013. Generation y value and lifestyle segments. USA. Journal of Consumer Marketing, 30: 7.
10. Dekhili, S.; d'Hauteville, F. 2009. Effect of the region of origin on the perceived quality of olive oil: An experimental approach using a control group. Food Quality and Preference. 20: 525-532.
11. Dekhili, S.; Sirieix, L.; Cohen, E. 2011. How consumers choose olive oil: The importance of origin cues. Food Quality and Preference. 22: 757-762.
12. Delgado, C.; Guinard, J. X. 2011. How do consumer hedonic ratings for extra virgin olive oil relate to quality ratings by experts and descriptive analysis ratings? Food Qual Preference. 22(2): 213-225.
13. Delgado, C.; Guinard, J. X. 2012. Internal and external quality mapping as a new approach to the evaluation of sensory quality-a case study with olive oil. J Sens Stud. 27(5): 332-343.

14. Del Giudice, T.; D'Elia, A. 2001. Valorizzazione dell' olio extravergine di oliva meridionale: Una proposta metodologica per l'analisi delle preferenze. Rivista di Economia Agraria. 56(4): 11-42.
15. Del Giudice, T; Panico, T.; Caracciolo, F.; Cicia, G. 2012. Le preferenze dei consumatori italiani nei confronti dell'attributo biologico nell' olio extra-vergine di oliva alla luce della nuova normativa sull'etichettatura. Available in: V Workshop Nazionale GRAB-IT. Ancona, (Date of consultation: 11/05/2012).
16. Del Giudice, T.; Cavallo, C.; Caracciolo, F.; Cicia, G. 2015. What attributes of extra virgin olive oil are really important for consumers: a metaanalysis of consumers' stated preferences. Agricultural and Food Economics. 3:20 DOI 10.1186/s40100-015-0034-5.
17. Detre, J. D.; Mark, T. B.; Clark, B. M. 2010. Understanding Why College-Educated Millennials Shop at Farmers Markets: An Analysis of Students at Louisiana State University. Journal of Food Distribution Research. 41(3): 14-24.
18. Duarte, A. 2015. Wine as a unique and valuable resource. British Food Journal. 117(11): 2757-2776.
19. Field, A. 2009. Discovering Statistics Using SPSS. Sage Publications.
20. Garrido, C. 2015. Análisis del comportamiento del consumidor de aceites de oliva en países emergentes: Brasil y Chile. [on line]. Master Olivar, Aceite de oliva y Salud. Andalucía. España: Universidad de Jaén.
21. Gázquez-Abad, J. C.; Sánchez-Pérez, M. 2009. Factors influencing olive oil brand choice in Spain: an empirical analysis using scanner data. Agribusiness. 25(1): 36-55.
22. Geraghty, S.; Torres, A. 2009. The Irish wine market: a market segmentation study", International Journal of Wine Business Research. 21(2): 143-154.
23. González, J.; Pazmiño, M. 2015. Cálculo e interpretación del Alfa de Cronbach para el caso de validación de la consistencia interna de un cuestionario, con dos posibles escalas tipo Likert. Revista Publicando, 2 (1). 2015, 62-77. Available from: https://www.researchgate.net/publication/272682754_Calculo_e_interpretacion_del_Alfa_de_Cronbach_para_el_caso_de_validacion_de_la_consistencia_interna_de_un_cuestionario_con_dos_posibles_escalas_tipo_Likert [accessed Sep 18 2019].
24. González-Yebra, O.; Aguilar, M. A.; Aguilar, F. J. 2019. A first approach to the design component in the agri-food industry of southern Spain. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 125-146.
25. Grunert, K.; Loose, S.; Zhou, Y.; Tinggaard, S. 2015. Extrinsic and intrinsic quality cues in Chinese consumers purchase of pork ribs. Food Quality and Preference. 42: 37-4726.
26. Güney, O.; Sangün, L. 2017. Seafood consumption attributes and buying behaviours according to the generations: a study on millennial generation in Turkish market. Turkish Journal of Agriculture. Food Science and Technology. 5(12): 1604-1608.
27. Hair, J.; Black, W.; Babin, B.; Anderson, R. 2014. Multivariate data analysis. Ed. Pearson.
28. Henchion, M.; McCarthy, M.; Resconi, V. 2017. Beef quality attributes: A systematic review of consumer perspectives. Meat Science. 128: 1-7.
29. Hu, F. 2003. The Mediterranean diet and modality: Olive oil and beyond. Waltham. MA. ETATSUNIS. Massachusetts Medical Society.
30. INE (Instituto Nacional de Estadística). 2002. Santiago. Chile. Available in:http://reportescomunales.bcn.cl/2012/index.php/La_Florida#Poblaci.C3.B3n_total_2002_y_proyectada_2012_INE (Date of consultation: 27/07/2015).
31. Jackson, D. L.; Gillaspy, J. A. (Jr.); Purc-Stephenson, R. 2009. Reporting practices in confirmatory factor analysis: An overview and some recommendations. Psychological Methods. 14(1): 6-23. Available in: http://dx.doi.org/10.1037/a0014694
32. Kline, R. 2011. Principles and practices of structural equation model. New York: The Guilford Press.
33. Kotler, P.; Kartajaya, H.; Seteiawan, I. 2010. Marketing 3.0. Cómo atraer a los clientes con un marketing basado en valores. México: LID Editorial.
34. Krystallis, A.; Ness, M. 2005. Consumer preferences for quality foods from a south European perspective: A conjoint analysis implementation on Greek olive Oil. International Food & Agribusiness Management Review. 8(2): 62-91.

35. Lee, P.; Lusk, K.; Mirosa, M.; Oey, I. 2015. An attribute prioritisation-based segmentation of the Chinese consumer market for fruit juice. Food Quality and Preference. 46: 1-8.
36. Matsatsinis, N.; Grigoroudis E.; Samaras, A. 2007. Comparing distributors' judgments to buyers' preferences. A consumer value analysis in the Greek olive oil market. Chania. Greece. Technical University of Crete. p. 21.
37. McCarthy, B.; Liu, H. B.; Chen, T. 2014. Trends in organic food consumption in China: opportunities and challenges for regional Australian exporters. In: Proceedings of the Sustainable Economic Growth for Regional Australia. From: SEGRA 2014: Sustainable Economic Growth for Regional Australia Conference. 8-10. Alice Springs. NT. Australia.
38. Medina Viruel , M. J.; Bernal Jurado, E.; Mozas Moral, A.; Fernández Uclés, D. 2018. Empresas agroalimentarias y adopción de las TIC: el caso del sector del aceite de oliva ecológico en España. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 233-251.
39. Metta, S.; Guinard, J. 2010. Means-end chains analysis of extra virgin olive oil purchase and consumption behavior. Food Quality and Preference. 22: 304-316.
40. Mora, M.; Magner, N. 2008. El mercado del aceite de oliva en la región Metropolitana: segmentación de mercados, un análisis cuantitativo. Facultad de Ciencias Agronómicas. Universidad de Chile. Revista Antumapu. 7(1/2): 46-49.
41. Mora, M.; Espinosa, J.; Schenettler, B.; Echeverria, G. 2011. Perceived quality in fresh peaches: an approach through structural equation modelling. Ciencia e Investigación Agraria. 38(2): 179-90.
42. Mora, M.; Schnettler, B.; Fichet, T.; Silva, C.; Estrada, L. 2013. Desarrollo estratégico del mercado del aceite de oliva en Chile. 9: 227-262. In: Aportes al cultivo del olivo, Universidad de Chile. Facultad de Ciencias Agronómicas. Santiago, Chile.
43. Morales, P. 2013. El Análisis Factorial en la construcción e interpretación de tests, escalas y cuestionarios. Madrid. Facultad de Ciencias Humanas y Sociales. Universidad Pontificia Comillas. p. 45.
44. Moreno, E.; Lezcano, S. 2015. Aceite de oliva: Piedra angular de la dieta Mediterránea. OLIVAE Revista Oficial del Consejo Oleícola Internacional. 121: 19-27.
45. Mtimet, N.; Ujiie, K.; Kashiwagi, K.; Zaibet, L.; Nagaki, M. 2011. The effects of information and country of origin on Japanese olive oil consumer selection. International Congress. August 30-September 2. European Association of Agricultural Economists. Zurich. Switzerland.
46. Mtimet, N.; Zaibet, L.; Zairi, C.; Hzami, H. 2013. Marketing olive oil products in the Tunisian local market: the importance of quality attributes and consumers' behavior. J Int Food Agribusiness Mark. 25(2): 134-145.
47. Mueller, S.; Hervé, R.; Yann, C. 2011. How strong and generalizable is the Generation Y effect? A cross-cultural study for wine. A cross-cultural study for wine. International Journal of Wine Business Research. 23(2): 125-144.
48. OLIVAE Revista Oficial del Consejo Oleícola Internacional. 2015. Available in: http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures?lang=es_ES (Date of consultation: 27/07/2016).
49. Olson, J.; Jacoby, J. 1972. Cue utilization in the quality perception process. In: Proceedings of the Third Annual Conference of the Association for Consumer Research. Ed. M. Venkatesan. Association for Consumer Research. Chicago. 167-179.
50. Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; W€urtele, G.; Spiegelhalder, B.; Bartsch, H. 2000. Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncology. 1: 107-112.
51. Pincheira, A.; Arenas, T. 2016. Caracterización de los profesionales de la "Generación Millennials" de Arica y Parinacota, Chile, desde una mirada del capital intelectual. Interciencia. vol. 41(12): 812-818.
52. Pladeco. 2009. Informe 2009-2016. Chile. Municipalidad de la Florida. Available in: http://www.laflorida.cl/web/wp-content/uploads/2010/12/37b-METODOLOGIAPLADECO-2009-2016.pdf (Date of consultation: 20/04/2017).

53. Reeves, T. C.; Oh, E. 2008. Generational differences handbook of research on educational communications and technology. Chapter: 25. Publisher: Taylor & Francis Group.
54. Romo, R.; Labrín, C.; Lizama, V.; Herrera, R. 2014. Caracterización de los consumidores chilenos de aceite de oliva: un análisis exploratorio aplicado a la Región del Biobío. Economía Agraria. 18: 47 -61.
55. Romo, R.; Lagos, M.; Gil, J. M. 2015. Market values for olive oil attributes in Chile: a hedonic price function. British Food Journal. 117(1): 358-370. https://doi.org/10.1108/BFJ-01-2014-0009
56. Salazar-Ordóñez, M.; Rodríguez-Entrena, M.; Cabrera, E.; Henseler, J. 2018. Understanding product differentiation failures: The role of product knowledge and brand credence in olive oil markets. Food Quality and Preference. 68(2018): 146-155.
57. Santosa, M.; Abdi, H.; Guinard, J. X. 2010. A modified sorting task to investigate consumer perceptions of extra virgin olive oils. Food Qual Preference 21(7): 881-892.
58. Santosa, M.; Guinard, J. X. 2011. Means-end chains analysis of extra virgin olive oil purchase and consumption behavior. Food Quality and Preference. 22: 304-316.
59. Santosa, M.; Clow, E. J.; Sturzenberger, N. D.; Guinard, J. X. 2013. Knowledge, beliefs, habits and attitudes of California consumers regarding extra virgin olive oil. Food Research International. 54: 2104-2111.
60. Scarpa, R.; Philippidis, G.; Spalatro, F. 2005. Product-country images and preference heterogeneity for Mediterranean food products: A discrete choice framework. Agribusiness. 21: 329-349.
61. Schawbel, D. 2015. 10 New Findings About The Millennial Consumer. Forbes 20 June. Available in: https://www.forbes.com/sites/danschawbel/2015/01/20/10-new-findings-about-the-millennial-consumer/#6776a1296c8f
62. Taylor, S.; Cosenza, R. 2002. Profiling later aged female teens: mall shopping behavior and clothing choice. Journal of Consumer Marketing. 19(5): 393-408.
63. Taylor, P; Keeter, S. 2010. "Millennials a portrait of generation next: confident, connected, open to change. Washington. DC: The Pew Research Center.
64. Vale-Nieves, O.; Rivera-Maldonado, M. 2015. Creación y validación del cuestionario sobre uso de mensajes de texto en el aula. Revista Puertorriqueña de Psicología. 26(1): 40-54.
65. Verbeke, W.; Van Wezemael, L.; de Barcellos, M.; Kügler, J.; Hocquette, J.; Ueland, Ø.; Grunert, K. 2010. European beef consumers' interest in a beef eating-quality guarantee: insights from a qualitative study in four EU countries. Appetite. 54: 289-296.
66. Zeithaml, V. A. 1988. Consumers perceptions of price, quality and value: A means-end model and synthesis of evidence. Journal of Marketing.

The need for extra-agrarian peasant strategies as a means of survival in marginal rural communities of Mexico

La necesidad de estrategias campesinas extra-agrarias como medio de supervivencia en comunidades rurales marginales en México

María Angélica Quintero Peralta *, Rosa María Gallardo-Cobos, Pedro Sánchez-Zamora

Originales: *Recepción:* 21/12/2018 - *Aceptación:* 02/11/2019

Abstract

The Mexican agrarian sector is currently facing a series of structural problems that have a direct impact on the potential of agricultural activities that provide rural families with food and economic livelihoods, particularly to those living in marginal rural commu- nities. A total of 132 interviews were conducted with farmers from marginal commu- nities in central Mexico. From the total, 64.2% had decreased their agricultural activity in order to engage in other activities and increase their income. Ninety-four percent (94.7%) of families spend between 50 and 100% of their income on food. The binomial logit model determined that there was a 95.4% probability of a family member securing employment outside the peasant production unit. Despite this, family income does not cover basic requirements satisfactorily. In the current context, peasants are subjected to food poverty and income instability. As a result, they look for livelihood options outside the agricultural activities that only allow them to subsist. It is highly probable that peasant families will continue to implement a variety of survival strategies with increasing frequency, to the detriment of Mexican family units and rural communities.

Keywords
peasant strategies • peasant production unit • Agricultural communities • non-farm income • Mexico

Universidad de Córdoba. ETSIAM. Departamento de Economía. Sociología y Política Agraria. Campus de Rabanales. Ctra. Nacional IV Km 396. Edificio Gregor Mendel 3ª planta. 14014 Córdoba. España. * es2qupem@uco.es

Resumen

El sector agrario en México enfrenta problemas estructurales que influyen directamente en la posibilidad de que las actividades agrícolas sean el sustento alimentario y económico de las familias campesinas, particularmente las que viven en comunidades rurales marginadas. Se aplicaron 132 entrevistas a campesinos de comunidades marginadas del centro del país, destacando que el 64,2% ha disminuido la actividad agrícola con el fin de realizar otras actividades y obtener ingresos, y el 94,7% de las familias destinan entre el 50 y 100% de sus ingresos a la alimentación. Utilizando un modelo logit binomial se determinó una probabilidad de que el 94,5% se emplee fuera de la unidad de producción campesina para obtener ingresos, aun así, no cubren satisfactoriamente sus necesidades alimenticias y otras necesidades básicas. En el entorno actual del sector, los campesinos muestran vulnerabilidad alimentaria e inestabilidad en la obtención de ingresos, por lo que buscan opciones de vida familiares más allá de las actividades agrícolas que les permitan subsistir. Existe la posibilidad de que las familias campesinas continúen implementando diversas estrategias, y que vayan en aumento, en detrimento de las propias familias y del campo mexicano.

Palabras clave

estrategias campesinas • unidad de producción campesina • comunidades agrícolas • ingresos no agrícolas • México

Introduction

Mexico is a country with an important rural sector. Agricultural and livestock production activities are performed in 57% of its territory (35), and 23% of its population lives in rural areas The Instituto Nacional de Estadística y Geografía (INEGI) defines a population as rural when it has less than 2,500 inhabitants (21).

Over the years, the Mexican agricultural sector has faced problems such as insufficient agricultural production, food dependency, lack of dynamism in rural employment, rural poverty, emigration, and devastation of natural resources. These problems are structural and historical, and cannot only be explained by specific policies, commercial treaties, or the vast process of globalization (31).

After facing macroeconomic disparities that led to an economic crisis and increasing external debt, Mexico declared a foreign debt moratorium in 1982. In order to obtain new loans, the Mexican Government had to adhere to several conditions set by the World Bank and the International Monetary Fund, which required implementing struc- tural adjustment policies in line with the neoliberal development model (2, 19, 30).

This situation was not exclusive to Mexico. Since the beginning of the economic crisis in 1980, and the debt crisis during that decade, Latin American and Caribbean countries were forced to implement drastic stabilization policies and structural adjustment programs to radically modify growth and development concepts (14, 22). Neoliberal policies in Latin America are characterized by fiscal adjustment, privatization, price adjustment, trade liberalization, attracting foreign investment, welfare state and labor market reforms (5).

The three main features of neoliberal restructuring in the Mexican agrarian sector were: 1) reducing state functions; 2) implementing the North American Free Trade Agreement, and 3) amending Article 27 of the Constitution (30). There was a clear anti-agricultural bias in the economic strategy during the first years of adjustment, as agriculture was not considered a priority sector. Important growth factors in the pre-neoliberal era, such as government expenses and investment, were substantially reduced, and as the results of economic reforms fell far short of expectations, agricultural growth was left behind (3, 32).

From 1988 to 1994, the political agenda for modernizing rural Mexico focused on redirecting government investment towards farmers with commercial and competitive potential in open market conditions. Small and medium-scale farmers weresoonclassified as "inefficient" and "non-competitive" and were excluded from government. As an alternative, they were provided with social programs, which to date supply food rations and stipends to poor households. The peasants were no longer classified as "farmers", but as "poor" (3).

The restructuring gave rise to a decrease in production units, loss of rural employment, a fall in rural wages, abandonment of farms by large numbers of farmers, and significant emigration to the United States (8, 34). In turn, this led to the restructuring of basic grain productive capacity (33), *i.e.*, maize, wheat, beans, and rice. Of these basic foodstuffs, maize is the most important and the main crop grown by most peasants.

Latin American countries have expressed concern about being able to mitigate the negative effects of globalization on inequality. In this context, programs such as conditional cash transfers play an important role in redistributing income to the poor, alleviating the negative effects of globalization on inequality. International remittances could become one of the largest financial inflows of resources to reduce poverty levels in countries such as Mexico and Brazil, which have increased levels of migration due to market-oriented political reforms. Income derived from the welfare state and other government transfers, has played a crucial role in mitigating the effects of "macroshocks" caused by globalization, specifically among the poorest segments of Brazilian society (28).

This study aimed to determine whether peasants need to engage in activities other than agriculture to obtain income, and verify if this strategy is sufficient to cover their basic needs.

In order to address the objective, the next section describes the most salient features of peasant production units, social reproduction and peasant strategies. This is followed by a detailed description of the methodology. The fourth section discusses the main results obtained from the binomial logit model that analyses the probability of peasant families working in activities other than the agricultural sector. The last section presents the most relevant conclusions drawn from the study.

Peasant production units, social reproduction strategies, and peasant strategies

The Mexican rural population is comprised primarily of peasants. Peasant Production Units (PPU) are distinguished by the following traits: small-scale production; minimum or null levels of investment in infrastructure and materials; limited access to resources and production services; reliance primarily on family labor; products are intended for family consumption and sometimes for limited sales, showing some degree of poverty. In addition, the PPU is an indivisible whole comprising a house, a backyard, a space adjacent to the house where families can grow crops or raise small livestock for household use, and a plot.

In a context wherein poverty primarily affects peasants, they are faced with the difficult task of situating themselves within the globalized economy. As such, the strategies they implement for the social reproduction of the family are different from those aimed at improving the level of wellbeing. Social reproduction strategies are a set of practices through which individuals or families strive to maintain or improve their social position in the class structure (9) by establishing a link between individual choices and social structures (25). When strategies are strongly conditioned by a context of inequality and vulnerability, they are referred to as survival strategies. These strategies cover the minimum satisfaction of needs to ensure the most immediate reproduction of life and are not guaranteed by the current mode of production or by the social policies implemented under the economic model. Strategies implemented in times of crisis can mitigate the crisis and guarantee subsistence, but little more. In short, strategies developed in situations of social vulnerability are an indicator of social inequality (18, 20, 25, 29).

In turn, peasant strategies are a complex set of behavior and actions concerning nature and society, in a certain historical and geographical context, in response to structural situations in which peasants must constantly readapt. They are targeted at renewing the material resources that enable social reproduction. For the majority of peasants, progressive participation in nonagricultural activities is a response to their social reproduction crisis and only enables them to survive (10, 16, 23, 25).

The continuity or collapse of the PPU depends, at least in part, on its capacity to navigate a complex network of farm and non-farm activities, within a continuously fluctuating environment (10, 16). The risk management strategies of the rural poor are based on income diversification, migration, and subsistence farming, giving rise to a relative increase in mercantile activities in relation to production for family consumption (6).

Neoliberal policies have stimulated peasant differentiation, given that increased impoverishment forces peasants to seek employment and income opportunities outside the PPU. There has been an increase in non-agricultural activities, multi-activities, multifunctionality in agriculture, emigration, and the proletarianization of the agricultural workforce. However, to this day, unemployment and an impoverished standard of living are recurring conditions in rural areas (23, 24). Public and private cash transfers, particularly remittances, have increased significantly and have an important impact on the total income of rural families, helping to alleviate poverty (32). In effect, the traditional resistance of peasants is continually renewed and reformed, given that must depend on their own initiatives when abandoned by governments (7, 10, 28).

This paper argues that peasant strategies are primarily for survival. They can be defined as a broad range of activities, based on experience, skills and individual knowledge, implemented to alleviate socioeconomic adversity resulting from structural adjustment. All family members participate in running the home and obtaining income outside the PPU. In general, the arrangements are not permanent but vary according to the options available, the family members who perform them, and the available economic resources.

MATERIALS AND METHODS

Primary data were obtained from 11 rural communities in the states of Hidalgo, Mexico, and Morelos located in the country's central region. Rural communities in these states have a high or very high degree of marginalization: 78.8% in Hidalgo, 76.8% in Mexico, and 63.6% in Morelos. Marginalized communities face a high degree of social vulnerability, with effects that are beyond personal or family control, given that they derive from a production model that does not provide the same opportunities for all. There are five degrees of marginalization: very high, high, medium, low, and very low, which are determined by the magnitude of deficiencies in education, housing, population distribution, and monetary income (13). In fact, all 11 communities included in this study have a high degree of marginalization (12). The sampling aimed to determine the situation and structure of the PPU; hence, the sampling units were peasants. The sampling was quasi-random (11). In the selection of individuals, the only requirement was that they performed or have performed agricultural activities for obtaining food or income. Direct interviews with 132 individuals were conducted, enough to make relationship inferences. The size of the sample for each state was determined by proportional affixation (11) *i.e.*, the size of the subsamples was proportional to the rural population with a high degree of marginalization. From April to June 2015, 45 interviews were conducted in Hidalgo, 78 in Mexico, and 9 in Morelos.

The questionnaire used consists of eight sections: Location; General data about the interviewee and their home; Agricultural activities; Food basket; Income; Diversification of activities; Public services, and Agrarian programs. The analyses performed in this study were based on the performance and intensity of agricultural activities; the composition and origin of the food basket consumed by families; income sources; the activities performed in addition to agriculture, and access to government programs.

The productive and socio-economic conditions of the peasant families were described, and the variables used in the statistical analysis were specified from the systematized primary data.

As food insufficiency and low-income forcesfamily members toseekemployment in several activities, peasant strategies relating to employment were explained using a logit model. A binomial regression model was chosen in which the explanatory variable has only two possible outcomes (17). The variable to be modeled or predicted is identified as the dependent variable *Y*, and the explanatory or independent variables are designated $X_1, X_2, ...X_k$.

The dependent variable is categorical and usually dichotomous (1). The relationship between the dependent variable and the explanatory variables is non-linear. The maximum likelihood method was used to estimate the dependent variable due to its dichotomous nature, solving the problem of heteroskedasticity. The probability distribution function defines a probability distribution from 0 to 1. Given that the interpretation of the coefficients is not immediate, an alternative measure to the coefficient of determination is required to measure the model's goodness of fit (26).

Considering the above, the dependent variable was defined as Peasant Strategies with Employment, which refers to whether or not income was supplemented by permanent or temporary employment of a family member. The categories defined were: Employment and Other situation. The first refers to whether or not a family member was temporarily or permanently employed outside the PPU. For farmers to be considered in the model estimation, they had to have performed agricultural activities, generating income or not. Only 123 out of 132 farmer responses were included because nine individuals had abandoned agricultural activities.

In order to find a significant relationship between the dependent variable and other variables, a bivariate analysis was performed, using contingency tables analyzed by the chi-square statistic. Thus, when the associated probability was less than 0.05, the null hypothesis of independence between the variables was rejected, and the conclusion drawn that the variables studied were related (4) SPSS version 15.0 for Windows was used. Based on the significance of the independent variables, only the variables that could be more relevant to the construction of the logit model were considered in order to determine which variables, acting together, might have a greater influence on the decision to diversify activities through employment (table 1, page 252).

A stepwise backward regression method was used for the likelihood ratio as it gives rise to different models for predictive purposes highlighting the most parsimonious ones. It involves starting with all the selected independent variables and eliminating those lacking statistical significance (1). To measure the goodness of fit, the percentage of correct estimates in the model was used, as it facilitates the comparison between the predicted values and the observed values. The cut-off point of probability *Y* to classify the individual variables was 0.5. The equation used to calculate the probabilities was as follows:

$$P = (Y = 1)\frac{1}{1 + e^{-(\beta_0 + \beta_1 X_1 + \beta_2 + X_2 + \dots \beta_k X_k)}}$$

where:

$\beta_0, \beta_1, \beta_2 \dots \beta_\kappa$ = Parameters of the model

$X_1, X_2 \dots X_\kappa$ = Independent variables

e = Exponential function. Raise the number *e* (Euler constant, whose approximate value to the thousandth is 2.718) to the power contained within the parenthesis (1).

Table 1. Variables included in the logit model.
Tabla 1. Variables incluidas en el modelo logit.

Variable	Name	Categories
Dependent		
Income supplemented via permanent or temporary employment of a family member	PSEmplo	Employment = 1 Other situation = 0
Independent		
Maize production	ProdMaize	Up to 2 t = 1 Over 2 t = 0
Maize production destination	DestProd	Consumption and sale = 1 Consumption = 0
Age of head of family	AgeHF	Over 50 = 1 20 to 49 = 0
Peasant production unit structure	PPU	Plot = 1 Plot and backyard = 0
Type of agricultural crops	ProdAgri	Basic grains = 1 Basic grains and others = 0
Decrease in agricultural activity	DecAct	Yes = 1 No = 0
Government support for production (*Proagro Productivo* Programme)	Proagro	Receives support= 1 No support = 0
Government support for health (*Seguro Popular* Programme)	SegPop	Receives support = 1 No support = 0
Source of maize for family consumption	SourMaize	Plot = 1 Plot and purchase = 0
Food sufficiency for family consumption	SufCon	Not always = 1 Frequently = 2 All year = 3
Percentage of income spent on food	SpeFood	50 to 100% = 1 Up to 50% = 0
Family members collaborating in agricultural activities	CollAA	Head of family = 1 Adults 20-45 years = 2 Adults 45-65 years = 3 Whole family = 4 None (labourers) = 5
Income sufficiency to cover family needs	SufInc	Insufficient = 1 Principal needs = 0

RESULTS

Peasant production units description

Agricultural activities

Agriculture in the PPUs focuses on the production of small-scale basic grains for family consumption or sporadic local sales (table 2). Activities are primarily performed in small, non-irrigated, family-owned plots, although it is common to rent or loan land, or to work in share-cropping, which are the means by which landless peasants produce. A decrease in agricultural activity was observed in 64.2% of the peasants studied; due to the decline in profitability of farming and the need to perform other activities to obtain income. For the rest (35.8%), there was no decrease in agricultural activity because farming is their main source of income; in addition to the fact that employment oppor- tunities lack in their communities. The main reason for performing agricul- tural activities is to obtain food (44.7%). Livestock husbandry primarily takes place in the backyard.

A total of 76.7% families grow between 0.5 and up to two t of maize, while 16.7% grow between two and four t. However, a minority (6.7%) grow more than four t, a volume that guarantees family consumption and a quantity for external sales. Practically all the interviewed families (99.2%) consume all or part of the harvested crops, and 66.7% sell small surpluses in local markets.

Food basket

Although a significant part of the maize and beans for family consumption comes from plots (60.6% and 27.3% respec- tively), families need to buy additional amounts to satisfy their food needs.

Table 2. Main characteristics of peasant production units.

Tabla 2. Principales características de las unidades de producción campesina.

Activities performed[1]			
Agricultural: 99.20%	Maize: 97.6%	**Livestock: 54.50%**	Poultry: 50.4%
	Beans: 59.3%		Sheep: 8.9%
	Other crops: 38.2%		Cattle: 7.3%
Access to land for production			
Landowners: 88.6%	Plot less than 1 ha: 19.3%	**Farmers without land: 11.4%**	
	From 1 to 2 ha: 46.2%		
	From 2 to 5 ha: 30.3%		
	From 5 to 10 ha: 4.2%		
Labour for agricultural activities			
Only family labor: 97.6%	The whole family collaborates: 39.0%	**Workers with wages: 2.4%**	
Spaces for farming			
Plot only: 47.2%			
Plot and backyard: 49.6%			
Backyard only: 3.2%			

[1] The sum of the breakdown of activities is greater than 100% because several activities are performed simultaneously.

[1] La suma del desglose de las actividades es mayor a 100% porque se realizan varias a la vez.

This reflects the decrease in agricultural activity and the insufficiency of the volumes obtained. The consumption of other types of food varies according to the season and availability of money. Fruit and vegetables are bought by 56.1% of families, while meat (chicken for 49.2%) comes mainly from the backyard; 2.3% never eat meat.

All the interviewees explained they only have enough supplies for two full meals per day. This situation, along with maize insuf- ficiency for the majority and infrequent consumption of other types of food needed for a balanced diet, shows major constraints in the availability of and access to food. This is supported by the fact that only 32.6% stated that the food produced or purchased was sufficient for the family food supply throughout the year - two meals per day. For 67.4%, it was insufficient.

Income and food expenditure

Since the late 1990s, predictions about neoliberal policies having long-term significant effects, especially on income distribution were made (5). Only 21.9% of families have a single source of income. Of this group, 9.1% depend on agricultural activities and the rest on permanent or temporary employment, or conditional cash transfers. Of the families who obtain income from various sources, 67.4% obtain income primarily from government support programs for production or social welfare; 61.4% from agricultural activities and various permanent or temporary employments; and to a lesser extent, 4.5% from remittances. Of the families receiving government support, 30.3% benefit from more than one program, usually *Proagro Productivo* and *Prospera*.

For 53.8% of families, income comes from various family members; for the rest, only from the head of the family. For 62.9%, income only covers food, and for 3.8%, their income is not enough for obtaining food. A significant proportion of income is spent on food; 94.7% of families spend 50 to 100% of their income on food. This highlights the inability of peasants to meet other basic needs that impact family welfare. Rural families are in a situation of food fragility because when income falls, food acqui- sition is directly affected in quantity, quality, and diversity. Peasants have to buy cheaper food, usually industrialized products with low nutritional value.

Diversification of activities. Peasant strategies

To a large extent, heads of families contribute to the family income by combining agricultural activities with temporary employment, or by exclusively securing employed outside the PPU (table 3, page 255). Whether as a supplementary source or a primary source of income, the main activities performed in order of importance are as follows: agricultural day laborers in Mexico or abroad, construction laborers, and any activity within the service or trade sectors. Those who emigrate do so mainly within the country, and those who emigrate abroad work in the United States and Canada.

Table 3. Diversification of activities of the head of the family.

Tabla 3. Diversificación de actividades del jefe de familia.

Activities performed	**%**
Only agricultural activities	26.5
Agricultural activities and temporary employment	49.2
Temporary or permanent employment	20.5
Emigrate (temporary, indefinite, definitive)	8.3
Other	3.8

For 83% of the interviewees, the reason for performing various activities is to supplement income. Other reasons are the decrease in the profitability of agricultural activities, low prices for produce (19.8%), creation of opportunities for young people or non-existent jobs in their communities (27.4%).

Diversification of activities highlights what various authors have analyzed about the effects of globalization on poverty, inequality and income distribution: the creation of winners and losers and increasing inequalities (28).

Binomial logit model results

The method used in this study reached results after 7 steps. The summary (the Cox and Snell R^2) indicated that 34.1% of the variation of the dependent variable is explained by the independent variables included in the model. Goodness of fit, determined through the Hosmer-Lemeshow test, indicates a good fit of the model in the seventh step (*p= 0.948*), given the high value of the predicted probability. Therefore, the hypothesis stating that the coeffi- cients are equal to zero is rejected. The model was able to accurately classify 82.2% of those employed outside the PPU as well as 77.9% who are either employed or in another situation (table 4).

Table 5 (page 256) shows the final variables in the model, the regression coefficients (B) with the corresponding standard errors (ET), the Wald chi-square test, the degrees of freedom, significance level, and the value of Exp(B) with its confidence intervals.

Table 4. Dependent variable classification table [(a)].

Tabla 4. Tabla de clasificación de la variable dependiente [(a)].

Observed			**Predicted**		
			PSEmplo		**Percentage correct**
			Other situation	**Employment**	
Step 7	PSEmplo	Other situation	35	14	71.4
		Employment	13	60	82.2
	Overall percentage				**77.9**

[(a)] The cut-off value is 0.500. / [(a)] El valor de corte es 0,500.

Table 5. Variables included in the equation.

Tabla 5. Variables incluidas en la ecuación.

		B	E.T.	Wald	gl	Sig.	Exp(B)	I.C. 95% for EXP(B)	
								Lower Limit	Upper Limit
Step 7[a]	ProdMaize	1.353	0.543	6.202	1	0.013	3.868	1.334	11.218
	DestProd	-1.607	0.592	7.365	1	0.007	0.200	0.063	0.640
	AgeHF	-1.536	0.537	8.177	1	0.004	0.215	0.075	0.617
	PPU	-0.953	0.499	3.638	1	0.056	0.386	0.145	1.027
	DecAct	1.567	0.571	7.547	1	0.006	4.794	1.567	14.667
	Proagro	-1.153	0.508	5.154	1	0.023	0.316	0.117	0.854
	SegPop	1.290	0.531	5.903	1	0.015	3.631	1.283	10.277
	Constant	1.062	0.958	1.228	1	0.268	2.891		

[a] Variables introduced in Step 1: ProdMaize, DestProd, AgeHF, PPU, ProdAgri, DecAct, Proagro, SegPop, SourMaize, SufCon, SpeFood, CollAA4, SufInc.

[a] Variables introducidas en el paso 1: ProdMaize, DestProd, AgeHF, PPU, ProdAgri, DecAct, Proagro, SegPop, SourMaize, SufCon, SpeFood, CollAA4, SufInc.

Based on these results and considering the order in which the independent variables show greater influence on the dependent variable, it can be argued that employment outside the PPU is higher when: i) agricultural activity decreases (DecAct); ii) maize production (ProdMaize) is less than 2 t, and iii) the family receives support from the *Seguro Popular* Programme (SegPop). In the opposite direction, employment outside the PPU is lower when: i) maize production (DestProd) is intended for consumption and sale; ii) the head of the family (AgeHF) is over 50 years old; iii) the PPU consists only of a plot, and iv) the family receives support from the *Proagro Productivo* Programme (Proagro). In turn, a predictive analysis was also performed using the regression coefficients to calculate probabilities. Thus, to calculate the probability of a family member being employed outside the PPU, the logit model equation is as follows:

$$P = (PS\ Employment) = \frac{1}{1 + e^{-z}}$$

where:

$$Z = (-1.062 - 1.353\,ProdMaize + 1.607\,DestProd + 1.536.AgeHF + 0.953\,PPU - 1.567\,DecAct) + 1.153\,Proagro - 1.290\,SegPop)$$

Taking into account the variables related to production and the categories that express the highest frequency among the farmers interviewed, the variables can be characterized as follows: maize production is less than 2 t, and is intended only for family consumption; the PPU consists of only the plot; agricultural activity has decreased in recent years; the family does not receive support for production (*Proagro Productivo*), and has no healthcare service (*Seguro Popular*). Under these conditions the probability is as follows:

$$P = (PS\ Employment) = 0.954$$

In other words, there is a 95.4% probability for a family member being employed outside the PPU in order to supplement income.

Similarly, assuming there is a significant improvement in the productive variables, *i.e.* that agricultural activity has not decreased, more than two t of maize are produced for consumption and sale, and that there is government support for production and family healthcare, then the probability is as follows:

$$P = (PS\ Employment) = 0.204$$

In other words, if such conditions were met there would be a 20.4% probability for a family member being employed outside the PPU.

In light of the above, it can be seen that agricultural production is an important determinant when it comes to implementing strategies involving permanent or temporary employment that can improve income levels and contribute to ensuring survival. Furthermore, government support is also important, if available, given that it decreases the probability of peasants implementing survival strategies involving employment outside the PPU. These results are in line with those obtained in other studies that show the potential of transfer programs (such as *Procampo/Proagro* and *Oportunidades* and *Seguro Popular*) to improve the living conditions of farmers (37).

However, it is also important to note that the implementation of these programs has benefited large producers more than small producers (29, 36). Consequently, there needs to be more progress made in the design of more flexible and democratic public support should be made, addressing the specific problems presented by small farmers and peasants in rural Mexico.

Although rural poverty in Latin America has declined over the past three decades, it is still exceptionally high. Some of the ways out of poverty are through agriculture, multi-activities, and assistance. The data is surprising given that among those households with land, 73% in Mexico and 34% in Nicaragua, obtain more than half of their income from non-agricultural activities. Poor house- holds are limited to easily accessible low-paid agricultural work. However, the most effective way out of poverty for the rural poor in Latin America is via multi-activities (15).

Conclusions

The environment where peasants currently live in rural Mexico has forced them to look for livelihood options outside of agricultural activities in order to survive. In the PPU, agricultural activities do not generate enough benefits given that production does not cover the basic needs of the families interviewed. In other words, farmers experience food vulnerability and income instability as well as difficulty in adequately satisfying basic requirements to achieve a minimum level of wellbeing or improve their quality of life.

The food fragility to which the interviewed peasant families are subjected is reflected in the limited number of full meals they consume per day and the limited consumption of the wide variety of foods required for a balanced and nutritious diet. This, along with the large percentage of income spent on food, and the involvement of more and more family members in different income-supplement activities, indicate that the various strategies rural families resort to, do not allow them to satisfy the primordial human need for food. As a result, there is little possibility of covering other basic needs. The food situation reflects the impoverishment of peasant families and the violation of a fundamental human right. Serious food deficiencies in the rural population can lead to high levels of malnutrition, with severe impacts on future generations.

The strategies implemented by the interviewed peasants are determined primarily by the conditions of the Mexican agrarian sector. If the current agrarian public policies approach is maintained, there will be little possibility of improving agricultural activities or generating employment opportunities for rural populations, with the risk of exacerbating and perpetuating the marginalization of peasants. The results of the peasant strategies observed through the inter- views, focused on increasing income, are insufficient for families to be able to live from their labor and in better conditions within their environment. Therefore, it is highly likely that this situation of poverty and marginalization will intensify, to the detriment of peasant families and rural Mexico.

It is highly probable that the conditions detected in this study, are present in innumerable cases among peasants in different regions of the country. This highlights the need to revitalize the productive capacity of PPUs via specific public policies that need to be designed from a different perspective than that of current policies. Despite reduced production, agricultural activity performed in PPUs remains a relevant survival strategy. Actions must be implemented to ensure this practice does not disappear from the rural family dynamic.

References

1. Aguayo, M. 2007. Cómo hacer una regresión logística con SPSS© "paso a paso", I. Fundación Andaluza Beturia para la Investigación en Salud. Docuweb Fabis. Dot. Núm 0702012. 16 p.
2. Aguilar, G. 2000. Desigualdad y pobreza en México, ¿son inevitables? México. Ed. Miguel Ángel Porrúa. 210 p.
3. Appendini, K. 2014. Reconstructing the maize market in rural Mexico. Journal of Agrarian Change. 14(1): 1-25. doi: 10.1111/joac.12013.
4. Arriaza, M. 2006. Guía práctica de análisis de datos. España. Ed. Junta de Andalucía. 198 p.
5. Baer, W.; Maloney, W. 1997. Neoliberalism and income distribution in Latin America. World Development. 25(3): 311-327.
6. Banco Mundial. 2005. Generación de ingreso y protección social para los pobres. México. Banco Mundial. 221 p.
7. Bartra, A. 2014. Campesinos del tercer milenio: aproximaciones a una quimera. Revista ALASRU. 10: 17-43.

8. Bello, W. 2008. Cómo generar una crisis mundial de los alimentos: Lecciones del Banco Mundial, el FMI y la OMC. Enfoque sobre Comercio. Edición especial. La crisis mundial de alimentos. 140: 2-14.
9. Bourdieu, P. 1988. La distinción. Criterios y bases sociales del gusto. Trad. Ruiz, M.C. Buenos Aires. Ed. Taurus. 597 p.
10. Cáceres, D. 1995. Estrategias Campesinas en Sociedades rurales contemporáneas. Revista de la Facultad de Agronomía. 15(1): 67-72.
11. Calatrava, J. 1998. Notas sobre muestreo y uso de técnicas de encuesta en investigación de mercados. España. Instituto Agronómico Mediterráneo de Zaragoza. 38 p.
12. CONAPO. 2010. Base de datos por entidad 2010. México. Consejo Nacional de Población.
13. CONAPO. 2013. Índice absoluto de marginación 2000-2010. México. Consejo Nacional de Población.
14. De Janvry, A.; Sadoulet, E. 1989. Investment strategies to combat rural poverty: A Proposal for Latin America. World Development. 17(8): 1203-1221.
15. De Janvry, A.; Sadoulet, E. 2000. Rural poverty in Latin America determinants and exit paths. Food Policy. 25: 389-409.
16. Espín, J. 1999. Estrategias campesinas de sobrevivencia y de reproducción social en la población negra del Valle de Chota, Ecuador. En: Espín, J.; Rivera, F.; Herrera, G.; Rodríguez, E.; Bassolet, B.; Songore, F.; Dansokho, M. y Coura, N. Estrategias de supervivencia y seguridad alimentaria en América Latina y en África. Buenos Aires. CLACSO. 5-27.
17. Fiuza, M. D.; Rodríguez, J. C. 2000. La regresión logística: una herramienta versátil. Nefrología. XX(6): 495-500.
18. Gutiérrez, A. B. 2003. La construcción social de la pobreza. Un análisis desde las categorías de Pierre Bourdieu. Anduli Revista Andaluza de Ciencias Sociales. 2: 29-44.
19. Herrera, F. 2009. Apuntes sobre las instituciones y los programas de desarrollo rural en México. Del Estado benefactor al Estado neoliberal. Estudios Sociales. 17(33): 8-39.
20. Hintze, S. 2004. Capital social y estrategias de supervivencia. Reflexiones sobre el 'capital social de los pobres'. En: Danani, C. (Coom.): Políticas sociales y economía social: debates fundamentales. Altamira, Argentina. UNGS-Fundación OSDE.
21. INEGI. 2015. Tabulados de la Encuesta Intercensal 2015. México. Instituto Nacional de Estadística y Geografía.
22. Janssen, W. G.; Sanint, L. R. 1991. Economic trends in Latin America. Roles for agriculture and new technology. Food Policy. 474-485.
23. Kay, C. 2007. Algunas reflexiones sobre los estudios rurales en América Latina. Íconos Revista de Ciencias Sociales. 29: 31-50.
24. Macías, A. 2013. Introducción. Los pequeños productores agrícolas de México. Carta Económica Regional. 25(111-112): 7-18.
25. Massa, L. 2010. Estrategias de reproducción social y satisfacción de necesidades. Parte I: Controversias conceptuales, polémicas prácticas. Revista Perspectivas Sociales. 12(1): 103-140.
26. Medina, E. 2003. Modelos de elección discreta. España. Universidad Autónoma de Madrid. 26 p.
27. Nissanke, M.; Thorbecke, E. 2010. Globalization, poverty, and inequality in Latin America: findings from case studies. World Development. 38(6): 797-802.
28. Palma, D. 1984. Entre la moda y la ciencia. Estrategias de sobrevivencia y participación. Revista Acción Crítica. 15: 1-14.
29. Piñera-Barajas, A.; Martínez-Saldaña, T.; Jiménez-Velázquez, M.A.; García-Cue, J. L. 2016. Política pública para el campo: PROCAMPO en el centro del país. Revista Mexicana de Ciencias Agrícolas. 7(1): 147-157.
30. Quintero, M. A.; Gallardo, R. M.; Ceña, F. 2016. Implicaciones de la disminución de la capacidad productiva de granos básicos sobre la alimentación en comunidades rurales pobres de México. Economía Agraria y Recursos Naturales. 16(2): 33-67.
31. Rello, F. 2008. Inercia estructural y globalización: la agricultura y los campesinos, más allá del TLCAN. Grupo de trabajo sobre desarrollo y medio ambiente en las Américas. Trabajo de Discusión No. 20.

32. Rello, F.; Saavedra, F. 2007. Implicaciones estructurales de la liberalización en la agricultura y el desarrollo rural. El caso de México. Ed. Banco Mundial. FLACSO. 215 p.
33. Rubio, B. 2006. Voces de la desesperanza: la desestructuración alimentaria en México (1994-2004). Gaceta Laboral. 12(001): 69-89.
34. Saavedra, F.; Rello, F. 2012. La problemática rural en México en perspectiva histórica: un marco de referencia para el análisis regional. En: Saavedra, F. y Rello, F. (Coord.): Integración y exclusión de los productores agrícolas. Un enfoque regional. México. Ed. FLACSO. 53-83.
35. SAGARPA. 2013. Manejo sustentable del suelo en México. México. Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación.
36. Valentín-Garrido, J. M; León-Merino, A.; Hernández-Juárez, M.; Sangerman-Jarquín, D. M.; Valtierra-Pacheco, E. 2016. Evaluación del programa PROAGRO productivo en comunidades rurales de la sierra norte de Puebla. Revista Mexicana de Ciencias Agrícolas. 7(2): 413-425.
37. Winters, P.; Davis, B. 2009. Designing a programme to support smallholder agriculture in Mexico: Lessons from PROCAMPO and oportunidades. Development Policy Review. 27(5): 617-642.

Spatio - temporal spread of *Plum pox virus* infecting European plum (*Prunus domestica* L. cv. D'agen) orchard in Mendoza, Argentina

Distribución espacial y temporal del *Plum pox virus* en un monte de ciruelo europeo (*Prunus domestica* L. cv D'agen) de Mendoza, Argentina

Angélica Dal Zotto [1*], Laura B. Porcel [2], Diana B. Marini [3], Cecilia N. Picca [2], Mariano Córdoba [4], Ingrid Teich [5]

Originales: *Recepción:* 20/09/2016 - *Aceptación:* 12/03/2019

Abstract

Sharka, caused by *Plum pox virus* (PPV), is considered one of the most serious viral diseases of stone fruits worldwide due to the great yield losses in orchards. In Rama Caída, Mendoza, a 5-year study (2007-2011) was conducted in a European plum cv D'agen orchard using samples from leaves and DAS-ELISA assay against PPV in order to determine incidence over time and spatial spread. Incidence significantly increased between 2007 and 2009, while for the next two years the increase was not statistically significant. Spatial point pattern of PPV at the plot was characterized by the occurrence of some heterogeneous clusters of infected trees located up to 65 m in the west-east direction of the rows over the five years. Point pattern and correlation type-approaches were undertaken using joint-count and Ripley's K function and showed that the detected infected plants had a disease aggregation pattern both in west-east and south-north directions, as well as within and between rows across the plot. This short-distance local dispersion would be associated with diverse factors, such as vector aphids, that were not evaluated in this study. Hence, this work can serve as a basis for further studies of sharka dispersion in Cuyo region.

Keywords

sharka • *Prunus domestica* cv. D'agen • temporal and spatial virus statistical analysis • PPV

1 Instituto de Patología Vegetal. CIAP-INTA. Av. 11 de Setiembre 4755. C. P. X5020 ICA- Córdoba. Argentina. * dalzotto.angelica@inta.gov.ar

2 Estación Experimental Agropecuaria Rama Caída. INTA. El vivero s/n. C. P. 5600. San Rafael. Mendoza. Argentina.

3 Estación Experimental Agropecuaria Junín. INTA. Carril Isidoro Busquets. La Colonia. C. P. 5573. Junín. Mendoza. Argentina.

4 Universidad Nacional de Córdoba. Facultad de Ciencias Agropecuarias. Cátedra de Estadística y Biometría. CONICET. Av. Valparaíso s/n. C. P. 5000. Córdoba. Argentina.

5 Instituto de Fisiología y Recursos genéticos vegetales (CIAP)-INTA. CONICET. 11 de Setiembre 4755. C. P. X5020 ICA. Córdoba. Argentina.

Resumen

Sharka causada por *Plum pox virus* (PPV) es considerada la enfermedad más nociva de los frutales de carozo debido a las pérdidas que produce en los montes frutales. En Rama Caída (Mendoza) se estudió la dispersión del PPV en un monte de ciruelo europeo cv D'agen durante 2007 a 2011 a través del análisis de muestras de hojas de árboles individuales por DAS-ELISA, para determinar la incidencia y distribución espacial de virus. Entre 2007 y 2009, el aumento en la incidencia fue estadísticamente significativo mientras que en 2010 y 2011 este no resultó significativo. El PPV se distribuyó en el lote, como un patrón de puntos caracterizado por agrupamientos heterogéneos de árboles infectados, ubicados hasta los 65 m en dirección oeste-este de las filas. A través del análisis de patrones de puntos y de correlación de árboles infectados, mediante la función K de Ripley y el estadístico Joint-count, se comprobó que las plantas infectadas presentaron un patrón espacial agregado, tanto en sentido oeste-este (entre filas), como sur-norte (dentro de fila), indicando una dispersión a corta distancia. Este escenario puede responder a múltiples factores no estudiados, como la presencia de áfidos vectores, y constituir las bases de futuros estudios de dispersión de PPV en Cuyo.

Palabras clave

Sharka • *Prunus domestica* cv. D'agen • análisis estadístico espacial y temporal del virus • PPV

Introduction

Sharka disease, caused by *Plum pox virus* (PPV, genus *Potyvirus*, family Potyviridae) is considered one of the most destructive diseases of stone fruit (apricots, Japanese and European plum, peach and cherry) worldwide (8, 15).

The disease causes important economic losses in stone fruit orchards affecting the appearance, flavour and texture of fruits, to the point of making them undesirable both for fresh consumption and industry. It may also cause premature fruit drop, thereby reducing production (15).

The virus was first detected in Bulgaria (1) and is present in countries of Western and Eastern Europe, Northern Africa and North and South America. In Argentina, it was detected in 2004 (12) in Japanese plum and apricot orchards in San Juan province and, more recently, in European plum in Mendoza province. PPV is disseminated via infected propagation material (buds, grafts) from one place to another, such as between countries, and/ or regions/locations within a country. Deficient or absent sanitary control between regions is considered the cause of PPV introduction to virus-free areas (2).

Once PPV is introduced to a geographical site, local dissemination among nearby plants can occur through vector aphids. There are about 20 aphid species that can transmit the virus in an uncontrolled, natural manner, carrying the virus from a diseased plant to healthy ones (23). There is no direct correlation between virus transmission capacity and vector *Prunus* colonization, because PPV can be efficiently transmitted both by species that do not colonize stone fruit trees (visitor aphids) and by species that form colonies in *Prunus*, with leaves and fruits being the main inoculum sources (24). While the disease expands rapidly in fruit orchards, progression rate can vary with the PPV strain (D or M, or others such as REC) and the *Prunus* species involved (plum, apricot or peach)(17, 24).

Studies on PPV dispersion in stone fruit trees have been conducted in different European countries over several years, such as Spain (18) France (11) and Greece (33), as well as USA and Canada in North America (20) involving different *Prunus* species. Their results showed that the spatial dispersion of PPV responds to different distribution patterns (8, 11, 18, 20, 24, 33). Data collected by intensive mapping include details of the spatial arrangement of sampling units.

The disease distribution pattern is a characteristic that can be attributed to a series of points (infected trees) and that describes their location in terms of relative distance of each point with respect to the remaining ones (32). Given that plant disease data can display spatial patterning in a number of different ways, it is useful to analyze spatial patterns using different approaches and search for congruence in the detected patterns (14, 28).

Accordingly, Join-count statistics (10) may be used to analyze spatial association for disease incidence data (26). This approach is appropriate when each individual plant is considered a sampling unit. Another robust method to measure the randomness of binary data of spatial points are the stochastic models proposed by Ripley (14, 30)widely used for analysis of spatial patterns, both in local dispersion (*i.e.* within row and across rows) (31) and regional dispersion(longdistancedispersion) (19, 20).

Models are also used to understand how plant diseases develop in a population over time. With models, the many individual observations are reduced to a few model terms, making it much easier to visualize and, ultimately, understand the studied phenomena (26).

Generalized linear mixed models (GLMMs) are an appropriate tool for evaluating virus incidence over time based on binary variables allowing taking into account possible correlations between observations measured on a single individual over time (25).

According to this, we postulate that a point pattern of infected PPV trees on orchards has an aggregated or clustered spread. A random distribution (complete spatial randomness, CRS) would be the null hypothesis for statistical analyses, whereas the alternative hypothesis postulates that the distribution pattern of infected trees is either regular or clustered (27). The aim of this work was to analyze and evaluate the spatial and temporal natural spread of Sharka virus in an European plum orchard in the southern area of Mendoza province, Argentina, from 2007 to 2011.

Materials and methods

Orchard sampling design

The study was conducted in a European plum (*Prunus domestica* L. cv D'Agen) orchard located in Rama Caída district (34° S, 68° W), San Rafael department, southern Mendoza province, Argentina. The orchard (1 ha) is characterized by a rectangular planting pattern of 3 x 4 m spacing between plant and row, comprising 750 trees on 25 rows (100 m) in west-east direction and 90 m in south-north direction. Sampling was performed during spring (October) from 2007 to 2011, and included all the trees in the orchard, some of which exhibited chlorotic symptoms and ring spots in leaves, as well as premature fruit drop.

Virus detection

Samples of expanded leaves (16 leaves per plant) were taken from branches oriented to the four cardinal points.

The double antibody sandwich-enzyme-linked immune sorbent assay (DAS- ELISA) was used, following the protocol of Clark and Adams (1977), using Inmunoglobulins and conjugates of PPV (Bioreba). Plates (NUNC 96-well polystyrene) were coated using 1:1000 IgG dilutions and conjugate. The extract was processed using 0.5 g of basal third of leaves diluted in 1/10 w/v extraction buffer.

Commercial positive controls (Bioreba) and negative controls (virus-free plum or healthy controls) were used. Plates were read at 405 nm in a Bio-TEK ELX800 Reader. Plants were considered healthy when their absorbance reading value was higher than twice the mean of healthy control plants.

Temporal analyses

Virus incidence and dispersion for the whole plot (750 trees) was assessed from 2007 to 2011. PPV infection was confirmed by DAS-ELISA, resulting in diseased trees (classified as D) and non-infected-healthy trees (classified as H). With these values of disease status, the incidence progress curve was fitted for the evaluated period with a generalized linear mixed model (GLMM) with the logit link function (25).

Year was included as a fixed effect and tree as a random effect in order to account for the intra-tree correlation along the years.

The estimated mean annual disease incidence was compared between years using the *a posteriori* test of Fisher's LSD (α=0.05). Analyses were performed using the software InfoStat (13).

Spatial analyses

Spatial distribution of PPV in Rama Caída orchard was analyzed via a spatial autocorrelation analysis, showing the spatial effects using georeferenced data, here applied at an individual tree level. We used two analyses in order to study the spatial pattern of PPV disease: 1) join-count statistics (10), and 2) Ripley's K function (30). Both methods describe the degree of clustering of individuals, in this case, in relation to PPV presence. The values of these statistics indicate if the pattern is clustered, random or dispersed.

Join-count

Join-count statistics is an area pattern method used to assess the spatial association of categories. We considered two point classes, D and H, where D represents virus presence in trees and H, absence of the disease in the tree, to determine the number of pairs of points with the same characteristic (presence or absence, DD or HH), within a neighbourhood defined by the distance between trees. Then the method calculates if the number of pairs of the same type, *e.g.*, DD (*i.e.*, both trees are infected) is higher or lower than the value expected by chance, considering the total number of Ds and Hs and the number of pairs of points defining the neighbourhood.

A similar approach is used for the events in which the pairs of HH trees (healthy plants) or DH trees (one healthy and one infected plant) are counted.

The neighbourhood was represented by a connectivity matrix (W) of N×N in size, where N is the number of trees and each W element (w_{ij}) has a value of 1 to 0, depending on whether the i^{th} tree is neighbour of the j^{th} tree (w_{ij} =1) or not (w_{ij} =0). For example, in cases of points of the same type (DD) the statistic J_{ij} is defined as equation (1):

$$J_{ij} = \frac{1}{2}\sum_{ij} w_{ij} f(D_i, D_j) \qquad (1)$$

where:
$f(D_i, D_j)$ is a function of a value of
1 if trees i and j are D type, and of
0 ($f(A_i, A_j)=0$) if any of the trees i and j are not type D, for all i≠j.

The indices considering the number of adjacent (neighbours) Joins of the same type (DD or HH) quantify the degree of positive spatial autocorrelation, whereas the index based on the number of adjacent regions that do not contain the same category (DH) describes the degree of negative autocorrelation. We defined neighbours using a distance-based connectivity matrix W, where neighbours were defined as locations within 4 m, from 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60, 64 up to 100 meters, which corresponds to the entire west-east length of the plot.

The results are expressed for each pair of connections (DD, HH, DH) as a standard normal deviate (SND), obtained by subtracting the expected paired values from the observed ones and dividing by the standard deviation. Thus, significance (α = 0.05) of SND would correspond to a ±1.96 threshold. To confirm the null hypothesis, a permutation procedure was used via Monte Carlo simulation, by which the categories are randomly reassigned with each simulation and the "join count" statistic of interest is calculated.

The fraction of simulations with a statistical value below the observed one, provides the *p* value. We used join count statistics to address whether the observed DD was considerably larger than expected in each evaluated year. The join-count statistic was calculated using the spdep library (6) in R (29).

Ripley's K function

The spatial concentration of points in the absence of randomness determines the interaction or spatial dependency among points across space, which is also described with the Ripley's K function. If "*y*" is the mean of infected trees per unit area (density), then $yK(d)$ is the number of infected trees within the distance (d) from an arbitrarily selected infected tree.

If a group of points is randomly distributed, for example via a Poisson process with λ density, the expected number of points in a circle of "d" radius is $\lambda\pi d^2$; thus, the Ripley's K function (30) can quantify the deviation from randomness (theoretical distribution) and reflect the type, intensity and range of the spatial pattern by analyzing the distances between all points. In this study, which is based on a rectangular plot, the equation (2) is fitted:

$$K(d) = n^{-2} A \sum_{i=1}^{n}\sum_{j=1}^{n} w_{ij}^{-1} I_{ij}(d) \qquad (2)$$

for i≠ j

where:

n = the number of points (trees) in the sample

A = the plot area in m^2

w_{ij} = the correction factor of the edge effect

$I_{ij}(d)$ equals 1 if $d_{ij} \leq d$ and 0 if $d_{ij} > d$, with d_{ij} being the distance between points *i* and *j*.

The factor w_{ij} is calculated following (16). The function L (modified Ripley's K function) $L(d) = \sqrt{K(d)/\pi}$ was used to linearize the function and stabilize the variance. This implied representation of L (d)-d against distance d, thereby fitting the null hypothesis to a value of zero (4).

Therefore, an aggregate pattern occurs when $L(d)$-d is significantly higher than zero and a regular pattern, when $L(d)$-d is significantly lower than zero (31). The null hypothesis of spatial randomness was proven via 100 Monte Carlo simulations. To ensure the performance of a significance Monte Carlo test at 0.05 level, 999 simulations were performed (5, 7). Ripley's K function was modelled using the spatstat library (3) in R (29).

Results

Temporal disease progression

The results of the DAS-ELISA analysis, showed 19 positive trees in 2007, 15 new infected plants in 2008 and 14 new infected plants in 2009, whereas in 2010 and 2011, only 6 and 10 new plants were infected, respectively (table 1).

The initial incidence was 2.53% in 2007, then 2% in 2008, 1.86% in 2009, 0.8% in 2010 and 1.34% in (2011), respectively. A total of 64 new plants infected with PPV were detected over the five years (table 1).

The fitted generalized linear mixed model (GLMM) showed significant increases in incidence between 2007 and 2008 and between 2008 and 2009 whereas in the last two years (2009-2011), the increase in incidence was not statistically significant.

The proportion of infected trees over time was 2.5 % (± 0.6) in 2007; 4.5% (± 0.8) in 2008, 6.4 % (± 0.9) in 2009; 7.2 % (± 0.9) in 2010 and 8.5 % (± 1.0) in 2011.

The disease cumulative incidence increased over time. However, between 2009 and 2011, the rate of increase was lower than at the beginning of the study, as shown by the disease progress curve (DPC) (figure 1, page 267).

Spatio-temporal point pattern analysis

The spatial distribution pattern of PPV infection in the European plum cv D'agen orchard showed that diseased trees were not randomly distributed. Rather, the pattern found was "heterogeneous aggregation" of infected plants within the plot.

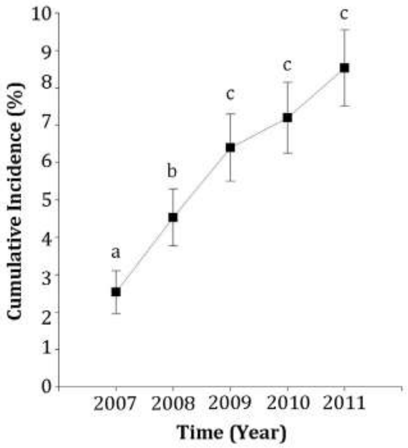
Table 1. Analysis of European plum cv D'agen for *Plum pox virus* by DAS- ELISA in a plot from Rama Caída, Mendoza, Argentina.

Tabla 1. Análisis por DAS-ELISA de *Plum pox virus* en ciruelo europeo cv D'agen en un monte de Rama Caída, Mendoza, Argentina.

Year	Number of analysed plants	Number of new infected plants/ year	Incidence/ year (%)	Cumulative number of infected plants	Cumulative Incidence*
2007	750	19	2.53	19	2.5 a
2008	750	15	2.0	34	4.5 b
2009	750	14	1.86	48	6.4 c
2010	750	6	0.8	54	7.2 c
2011	750	10	1.35	64	8.5 c

* Different letters indicate statistically significant differences.

* Letras diferentes indican diferencias estadísticamente significativas.



Different letters indicate statistically significant differences (Fisher's LSD at α=0.05). Bars represent standard deviations (SD) of data.

Letras diferentes indican diferencias estadísticamente significativas (prueba de Fisher α=0,05). Las barras representan los desvíos estándar (DS).

Figure 1. Disease progression curve (DPC) of sharka disease (*Plum pox virus* infection) in a plot of European plum cv D'agen in Rama Caída, Mendoza, during 2007-2011. The figure shows the cumulative incidence of PPV over the 5-year study period.

Figura 1. Curva de progreso de la enfermedad de sharka (DPC) (*Plum pox virus*) en un monte de ciruelo europeo cv D' agen de Rama Caída (Mendoza) estudiado entre 2007 y 2011. Se muestra la incidencia acumulada (%) en cada año.

The distribution map of infected trees and healthy trees of the studied orchard is represented in a scatter plot (figure 2, page 268-270), with each point representing a tree. At the beginning of the study (2007), the disease was located in the first five rows of plants (up to 20 m), with west-east direction in the plot (figure 2A, page 268).

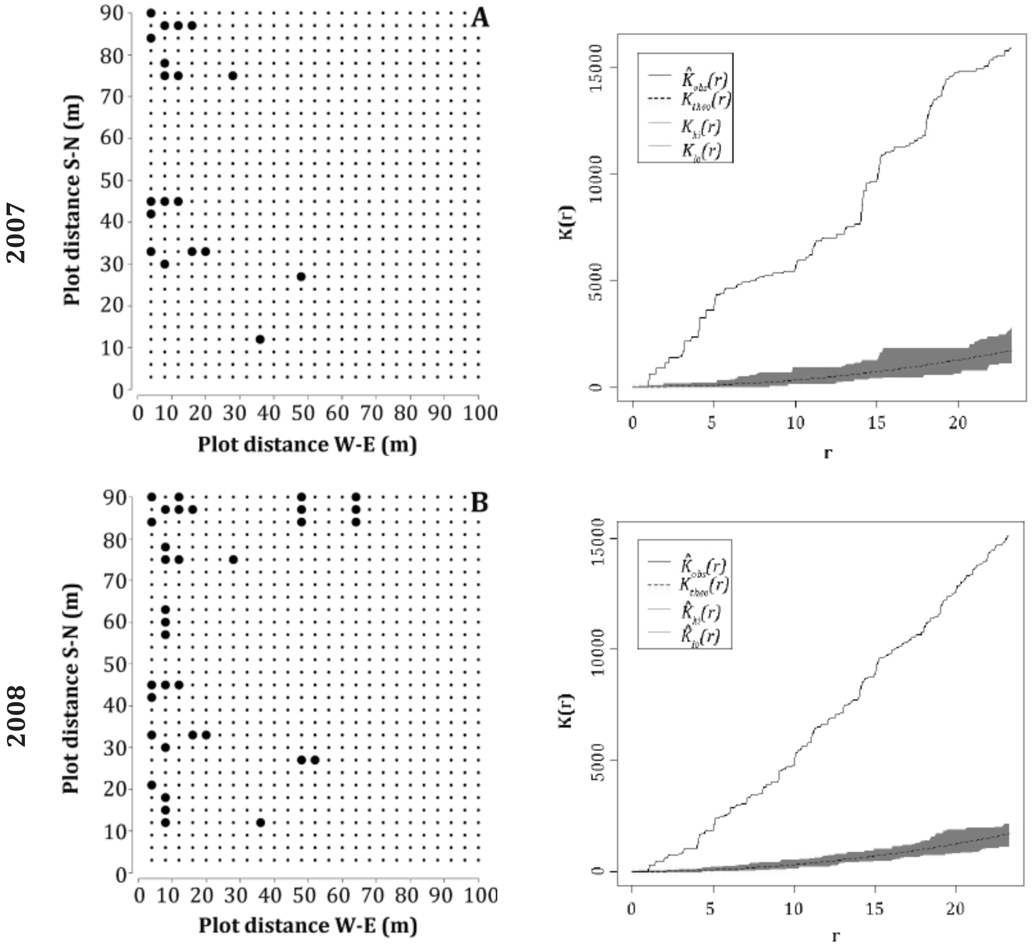
In the second year (2008), new diseased trees were observed around the initially infected trees, forming heterogeneous associations, which did not extend more than 20 m (figure 2B, page 268), and another group of isolated plants was observed at between 45 and 65 m during the third year (2009) (figure 2C, page 269), New plants in the clustered group around the trees detected in the first years were detected in the last study year (2011), as well as other isolated plants or weakly clustered plants, which appeared at a distance of 70 to 100 m (figure 2D, page 269; figure 2E page 270; table 1, page 266).

Evaluation of the spatial point pattern by join-count method

The results obtained using the join-count statistic showing the joins of the same type category (DD), and adjacent (neighbours) to the presence-presence (DD) infection class, are presented in table 2 (page 271). They reject the null hypothesis of a random distribution of diseased plants (p-value<0.01) and indicate aggregated clustered disease distribution pattern. Autocorrelation was observed between individuals close to a given tree with respect to those located at a distance between 0 and 96 m in east-west direction.

In table 2 (page 271), we present only the DD presence-presence category (join-count statistic data), which was considerably larger than the expected value (value under complete randomness) in the five years evaluated.

The analyses show that the calculated join-count statistic was higher than expected for each year and for each one of the distances considered to define the neighbourhoods for each tree.

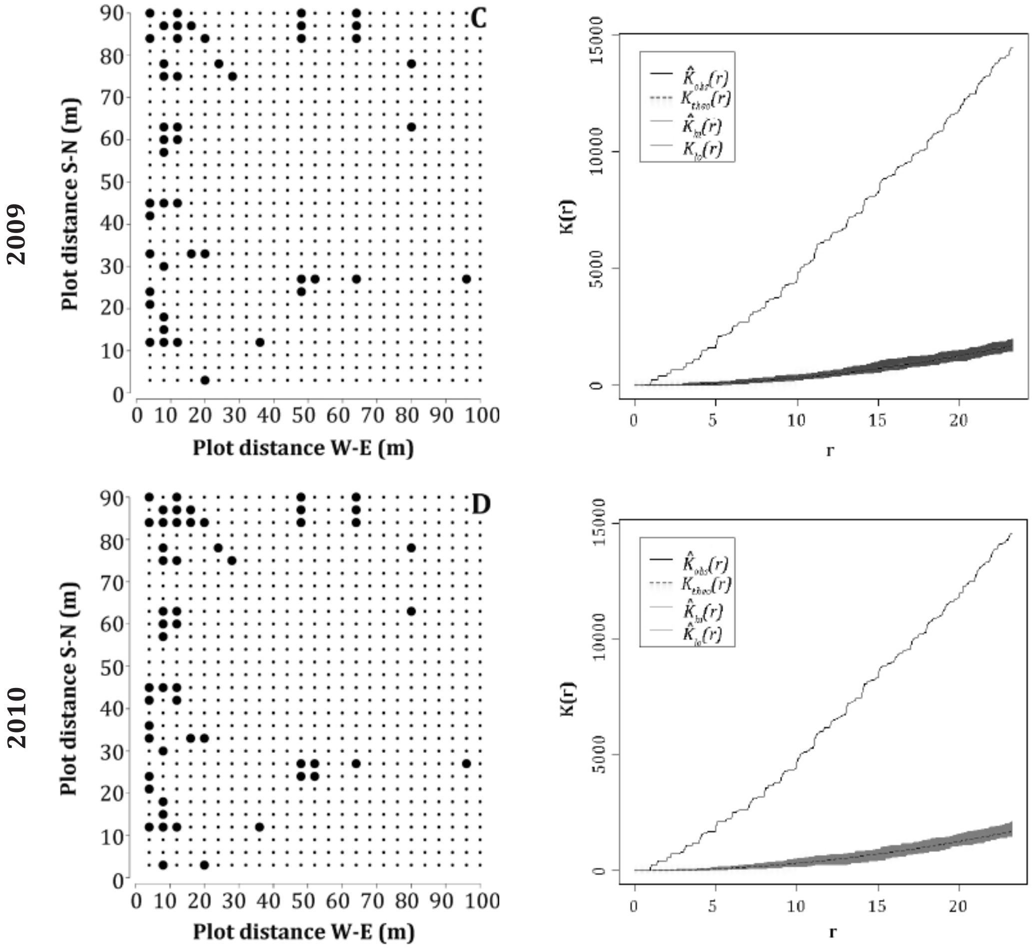


Left: Spatial pattern of PPV-diseased trees (large dots) and healthy (PPV-free) trees (small dots). Right: Ripley K-function *versus* distance in m (r). The black solid line corresponds to the observed K function ($_{Kobs}$), the dotted line to the theoretical K-function ($_{Ktheo}$) and the grey area to the envelope obtained through Monte Carlo simulation. K_{obs} is higher that the envelope simulated for a random point pattern indicating spatial dependency between trees in a short distance (clustered spatial pattern).

Izquierda: Patrón espacial de árboles infectados con PPV (círculos grandes) y árboles sanos (círculos pequeños). Derecha**:** Función K de Ripley en función de la distancia en m (r). La línea continua corresponde a la función empírica K_{obs}, la línea discontinua a la función K teórica ($_{Ktheo}$) y el área gris corresponde a los intervalos obtenidos mediante simulación Monte Carlo para la hipótesis de aleatoriedad espacial. La función de distribución empírica K_{obs} se encuentra por encima del límite del intervalo obtenido por simulación para una distribución al azar, indicando dependencia espacial entre los árboles infectados en distancias cortas (patrón agregado).

Figure 2. Spatial and temporal analysis of *Plum pox virus* (PPV) infection in a plot of European plum D'agen during 5 years (2007-2011).

Figura 2. Análisis de la distribución espacial y temporal del *Plum pox viru*s (PPV) en un lote de ciruelo europeo durante 5 años (2007-2011).



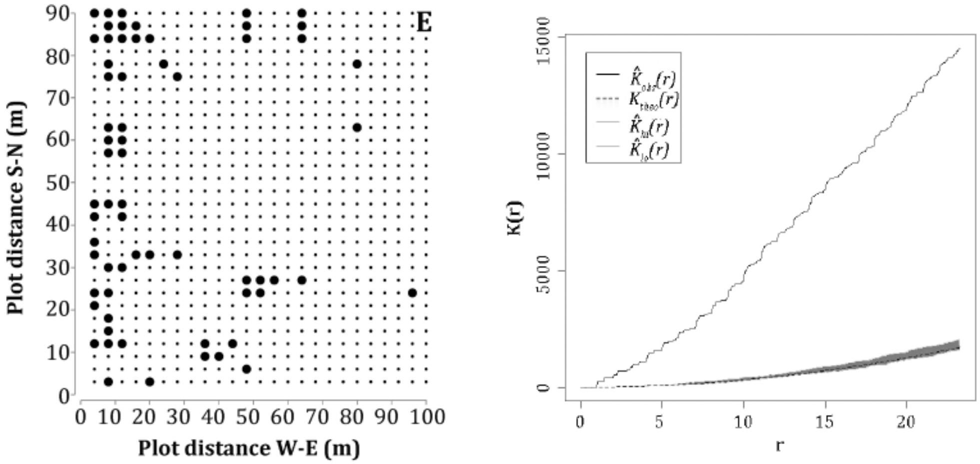
Left: Spatial pattern of PPV-diseased trees (large dots) and healthy (PPV-free) trees (small dots). Right: Ripley K-function *versus* distance in m (r). The black solid line corresponds to the observed K function ($_{Kobs}$), the dotted line to the theoretical K-function ($_{Ktheo}$) and the grey area to the envelope obtained through Monte Carlo simulation. K_{obs} is higher that the envelope simulated for a random point pattern indicating spatial dependency between trees in a short distance (clustered spatial pattern).

Izquierda: Patrón espacial de árboles infectados con PPV (círculos grandes) y árboles sanos (círculos pequeños). Derecha**:** Función K de Ripley en función de la distancia en m (r). La línea continua corresponde a la función empírica K_{obs}, la línea discontinua a la función K teórica ($_{Ktheo}$) y el área gris corresponde a los intervalos obtenidos mediante simulación Monte Carlo para la hipótesis de aleatoriedad espacial. La función de distribución empírica K_{obs} se encuentra por encima del límite del intervalo obtenido por simulación para una distribución al azar, indicando dependencia espacial entre los árboles infectados en distancias cortas (patrón agregado).

Figure 2 (cont.). Spatial and temporal analysis of *Plum pox virus* (PPV) infection in a plot of European plum D'agen during 5 years (2007-2011).

Figura 2 (cont.). Análisis de la distribución espacial y temporal del *Plum pox viru*s (PPV) en un lote de ciruelo europeo durante 5 años (2007-2011).

2011



Left: Spatial pattern of PPV-diseased trees (large dots) and healthy (PPV-free) trees (small dots). Right: Ripley K-function *versus* distance in m (r). The black solid line corresponds to the observed K function ($_{Kobs}$), the dotted line to the theoretical K-function ($_{Ktheo}$) and the grey area to the envelope obtained through Monte Carlo simulation. K_{obs} is higher that the envelope simulated for a random point pattern indicating spatial dependency between trees in a short distance (clustered spatial pattern).

Izquierda: Patrón espacial de árboles infectados con PPV (círculos grandes) y árboles sanos (círculos pequeños). Derecha: Función K de Ripley en función de la distancia en m (r). La línea continua corresponde a la función empírica K_{obs}, la línea discontinua a la función K teórica ($_{Ktheo}$) y el área gris corresponde a los intervalos obtenidos mediante simulación Monte Carlo para la hipótesis de aleatoriedad espacial. La función de distribución empírica K_{obs} se encuentra por encima del límite del intervalo obtenido por simulación para una distribución al azar, indicando dependencia espacial entre los árboles infectados en distancias cortas (patrón agregado).

Figure 2 (cont.). Spatial and temporal analysis of *Plum pox virus* (PPV) infection in a plot of European plum D'agen during 5 years (2007-2011).

Figura 2 (cont.). Análisis de la distribución espacial y temporal del *Plum pox virus* (PPV) en un lote de ciruelo europeo durante 5 años (2007-2011).

For example, in the second study year (2008), the value recorded at 4 m was 4.75 *vs* 0.75 (expected value), at 8 m (2.93 *vs* 0.75), showing that this significant difference is large up to 60 m (1.05 *vs* 0.75), with a statistical significance p-value of 7.9×10^{-16}.

The difference starts decreasing at 64 m and up to 96 m (data not shown), with a join-count of 0.79 at 96 m in 2008, which is close to the expected value (0.75) under randomness, while p-value increases to 4.4×10^{-16}.

The same trend is observed in the following years: 2009 (1.88 *vs*. 1.51); 2010 (1.84 *vs*. 1.77) and 2011 (2.63 *vs*. 2.52).

A reduction of the statistic value is observed with increasing distance, indicating a lower significant difference between the observed and the expected values at 96 m. However, the difference remains significant, indicating aggregation of diseased plants (p value<0.05) and positive autocorrelation between nearby individuals.

Table 2. Spatial analysis of *Plum pox virus* dispersion in a European plum cv D'agen plot using the join-count autocorrelation statistic.

Tabla 2. Análisis espacial de la dispersión del *Plum pox virus* en un lote de ciruelo europeo cv D'agen a través del estadístico de autocorrelación Join-count.

Year	Distance (m)	Statistic (Join count)*	Expected Value**	Variance	P-value
2007	4	2.54	0.25	0.063	2.2×10^{-16}
	8	1.56	0.25	0.0014	2.2×10^{-16}
	12	1.40	0.25	0.007	2.2×10^{-16}
	16	1.34	0.25	0.004	2.2×10^{-16}
	20	1.20	0.25	0.003	2.2×10^{-16}
	24	0.88	0.25	0.002	2.2×10^{-16}
	36	0.57	0.25	0.00089	2.2×10^{-16}
	40	0.50	0.25	0.0007	2.2×10^{-16}
	60	0.44	0.25	0.0004	2.2×10^{-16}
	96	0.27	0.25	1.3×10^{-5}	4.4×10^{-16}
2008	4	4.75	0.75	0.180	2.2×10^{-16}
	8	2.93	0.75	0.046	2.2×10^{-16}
	12	2.35	0.75	0.022	2.2×10^{-16}
	16	2.29	0.75	0.012	2.2×10^{-16}
	20	1.98	0.75	0.008	2.2×10^{-16}
	24	1.70	0.75	0.006	2.2×10^{-16}
	36	1.29	0.75	0.0028	2.2×10^{-16}
	40	1.17	0.75	0.0024	2.2×10^{-16}
	60	1.05	0.75	0.0014	7.9×10^{-16}
	96	0.79	0.75	4.9×10^{-5}	1.2×10^{-09}
2009	4	6.87	1.51	0.346	2.2×10^{-16}
	8	5.14	1.51	0.090	2.2×10^{-16}
	12	4.13	1.51	0.043	2.2×10^{-16}
	16	3.88	1.51	0.023	2.2×10^{-16}
	20	3.47	1.51	0.015	2.2×10^{-16}
	24	3.08	1.51	0.011	2.2×10^{-16}
	36	2.40	1.51	0.0058	2.2×10^{-16}
	40	2.19	1.51	0.0052	2.2×10^{-16}
	60	1.88	1.51	0.0035	1.2×10^{-10}
	96	1.56	1.51	0.0001	1.3×10^{-7}
2010	4	9.50	1.77	0.402	2.2×10^{-16}
	8	6.52	1.77	0.105	2.2×10^{-16}
	12	5.45	1.77	0.050	2.2×10^{-16}
	16	4.77	1.77	0.017	2.2×10^{-16}
	20	4.10	1.77	0.018	2.2×10^{-16}
	24	3.69	1.77	0.013	2.2×10^{-16}
	36	2.80	1.77	0.0070	2.2×10^{-16}
	40	2.59	1.77	0.0062	2.2×10^{-16}
	60	2.24	1.77	0.0044	6.9×10^{-13}
	96	1.84	1.77	0.0001	3.3×10^{-9}
2011	4	12.33	2.52	0.557	2.2×10^{-16}
	8	8.68	2.52	0.145	2.2×10^{-16}
	12	7.17	2.52	0.070	2.2×10^{-16}
	16	6.21	2.52	0.038	2.2×10^{-16}
	20	5.34	2.52	0.025	2.2×10^{-16}
	24	4.83	2.52	0.019	2.2×10^{-16}
	36	3.86	2.52	0.0102	2.2×10^{-16}
	40	3.59	2.52	0.0094	2.2×10^{-16}
	60	3.14	2.52	0.0070	14.7×10^{-13}
	96	2.63	2.52	0.0002	3.6×10^{-12}

* The calculated (join-count statistic) and **expected statistic values for disease presence category (DD) and for W-E distances between rows every 4 m, (from 4 m to 40) as well as for 60 m and 96 m, in the plot are shown for each year from 2007 to 2011.

* El estadístico Join Count calculado y el **estadístico de valores esperados para la categoría presencia de enfermedad (DD) y para la distancia oeste-este entre filas cada 4 m (desde 4 hasta 40 m) y también para los 60 m y 96 m de distancia se muestran en la tabla, para cada año desde 2007 hasta 2011.

Evaluation of the spatial point pattern by Ripley's K-function method

The analysis of the PPV virus spatial spread via Ripley's K statistic indicated a non-random distribution of diseased trees as well as a clustered pattern in the plot. Ripley's K function was fitted for each sampling year (2007 to 2011) and data of virus presence in trees were compared as a function of the distance between them.

Figure 2 (page 268-270), shows the graphs of the theoretical Ripley's function and the fitted function for a maximum distance (r) of 23 m, corresponding to a quarter of the total distance between trees 90 m in south-north direction.

The envelope $\propto = 0.05$ (K_{hi} (r)-K_{lo} (r)) for the theoretical distribution under Complete Spatial Randomnes, obtained by Monte Carlo simulation, is shown in grey. The empirical function $K_{obs}(r)$ obtained from the data collected for each year evaluated was above the theoretical function $K_{theo}(r)$ and the simulated envelopes, suggesting that trees infected with PPV show a clustered pattern. The separation between the collected K_{obs} data and the K_{theo} theoretical curve was greatest in 2007, decreasing in the following years, even in 2011. Therefore, in the first year there would be a higher spatial dependency in the distribution of infected plants. This distribution is observed along the distance (23 m) analyzed by the Ripley's K function. Spatial dependency was higher during the first two years of study, when incidence was higher.

DISCUSSION

The European plum plot evaluated for PPV presence in Rama Caída is the only plot where PPV has been detected in San Rafael up to now.

Sample collection was made under controlled management conditions during the study period. After the end of this study, the process of eradication of positive plants, following the regulatory process for diseases of quarantine concern, was implemented.

In the D'agen plum plot, PPV positive trees were serologically detected by DAS-ELISA during the 5-year study period (2007-2011), showing increasing number of diseased plants over time. This finding indicates that the virus dispersed to healthy trees in the plot over time, but with a significantly lower number of infected plants in the last years than in the first two years, suggesting that PPV epidemic was at very early stage during the study.

PPV distribution observed by the spatial and temporal pattern suggests that PPV has spread naturally, forming heterogeneous aggregations of diseased trees in the orchard. The number of infected plants that appeared with increasing distance, gradually decreased from the focus of the earliest infected plants and especially in the last two years of study with respect to the plants detected in the first three years.

In the plum orchard in Rama Caída, spatial clusters were observed at 4 m within the row (from initial focus) and up to 65 m across rows. These small clusters indicated a relationship between sharka diseased trees (D) that were at a close distance. Over time, other isolated or weakly clustered plants appeared at a distance of 70 to 100 m (W-E) in 2010-2011, indicating a lower disease progression over space and time.

Heterogeneous aggregated spatial associations occurred close in space and time, which was confirmed with similar approximation by autocorrelation spatial analysis with Ripley's K function and Join-count. This result supports the hypothesis of tree aggregation and is in agreement with Madden *et al.* (2007). Our results indicate an aggregated point pattern that has a positive correlation, *i.e.* between the infected tree and the distance between each one.

The results also suggest that the spatially closest trees had a greater probability of becoming infected than healthy trees separated from diseased ones (at least up to 96 m), which is in agreement with Madden *et al.* (2007). Accordingly, studies of PPV-D spread in orchards from Pennsylvania (USA), conducted using autocorrelation analyses between 1999 and 2000, reported the presence of clusters, which were stronger within rows than between rows, with fewer and smaller aggregations with increasing distance (17, 20). Similarly, in apricot orchards infected with PPV-D in France, new symptomatic trees were sometimes found to be close to previously infected ones, forming infection clusters over time (24).

Other findings of PPV-D spread reported for orchards in Pennsylvania and Canada indicated that a few clusters of new infected trees appeared around already infected trees, within a range of 25-150 m (17, 20). All of these findings are in agreement with or similar to our results.

By contrast, in eastern Spain, no clusters of trees infected with PPV-D were observed between adjacent trees or within rows in apricot and peach orchards. Rather, the clusters were formed at greater distances and with no specific direction, *i.e.*, no spatial disease gradients were observed over time (18). In Rama Caída orchard, we observed a west-east trend of PPV spread. However, the formation of new associations of positive trees occurring over time did not show a disease gradient in the plot.

Studies on the spatial distribution of PPV-D conducted in different countries attributed the formation of spatial aggregations at a short distance (less than 100 m) to the virus transmission by viruliferous aphids. Distribution can result in aggregations or groups of trees that are both far from the initially infected trees and around the trees immediately adjacent to an infected tree (8, 18).

Our study shows that the area infected with PPV has expanded from west to east, coinciding with the prevailing wind direction in the area. This fact, along with results on the distribution pattern and the short distance evaluated, would suggest that the observed spread might be associated with movement of aphid vectors, a fact that has not been evaluated in this work. It has been suggested that PPV expansion would depend both on the presence of and proximity to viruliferous aphids sources (34).

The fruit species and the cultivar would also contribute to susceptibility to PPV and to the aphid, as observed in Chile (21, 22) and in France (23).

Our future research on sharka disease and PPV spread will focus on vector presence and aphid species disseminating PPV, their population dynamics and virus transmission efficiency.

Conclusion

The natural dispersion of *Plum pox virus* in European plum analyzed and quantified using mixed generalized models and functions with autocorrelation analysis, was led to determine the movement of infection among close spatially plants. These tools allowed to contrast the hypothesis of randomness of the disease in the infected bush, resulting in an aggregate distribution of the disease in aggregate, under the presence of clusters of infected trees. In turn the incidence of the disease was higher at the beginning of the study and lower for the following years, meaning that the infection rate among nearby trees decreased over time as dispersion distance increased from the first test plants. These results allowed us to evaluate the movement of the disease in European plum cv D'agen, which could be used in future studies of distribution of the virus in other cultivars or species of stone fruit and other growing regions in Argentina, as well as to improve disease management and control measures.

References

1. Atanasovv, D. 1932. *Plum pox*. A new virus disease. Annals of the University of Sofia. Faculty of Agriculture and Silviculture. 11: 49-70.
2. Auger, J.; Esterio, M. 1995. La enfermedad de la Sharka (*Plum pox Virus*) en Chile. Revista Aconex. 47: 25-8.
3. Baddeley, A.; Turner, R. 2008. spatstat: An R package for analyzing spatial point patterns. Journal of Statistical Software. 12 1-42.
4. Besag, J. 1977. Contribution to the discussion of Dr. Ripley's paper. J R Statist Soc B 39 193-5.
5. Besag, J.; Diggle, P. J. 1977. Simple Monte Carlo tests for spatial pattern. Appl Stat. 26: 327-33.
6. Bivand, R. 2014. Spdep: Spatial dependence: weighting schemes, statistics and models version 0.5-74/r555 http://R-Forge.R-project.org/projects/spdep/
7. Bryan, F.; Manly, J. 1997. Randomization, Bootstrap and Monte Carlo Methods in Biology. London. ed. CH CRC.
8. Cambra, M.; Capote, N.; Cambra, M. A.; Llácer, G.; Botella, P.; Lopez-Quilez, A. 2006. Epidemiolgy of sharka disease in Spain. Bulletin EPPO. 36: 271-5.
9. Clark, M.; Adams, A. N. 1977. Characteristics of the microplate method of enzyme-inmunosorbent assay for the detection of Plant Virus. Journal of General Virology. 34: 475-83.
10. Cliff, A. D.; Ord, J. K. 1981. Spatial Processes: Models and Applications. London. ed. 266 p.
11. Dallot, S.; Gottwald, T.; Labonne, G.; Quiot, J. B. 2003. Spatial pattern analysis of sharka disease, *Plum pox virus* Strain M, in Peach Orchards of Southern France. Phytopathology. 93: 1543-52.
12. Dal Zotto, A.; Ortego, J. M.; Raigón, J. M.; Callogero S.; Rosssini M.; Ducasse D. A. 2006. First report in Argentina of *Plum pox virus* causing Sharka disease in Prunus. Plant Disease. 90: 523.
13. Di Rienzo, J. A.; Casanoves, F.; Balzarini, M. G.; Gonzalez, L.; Tablada, M.; Robledo, C. W. 2014. InfoStat version 24-03-2014 Grupo InfoStat. FCA Universidad Nacional de Córdoba Córdoba. Argentina. Available in: http://www.infostat.com.ar/
14. Dixon, P. M. 2002. Ripley's K function En: AH El-Shaaraui, WW Piergorsch (Ed).The encyclopedia of environmetrics. New York. Wiley. 1796-803.
15. García, J. A.; Glasa, M.; Cambra, M.; Candresse, T. 2014. *Plum pox virus* and sharka: a model potyvirus and a major disease. Molecular Plant Pathology 15: 226-41.
16. Getis, A.; Franklin, J. 1987. Second-order neighborhood analysis of mapped point patterns. Ecology. 68: 473-7.
17. Gottwald, T. 2006. Epidemiology of sharka disease in North America. Bulletin EPPO. 36: 279-86.
18. Gottwald, T.; Avient, L.; Llácer, G.; Hermoso de Mendoza, A.; Cambra, M. 1995. Analysis of the spatial spread of Sharka (*Plum pox virus*) in apricot and peach orchards in eastern Spain. Plant disease. 79: 266-78.
19. Gottwald, T. R.; Sun, X.; Riley, T. R.; Graham, J. H.; Ferrandino, F.; Taylor, E. 2001. Geo-Referenced spatiotemporal analysis of the urban citrus canker epidemic in Florida. Phytopathology. 92: 361-77.

20. Gottwald, T. R.; Wierenga, E.; Luoa, W.; Parnell, S. 2013. Epidemiology of Plum pox 'D' strain in Canada and the USA. Canadian Journal of Plant Pathology. 35: 442-7.
21. Herrera, G. 2013. Investigations of the *Plum pox virus* in Chile in the past 20 years. Chilean journal of agricultural research. 73: 60-5.
22. Herrera, G.; Madariaga, M. 2003. Diseminación natural del virus causante de la enfermedad de sharka (*Plum pox virus*) en tres termporadas en un huerto de damasco. Agricultura técnica. 63.
23. Labbone, G.; Dallot, S. 2006. Epidemiology of sharka disease in France. Bulletin EPPO. 36: 267-70.
24. Labbone, G.; Yvon, M.; Quiot, J. B.; Avinent, L.; Llacer, G. 1995. Aphids as potential vectors of *Plum pox virus*: comparison of methods of testing and epidemiological consequences. Acta Horticulturae. 386: 207-18.
25. Lee, Y.; Nelder, J. A.; Pawitan, Y. 2006. Generalized linear models with random effects: unified analysis via H-likelihood. CRC Press. 416 p.
26. Madden, L. V.; Hughes, G.; van den Bosch, F. 2007. The Study of plant disease epidemics. St. Paul. MN USA. ed. 421 p.
27. Parnell, S.; Gottwald, T. R.; Irey, M. S.; Luo, W.; van den Bosch, F. 2011. A stochastic optimization method to estimate the spatial distribution of a pathogen from a sample. Phytopathology. 101: 1184-90.
28. Perry, G. L.; Mille, W. B. P.; Enright, N. J. 2006. A comparison of methods for the statistical analysis of spatial point patterns in plant ecology. Plant Ecology. 187: 59-82.
29. R Core Team. 2014. R: A language and environment for statistical computing. Vienna. Austria. Available in: http://www.R-project.org/
30. Ripley, D. B. 1977. Modeling spatial patterns. J. Royal Stat. Soc. Bulletin. 39: 72-212.
31. Rozas, V.; Camarero, J. 2005. Técnicas de análisis espacial de patrones de puntos aplicadas en ecología forestal. Invest. Agrar: Sist. Recur. Forest. 14: 79-97.
32. Upton, G. J. G.; Fingleton, B. 1985. Spatial data analysis by example. Point Pattern and Quantitative Data. ed. 422 p.
33. Varveri, C. 2006. Epidemiology of *Plum pox virus* strain M in Greece. Bulletin EPPO. 36: 276-8.
34. Varveri, C.; Dimou, D.; Zintzaras, E.; Di Terlezzi, B. 2001. Monitoring and spatiotemporal analizis PPV-M spread in two apricot orchards in southern Greece. Acta Horticulturae. 550: 129-31.

Acknowledgments

This work was supported by INTA. We are grateful to the members of the Statistics and Biometry group of FCA-UNC-CONICET, directed by Dr. M. Balzarini, for their support in statistical analyses.

Rev. FCA UNCUYO. 2020. 52(1): 276-292. ISSN (en línea) 1853-8665.

Fungal diversity and *Fusarium oxysporum* pathogenicity associated with coffee corky-root disease in Mexico

Diversidad de hongos y patogenicidad de *Fusarium oxysporum* asociados a la corchosis de la raíz del cafeto en México

Daniel López-Lima [1], Gloria Carrión [1*], Petra Sánchez-Nava [2], Damaris Desgarennes [1], Luc Villain [3]

Originales: *Recepción:* 18/10/2017 - *Aceptación:* 01/06/2018

Abstract

The disease known as coffee corky-roots associated to the infection by the root-knot nematode *Meloidogyne paranaensis* is an important issue for coffee crop in several countries. In Mexico, particularly in the Veracruz state, considerable loses are recorded annually in *Coffea arabica* plantations by corky-root disease. Previous studies have revealed the presence of fungi in coffee corky-root tissues. However, these fungi have not been yet identified. This work aimed to identify at species level the fungi associated to the coffee corky-root symptoms and determine their pathogenicity on coffee plants. Coffee roots with corky-root symptoms were collected in eight sites distributed through the major coffee growing region of Veracruz. Observations of inside cortical root tissues under scanning electron microscope revealed abundant mycelium and conidia in corky-root samples in contrast with absence of any fungi development in healthy roots. Forty-nine fungi strains from internal corky-root tissue were isolated and identified at species level by ITS sequences. *Fusarium oxysporum* was the most frequent species and the only present in all of the corky-root samples. These strains were selected for the pathogenicity test. All *F. oxysporum* strains colonized the vascular system of coffee plants although none caused wilting symptoms.

Keywords
Coffea arabica • root-knot nematodes • filamentous fungi

1 Instituto de Ecología, A.C.-Cluster Biomimic. Carretera antigua a Coatepec 351. Xalapa 91070. Veracruz. México. * gloria.carrion@inecol.mx
2 Universidad Autónoma del Estado de México. Instituto Literario 100. Toluca 50000. Estado de México. Mexico.
3 Cirad. UMR RPB, F- 34394 Montpellier. France.

Resumen

La corchosis de la raíz del café asociada a la infección del nematodo agallador de la raíz *Meloidogyne paranaensis* es un importante problema para el cultivo de café en varios países. En México, particularmente en el estado de Veracruz, se registran considerables pérdidas anuales en las plantaciones de *Coffea arabica* por esta enfermedad. Estudios anteriores han revelado la presencia de hongos en los tejidos afectados con corchosis de la raíz del café. Sin embargo, estos hongos aún no han sido identificados. El objetivo de este trabajo fue identificar a nivel de especie los hongos asociados a la corchosis de la raíz y determinar su patogenicidad en plantas de café. Se recolectaron raíces de cafetos con síntomas de corchosis en ocho sitios distribuidos a través de la principal región cafetalera de Veracruz. Las observaciones de los tejidos internos de las raíces bajo el microscopio electrónico de barrido revelaron abundante micelio y conidios en muestras de raíz con corchosis, en contraste con su ausencia en raíces sanas. Se aislaron 49 hongos de los tejidos internos afectados con corchosis y se identificaron a nivel de especie mediante secuencias de ITS. *Fusarium oxysporum* fue la especie más frecuente y la única presente en todos los sitios de muestreo, por lo que estas cepas fueron seleccionadas para la prueba de patogenicidad. Todas las cepas de *F. oxysporum* fueron capaces de colonizar el sistema vascular de las plantas de café, aunque ninguna causó síntomas de marchitez.

Palabras claves

Coffea arabica • nematodo agallador • hongos filamentosos

Introduction

Coffee takes the second place among the most worldwide traded products (after oil) providing economic livelihood to more than 125 million people. During the coffee cycle 2015/2016 the producing countries all together, exported more than 110 million, 60-kg bags (22).

In Mexico, Arabica coffee plantations represent almost the 90% of the coffee production and still plays an important socio economic role in many rural areas with high level of poverty. Additionally, Arabica plantations provide many important ecosystemic services to the country, since this crop is predominantly grown in agroforestry systems and in ecologically sensible mountainous areas (20). However, Mexico coffee exportable production has been decreasing, almost constantly, since the end of the 90's falling from around 4 million to less than half million of 60-kg bags for the last harvest, 2015/2016 (22). This dramatic decrease in production is caused by different reasons like the aging of most coffee plantations and biotic stresses. In this sense, the coffee leaf rust has affected Mexican coffee crop mainly during the last three years, in addition, to plant-parasitic nematodes which have wide distribution in all coffee growing regions of Mexico (21). Today, coffee leaf rust and plant-parasitic nematodes are the two major phytosanitary problems affecting Arabica coffee plantations throughout Latin America (1, 42).

Nonetheless, while coffee leaf rust incidence and damage are determined by many factors such as, micro and macroclimate conditions and agronomic practices, plant-parasitic nematodes represent a continued and underlying threat for both Arabica and Robusta plantations with a high potential damage. Due to the lack of analysis and detection of plant-parasitic nematodes in nurseries and the fact that seedlings show symptoms after high nematode densities are reached, field nematode infestations continue to expand.

Moreover plant-parasitic nematodes create a continued stress during the entire lifetime of the plantation. No complete eradication by control methods is possible, in addition to the unsafe usage of susceptible germplasm (41). The major nematode damages in Latin America are caused by root-knot nematodes (RKN), *Meloidogyne* spp. (42), particularly by two species associated with a devastating syndrome called coffee corky-root disease: *Meloidogyne arabicida*, to date only detected in Costa Rica (24) and *M. paranaensis*, with a wider distribution, in Brazil (11), Guatemala (42), and Hawaii (11).

In Mexico, coffee corky-root disease has been detected since the 1960s in the state of Veracruz (the second national coffee producing state) and *M. paranaensis* has been confirmed by using specific SCAR molecular markers as the RKN species linked to coffee corky-root symptoms (25).

The affected coffee trees show a progressive decline, starting with chlorosis followed by flower, leaf and fruit fall, until the death of plants. This occurs in a period between two to four years depending on agro-ecological conditions and mainly when plants begin to produce (5).

The root system of infested plants shows numerous small elongated galls on young white roots and large swelling on older and more lignified roots accompanied by large, deep and cracked cortical tissues, reminding of cork aspect (5). These corky symptoms can affect the primary roots including the taproot, up to the plant crown and even reaching the first centimeters of the stem as observed in this work. Cuttings of these corky root swellings reveal numerous *M. paranaensis* females with their egg masses (25) It is noteworthy that, in Mexico, the fungus *Fusarium oxysporum* has been strongly associated to coffee plants with corky-root symptoms (16) along with other fungi like *Cylindrocladium* sp., *Fusarium solani*, *Trichoderma* sp. and *Verticillium* sp. (36).

After the aforementioned to research those fungi directly associated with internal tissues of coffee roots damaged by nematodes, collected from different sites of coffee regions, tuns necessary. Therefore, the objectives of this work were to: i) observe the presence of fungi in the affected tissues, ii) isolate and identify the fungal community associated with the coffee corky-root disease using molecular methods and iii) conduct pathogenicity test of isolated fungi on coffee plants without the presence of the nematode *M. paranaensis.*

Materials and methods

Sampling

The sampling of coffee corky-roots was done on eight coffee plantations distributed in the main coffee cropping area of the Veracruz state, located between the eastern slope of the Mexican Trans Volcanic Belt and the southern slope of the Sierra Madre Oriental.

The sampled coffee plantations were selected based on field technical information and on previous studies that registered the presence of the corky-root disease or spots in coffee plantations with affected roots and aerial symptoms such as, chlorosis, deficient growth, defoliation and premature death of plants. On each plantation, roots were taken from 8-9 coffee plants with corky-root symptoms to form one composite sample of each sampling site. In a previous study, it was determined that in all coffee corky-root collected samples the only present RKN was *M. paranaensis* (25).

Scanning electron microscope observations

For the scanning electron microscope (SEM) observations, 3 months old seedlings of an *in vitro* propagated F1 intraspecific hybrid line of *Coffea arabica* (7) cultivated in 6 litres pots filled with previously sterilized substrate were infested with a population of *M. paranaensis* reared on tomato plants in greenhouse. This population was initially collected on coffee at one of the eight sampling sites of this study, Jilotepec site (table 1, page 281-282).

The plants were kept in a greenhouse for one year to obtain numerous corky-roots. The roots were washed with tap water to remove the excess of soil. Longitudinal cuts of corky-roots were made with scalpel, and 1 mm thickness rectangular sections (2 mm x 5 mm) of inner tissues of the corky-root parts were collected and fixed in glutaraldehyde at 2% for 5 days to preserve the structural integrity. Subsequently, the samples were submitted to a dehydration process with increasing concentrations of ethanol (10, 30, 50, 70 and 90% from 15 to 25 min in each concentration) until conserving the tissue root sections in absolute alcohol.

The samples were placed in a filter paper bag and dehydrated in a critical point camera. Then the samples were mounted on aluminum cylindrical stubs and coated with gold-palladium for its further observation under SEM. Root sections of healthy plants of same age (15 months) were collected and processed in the same way as controls.

Isolation and identification of fungi

For the fungi isolations, roots from the eight sampling sites, apparently with recently formed corky swelling, were selected to avoid saprophytic fungi that may be present in old corky-root formations. Roots were carefully washed with tap water to remove adhered soil, then disinfected by consecutively soaking in 70% alcohol (during 1 minute), 3% NaCIO (1 min), 96% alcohol (30 seconds); and then rinsed tree times with distillated sterile water. Longitudinal cuts of the corky-root tissues were made and fragments of the inner tissues were extracted and placed in Petri dishes prepared with potato dextrose agar (PDA) and chloramphenicol (1 mg mL^{-1}).

Fungi mycelia that grew from the extracted inner part of corky-root tissue fragments were transferred to other Petri dishes with PDA, until pure cultures from each isolate were obtained. To identify the fungi at species level, DNA was extracted from 25 mg of mycelia of each strain using the extraction kit: Fungal/Bacterial DNA MiniPrep Zymo Research. A molecular marker of 500 bp, that encompasses the Internal Transcriber Spacer (ITS) 1, the 5.8 rDNA, and the ITS2 molecular markers, was used and amplified by PCR (34).

The PCR products were analyzed on a 1.2 % agarose gel; and the DNA was purified and sent to Macrogen INC for sequencing.

The obtained sequences were edited in the e-Biox program and compared by BLAST analysis to the database of the National Center for Biotechnology Information (NCBI).

Pathogenicity tests of fungi in coffee plants

The 27 *Fusarium oxysporum* strains previously isolated and identified were selected for pathogenicity test because they were the only species found at all sampling sites. The inoculum was prepared by culturing mycelium of each strain in flasks with oat-yeast extract (10 g L^{-1} and 1 g L^{-1}) liquid medium. The flasks were incubated in an orbital shaker at 150 rpm and 25°C during 5 days. The conidia concentration was determined with a Neubauer chamber and was adjusted to 1.10^6 spores per mL.

Ex vitro plantlets of *Coffea arabica* with two or three pairs of leaves of a F1 intraspecific hybrid H18 (ET06 wild Ethiopian accession x introgressed Cv. Naryelis) were used for this experiment. Besides the fact that *ex vitro* plantlets acclimated in horticultural trays filled with sterilized peat-moss allowed working with pathogen-free vegetal material this germplasm micropropagated by somatic embryogenesis provided strongly homogeneous material (8). Plantlets were extracted from horticultural trays and roots were carefully washed in distilled sterile water. Two different groups of plantlets were prepared for *F. oxysporum* inoculation.

The first group of plantlets was predisposed to the fungus infection by cutting the roots 1 cm from their apex with a sterile scalpel (13, 35). In the second group, plantlets were kept with intact roots. Each strain of *F. oxysporum* was inoculated on 5 plantlets of each of the two groups by submerging rootlets in 75 mL of a conidia suspension for 20 min (17). In each case, a group of plantlets without *F. oxysporum* inoculation was used as control. Subsequently all coffee plantlets were sowed in 100 mL pots filled with a sterilized (twice autoclaving) peat moss-sand 2:1 mix and placed in a greenhouse at 25 ± 2°C with relative humidity of 80-90% a 12 hours photoperiod. The experiment was arranged under a completely randomized design. The plantlets were manually watered every 72 hours with sterile water.

45 days after the inoculation, the plants were extracted from the pots, 45 days after the inoculation. Roots were washed with sterile distilled water to remove the substrate. Symptoms like lesions, root necrosis and wilt were annotated through a scale from 1 to 5 in order to determine the severity rate of disease according to Parke and Grau (1993) and Reis and Boiteux (2007) where: 1 = Plant without symptoms; 2 = Plants without wilting symptoms, but with light brown spots on the root; 3 = Plants with vascular necrosis symptoms and wilting symptoms, but without yellowing of the leaves; 4 = Generalized necrosis in the root, wilting and severe chlorosis; 5 = Dead plant.

To detect the vascular colonization of the different *F. oxysporum* strains along the root and the stem, three plants of each strain were selected in each group of plants. The surface of both parts of the plantlets was subsequently disinfected with 70% alcohol (during 1 min), 3% NaClO (1 min) and 96% alcohol (30 seconds); then rinsed three times with sterile distilled water.

The first 3 mm next to the collar plant cutting were removed from root and stem parts.

Table 1. Geographic data of sampling sites and molecular identification of fungal species associated with coffee corky-root disease.

Tabla 1. Datos geográficos de los sitios de muestreo e identificación molecular de las especies de hongos asociadas a la corchosis de la raíz del cafeto.

Location	Municipality/ Locality	Altitude m a. s. l.	Host species, and cultivar	Isolate code	Molecular species diagnostic	% identity	NCBI accession number
N 19°54'13.1" W 97°13'28.4"	Atzalan, Napuala	689	*Coffea arabica*cv. Costa Rica 95	Na1	*Fusarium oxysporum*	99	KU847855
				Na2	*Purpureocillium lilacinum*	99	KC157741
				Na3	*Penicillium citrinum*	99	KX090324
				Na4	*Fusarium oxysporum*	99	KU847855
				Na5	*Stereum complicatum*	99	KJ140584
				Na6	*Fusarium oxysporum*	94	KY073258
N 19°51'50.8" W 97°10'06.8"	Atzalan, Chachalacas	664	*Coffea canephora* cv. unknown	Ch1	*Fusarium oxysporum*	99	LT571434
				Ch2	*Fusarium oxysporum*	99	KC304813
				Ch3	*Purpureocillium lilacinum*	100	KM458848
				Ch4	*Purpureocillium lilacinum*	99	KX347471
				Ch5	*Fusarium oxysporum*	100	KF718222
N 19°50'27.6" W 96°47'05.8"	Yecuatla, La Victoria	559	*Coffea arabica* cv. Typica	Ye1	*Beauveria bassiana*	99	KX219590
				Ye2	*Purpureocillium lilacinum*	99	FJ765023
				Ye3	*Fusarium oxysporum*	100	KX196809
				Ye4	*Alternaria longissima*	99	DQ865104
				Ye5	*Fusarium solani*	99	KU377471
				Ye6	*Alternaria longissima*	96	DQ865104
				Ye7	*Fusarium oxysporum*	100	KC304807
				Ye8	*Fusarium solani*	98	KU878142
N 19°35'42.2" W 96°53'01.4"	Jilotepec, Paso San Juan	996	*Coffea arabica* cv. Costa Rica 95	Ji1	*Penicillium citrinum*	100	KX090324
				Ji2	*Fusarium oxysporum*	100	KU680363
				Ji3	*Fusarium oxysporum*	100	KX196807
				Ji4	*Fusarium oxysporum*	100	KX058057
				Ji5	*Fusarium solani*	100	HQ176441
				Ji6	*Penicillium citrinum*	100	KX090324
				Ji7	*Penicillium citrinum*	100	KX090324

Table 1 (cont.). Geographic data of sampling sites and molecular identification of fungal species associated with coffee corky-root disease.

Tabla 1 (cont.). Datos geográficos de los sitios de muestreo e identificación molecular de las especies de hongos asociadas a la corchosis de la raíz del cafeto.

Location	Municipality/ Locality	Altitude m a. s. l.	Host species, and cultivar	Isolate code	Molecular species diagnostic	% identity	NCBI accession number
N 19°19'45.4" W 96°58'20.1"	Cosautlán, La Lagunilla	1152	*Coffea canephora* cv. unknown	Co1	*Fusarium oxysporum*	100	KT896661
N 19°19'45.4" W 96°58'20.1"	Cosautlán, La Lagunilla	1152	*Coffea canephora* cv. unknown	Co2	*Gliocladiopsis curvata*	99	JX500723
N 19°19'45.4" W 96°58'20.1"	Cosautlán, La Lagunilla	1152	*Coffea canephora* cv. unknown	Co3	*Fusarium oxysporum*	96	KC282839
N 19°19'45.4" W 96°58'20.1"	Cosautlán, La Lagunilla	1152	*Coffea canephora* cv. unknown	Co4	*Fusarium oxysporum*	100	KC304799
N 19°19'45.4" W 96°58'20.1"	Cosautlán, La Lagunilla	1152	*Coffea canephora* cv. unknown	Co5	*Fusarium oxysporum*	100	KC304807
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So1	*Fusarium oxysporum*	98	KY073257
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So2	*Fusarium oxysporum*	100	KJ699122
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So3	*Fusarium oxysporum*	100	KC304800
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So4	*Fusarium solani*	100	JQ676178
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So5	*Fusarium oxysporum*	100	KC787019
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So6	*Fusarium oxysporum*	98	XR001936475
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So7	*Fusarium oxysporum*	99	KU847855
N 19°03'16.5" W 96°55'41.3"	Ixhuatlán del Café, Moctezuma	1186	*Coffea arabica* cv. Garnica and Pacamara	Mo1	*Fusarium oxysporum*	100	EU715659
N 19°03'16.5" W 96°55'41.3"	Ixhuatlán del Café, Moctezuma	1186	*Coffea arabica* cv. Garnica and Pacamara	Mo2	*Fusarium oxysporum*	100	KC304813
N 19°03'16.5" W 96°55'41.3"	Ixhuatlán del Café, Moctezuma	1186	*Coffea arabica* cv. Garnica and Pacamara	Mo3	*Penicillium citrinum*	100	KX090324
N 19°03'16.5" W 96°55'41.3"	Ixhuatlán del Café, Moctezuma	1186	*Coffea arabica* cv. Garnica and Pacamara	Mo4	*Penicillium citrinum*	100	KX090324
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne1	*Fusarium solani*	99	JQ712137
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne2	*Purpureocillium lilacinum*	99	KX347471
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne3	*Fusarium oxysporum*	99	EF590327
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne4	*Fusarium oxysporum*	100	KX196809
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne5	*Fusarium oxysporum*	99	KC304802
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne6	*Pochonia chlamydosporia*	99	KT583165
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne7	*Fusarium oxysporum*	100	KT896661

The remaining root and stem parts were cut over a 50 mm length from the base into 10 sections of equal length. Root and stem sections of each plantlet were placed horizontally clockwise arranged into 90 mm Petri dishes with PDA-Chloramphenicol.

The Petri dishes with root and stem sections were accommodated in the laboratory under a totally randomized design and were incubated at 25°C for 8 days and examined every day for outgrowths of the fungi from the vascular ring of each root or stem section. The mycelia that grew from the root or stem fragments was transferred to Petri dishes with PDA to obtain pure cultures and to molecularly identify them in accordance with the methodology described above. The depth or height reached by the fungus inside the root or stem was determined from the re-isolation data for the root or stem sections for each plant (35).

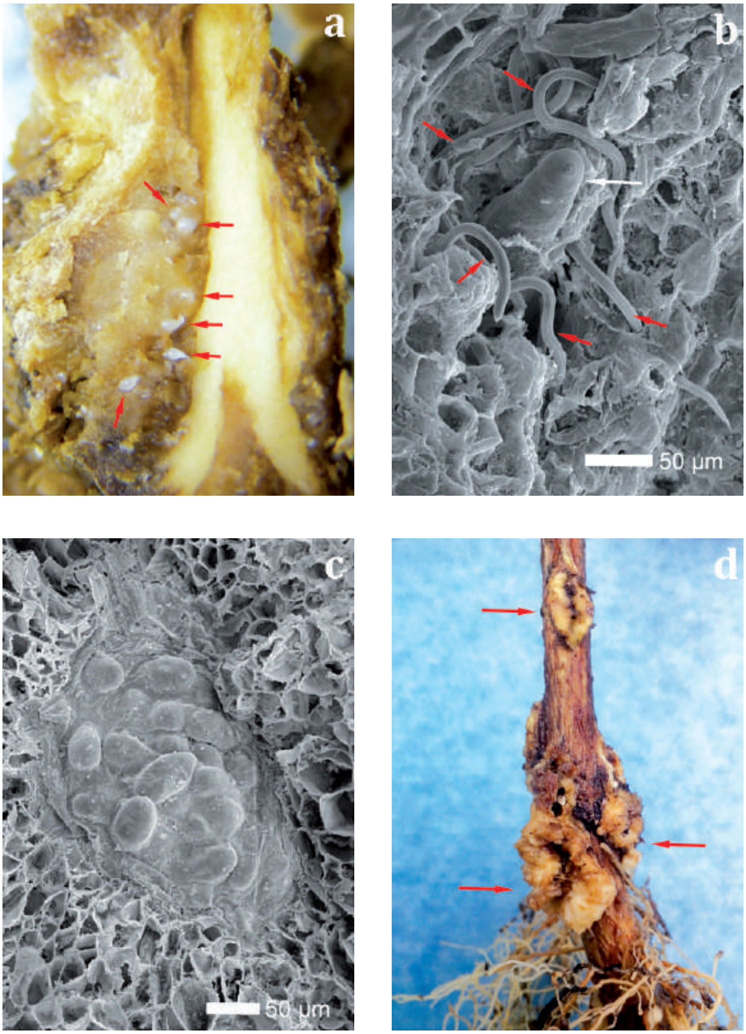
Data on symptoms and plant development were analized by one-way ANOVA for each group of plants (wounded and healthy roots). The data obtained from the frequency of re-isolation between the two groups of plants (wounded and healthy roots) and between plant organ (root and stem), were used to build a distance matrix, calculating statistical distances with the Bray-Curtis method. To assess the effect of the groups of plant and plant organ on the frequency of fungal re-isolation, the distance matrix was analyzed with a permutational analysis of variance (PERMANOVA). To compare the vascular colonization between each isolate, the data was evaluated as a function of the frequency of re-isolations in the stems and roots in the ten sections arranged from the base of the stem or root every 5mm up to 50 mm. Percentage values of re-isolations were submitted to one-way ANOVA.

Results and discussion

Coffee corky-root disease, tissue observations

Many females, eggs and second juvenile stage (J2) of *M. paranaensis* were observed in the roots affected with the coffee corky-root disease (figure 1a, 1b and 1c, page 284). Cell lesions caused by the movement of the J2 through the root tissues were also observed (figure 1b, page 284). Corky protrusions with presence of numerous *M. paranaensis* individuals (females, J2 and egg masses) were observed on the stem up to about 5 cm above ground (figure 1d, page 284). To our knowledge, this the first report of RKN presence and symptoms at this above ground distance in plant stem. The pericycle and cortical tissues of corky-roots and stem lower parts of infested coffee plants showed cell distortions and corrugations, as well as some cell wall thickenings (figure 1b and 1c, page 284; figure 2a and 2b, page 285).

No change in cortical cell volume was observed in corky-root tissues compared to healthy tissues, but hyperplasia like process was observed in cortical cell layers leading to a lateral expansion of the root or stem cortex. Presence of many conidia was observed in the cells of the infested tissues, as well as abundant mycelium crossing the cell walls, even in tissue areas where nematodes were not observed (figure 2c, d, page 285).

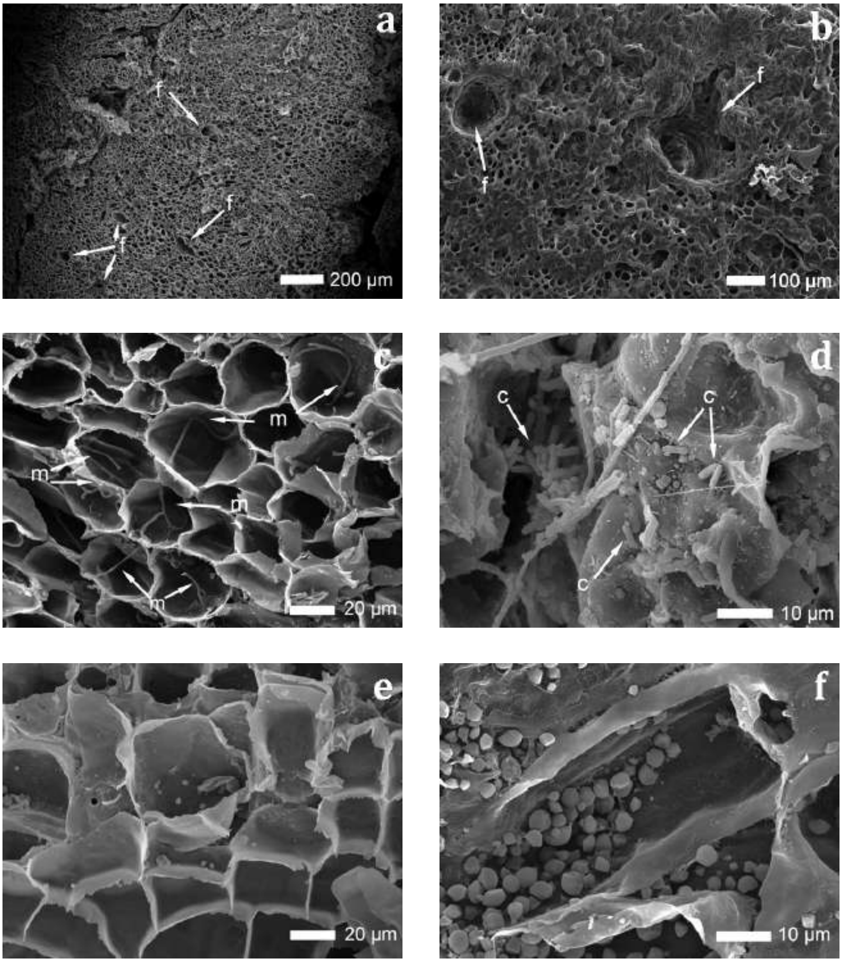


a) Longitudinal section of an infested root showing numerous *M. paranaensis* females (red arrows) inside the corky tissues 10X; **b)** Detail of a root inside corky tissue with a female (white arrow) and J2 stage juveniles (red arrows) of *M. paranaensis* 350X; **c)** Egg mass of *M. paranaensis* (center of the picture) with the eggs already wrapped by the gelatinous matrix and surrounded by distorted and corrugated tissues 270X; **d)** Corky-root symptoms on the stem base of a 12 month aged coffee seedling.

a) Sección longitudinal de una raíz infestada que muestra numerosas hembras de *M. paranaensis* (flechas rojas) en el interior del tejido afectado 10X; **b**) Detalle de una raíz dentro del tejido corchoso con una hembra (flecha blanca) y juveniles J2 (flechas rojas) de *M. paranaensis* 350X; **c)** Masa de huevos de *M. paranaensis* (centro del cuadro) con los huevos envueltos por la matriz gelatinosa y rodeados de tejidos distorsionados y corrugados 270X; **d)** Síntomas de corchosis en la base del tallo de una plántula de café de 12 meses.

Figure 1. Coffee corky-root disease symptoms associated to *Meloidogyne paranaensis* parasitism on *Coffea arabica*.

Figura 1. Síntomas de la corchosis de la raíz del cafeto asociada al parasitismo de *Meloidogyne paranaensis* en *Coffea arabica*.



a) Transversal section of a corky formation on the stem 60X; **b)** Transversal section of a corky-root 120X; **c)** Pericycle cells in an infected root with presence of mycelia 700X; **d)** Transversal section of the vascular system of a coffee corky-root with presence of mycelium and conidia 1600X; **e)** Pericycle cells in a healthy root 700X; **f)** Longitudinal section of a vascular system of a healthy coffee root with presence of numerous sap organic particles 1600X. f= lesions left by the growth of females; m= mycelium; c = conidia.

a) Sección transversal de una formación corchosa en el tallo 60X; **b)** Sección transversal de una raíz con corchosis 120X; **c)** Células del periciclo en una raíz infectada con presencia de micelio 700X; **d)** Sección transversal del sistema vascular de una raíz de café con corchosis con presencia de micelio y conidios 1600X; **e)** Células de periciclo en una raíz sana 700X; **f)** Sección longitudinal de un sistema vascular de una raíz de café sana con presencia de numerosas partículas orgánicas de savia 1600X. f = lesiones dejadas por el crecimiento de las hembras; m = micelio; c = conidios.

Figure 2. Sections of healthy and infested coffee roots and stems observed under scanning electron microscope.

Figura 2. Secciones de raíces y tallos de café sanos e infestados observados bajo microscopio electrónico de barrido.

Many organic particles which nature was confirmed by energy-dispersive X-ray spectroscopy (72.1% C and 27.9% O) (figure 2e, f, page 285), were observed in the vascular system of healthy roots. This material was not observed in the vascular systems of diseased roots, revealing a dysfunction in the vascular nutrient transport. Numerous bacteria were observed in the corky root tissues (figure 3a, b) while no bacteria were observed in healthy tissues. To date no bacteria has been reported as associated to the coffee corky-root disease. However, after these observations it seems necessary to investigate if some of these bacteria detected in the inner corky-root tissues could be involved in the pathogenesis of the disease as being part of the corky-root pathobioma or if they just have an opportunistic role as saprophytes developing on decaying tissues. Studies on tomato indicate that the communities of endophyte bacteria are significantly affected by the infection of the nematode *M. incognita bringing* some new groups of bacteria, particularly those that contribute to the nematode infection process by degrading the plant cell walls or allowing a mutualistic relation with the provision of nutrients (37).

Diversity of fungi associated to coffee corky root disease

Forty-nine fungi strains were obtained from the coffee corky-root inner tissues. According to the molecular identification, 55% of the isolates correspond to *Fusarium oxysporum*; 12% to *Penicillium citrinum*; 10% to *F. solani;* 10% to *Purpureocillium lilacinum;* 4% to *Alternaria longissima* and the remaining 8% to the following species: *Baeuveria bassiana, Gliocladiopsis curvata, Pochonia chlamydosporia* and *Stereum complicatum*.

The isolation of all fungi for each sampling site is summarized in table 1 (page 281-282). Except for *F. oxysporum* and *F. solani*, all fungi species found in this work are registered for the first time on coffee corky-roots. *Alternaria* sp., *B. bassiana, F. oxysporum* and *P. citrinum* have been registered as endophyte of healthy coffee plant roots (30, 39, 40).

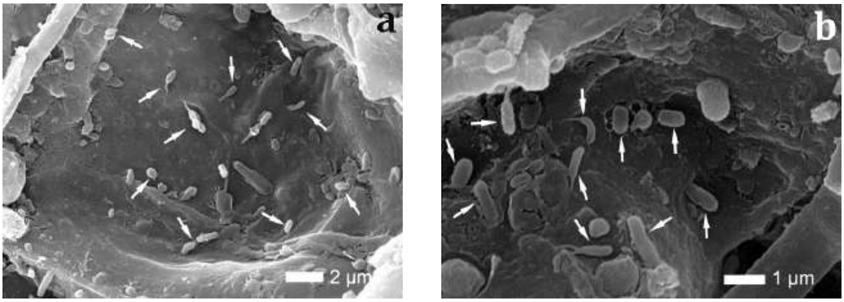


Figure 3. Bacteria inside cells of coffee corky-root tissues: **a)** 6000 and **b)** 13000X.

Figura 3. Bacterias dentro de las células de los tejidos de una raíz de café con corchosis: **a)** 6000 y **b)** 13000X.

G. curvata is a fungus previously isolated from soil and plants debris, although its ecology or role as potential pathogen of plants is less known (23). *S. complicatum* is a saprophytic fungus commonly found in decaying wood tissues (3).

P. lilacinus and *P. chlamydosporia* are fungi commonly associated to nematodes. They may be found parasitizing *M. paranaensis* (19).

F. oxysporum was the only species found in all sampling sites of this study. In Costa Rica, the simultaneous role of *F. oxysporum* and the RKN, *M. arabicida* as causal agents of a similar coffee corky-root disease was demonstrated (4). In Puerto Rico, strains identified as *F. oxysporum* f. sp. *coffeae,* have been registered as pathogen, causing vascular wilting in coffee plants infested with the RKN, *M. incognita*, but without corky-root symptoms (27).

In Brazil, this same *F. oxysporum* f. sp. *coffeae* was reported for causing vascular wilting without the presence of nematodes (10). It has also been registered in abundance in the rhizosphere of coffee plants infected with the RKN *M. exigua,* without causing any symptoms of corky-root or vascular disease. I has even been checked that some of this strains could have nematicide activity (15).

On the other hand, though *F. solani* has only been detected on four sampling sites, this fungus has also been previously detected in coffee corky-roots in the State of Veracruz (36). However, the only report of *F. solani* as a confirmed causal agent of a coffee disease is from Kenya, causing a coffee root rot (2). *F. oxysporum* and *F. solani* are considered separately as a complex of species including numerous plant pathogenic strains referred as special forms, related to some host plant(s); opportunistic strains that cause infections in humans and animals and saprophytic populations that are found commonly in soil, roots in senescence and vegetal debris (12, 38).

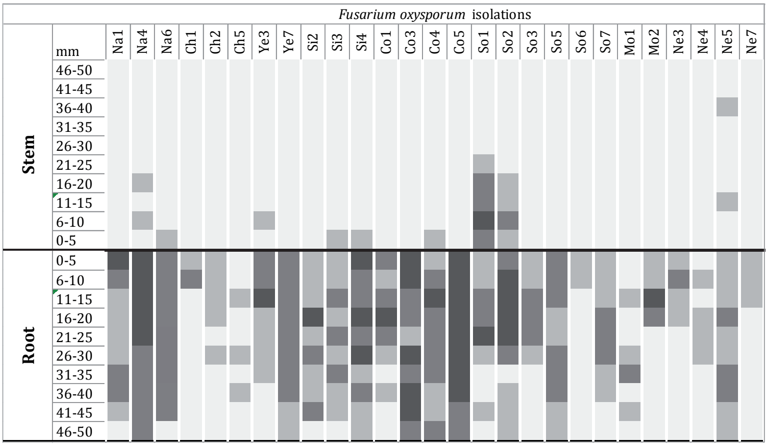
Pathogenicity and vascular colonization of *Fusarium oxysporum* on *Coffea arabica* plants.

Six weeks after inoculation, plants with the healthy and wounded roots did not show any wilting symptoms of wilting. Of the 1680 fragments examined in each group of plants, 354 re-isolations of F. oxysporum were achieved in plants with healthy roots and 288 in the plants with injured roots (0.06868, p = 0.001). No re-isolate was obtained from control plants. In both groups of plants, the fungi strains colonized the root, but not all the stems (0.11666 p = 0.001) and the re-isolation of the strains was discontinuous (figure 4 and 5, page 288).

All *F. oxysporum strains* were re-isolated in healthy roots, where 330 (93.2%) re-isolations corresponded to root and 24 (6.8%) to stems.

The strains Co5, Na4 and Co3 presented the highest (F = 7.8995, p = 0.00) frequency along the root with 93, 83 and 80% respectively. Ten strains of *F. oxysporum* were re-isolated from the stem of the plants with healthy roots.

The strain So1 presented the highest (F = 3.0639, P = 0.00) frequency of re-isolations in the stem (33%) to 30 mm hight, although the NE 5 strain was isolated at 40 mm from the stem base. In the plants with injured roots 25 of the 27 strains of *F. oxysporum* were re-isolated from the roots, 270 (93.75%) re-isolations corresponded to the root and 18 (6.25%) to stems.

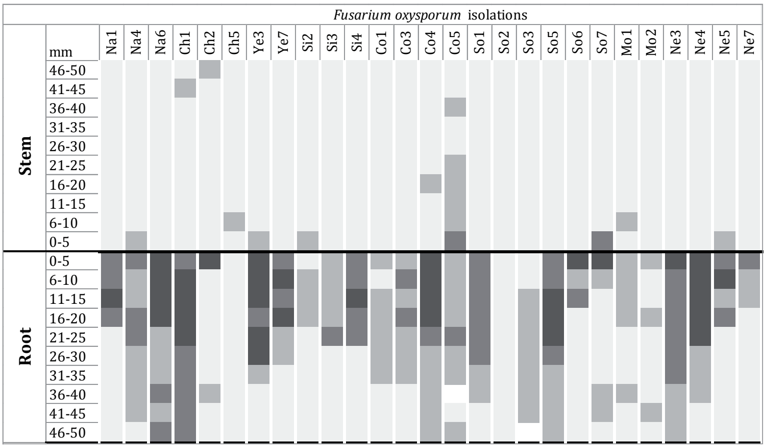


The colors on the bars indicate the frequency of re-isolation in each longitudinal section of the stem or root: 0= ■, 1= ■, 2=■, 3=■

Los colores en las barras indican la frecuencia de reaislamientos en cada sección longitudinal del tallo o de la raíz: 0= ■, 1= ■, 2=■, 3=■

Figure 4. Vascular colonization of *Fusarium oxysporum* strains in root and stem, 45 days after inoculation in *Coffea arabica* plants with not wounded roots.

Figura 4. Colonización vascular en raíz y tallo de las cepas de *Fusarium oxysporum* 45 días después de la inoculación en plantas de *Coffea arabica* con raíces sin heridas.



The colors on the bars indicate the frequency of re-isolation in each longitudinal section of the stem or root: 0= ■, 1= ■, 2=■, 3=■

Los colores en las barras indican la frecuencia de reaislamientos en cada sección longitudinal del tallo o de la raíz: 0= ■, 1= ■, 2=■, 3=■

Figure 5. Vascular colonization of *Fusarium oxysporum* strains in root and stem, 45 days after inoculation in *Coffea arabica* plants with wounded roots.

Figura 5. Colonización vascular en raíz y tallo de las cepas de *Fusarium oxysporum* 45 días después de la inoculación en plantas de *Coffea arabica* con raíces con heridas.

The Ch1 and Na6 strains presented the highest (F=3.8618, P= 0.000) percentage of re-isolations in root, with 80 and 67%, respectively. Eleven strains of *F. oxysporum* were re-isolated from the stems of this group of plants with injured root. The Co5 strain presented the highest percentage of the re isolation (23%) to the height of 40 cm (F = 3.2517, P = 0.00). However, the maximum height was recorded for the strains Ch1 and Ch2 which were found at 45 and 50 mm from the stem base, respectively.

Different studies mention that the phytopathogenic fungi that causes withering, enter the roots by mechanic wounds, like the ones caused by nematode penetration (14) and in the case the RKN by the wounds caused by the expulsion of the eggs masses (18). Nevertheless, our results it is prove that this wounds are not necessary for *F. oxysporum* to colonize the vascular systems of coffee plants, since *F. oxysporum* isolated from corky root disease, colonized coffee plant roots with and without wounds.

All the studied strains colonized the root but only some got to the stem. The movement of the fungus to the stem is considered a pathogenicity indicator. Nevertheless, in this work, none of the isolations of *F. oxysporum* caused symptoms of vascular withering (9, 26, 32).

Some studies suggest that the phytopathogenic fungi can colonize their host and behave as endophytes long before presenting any symptoms of disease, which are expressed when the host plant goes into stress (33).

In coffee plants, it might possible that the strains of *F. oxysporum* present in the roots with the corky root disease enter the plant as endophytes and remain without causing any symptoms until the plant goes into any kind of stress (29). However this hypothesis must be studied.

Conclusions

According to the observations, tissues of coffee infested with corky-root diseases have nematodes, fungi and bacteria interacting at once. *F. oxysporum* is a major fungus associated with coffee corky-root disease.

F. oxysporum strains isolated from corky-root disease do not cause wilting symptoms in coffee plants in the absence of nematode *M. paranaensis*. Nevertheless, more studies *in vivo* and at molecular level should be conducted, detecting pathogenicity genes and determining if these strains are latent pathogens or saprophytes in coffee roots before being affected by nematodes.

References

1. Avelino, J.; Cristancho, M.; Georgiou, S.; Imbach, P.; Aguilar, L.; Bornemann, G.; Läderach, P.; Anzueto, F.; Hruska, A. J.; Morales, C. 2015. The coffee rust crises in Colombia and Central America (2008-2013): impacts, plausible causes and proposed solutions. Food Security. 7(2): 303-321.
2. Baker, C. J. 1972. *Fusarium solani* associated with a wilt of *Coffea arabica* in Kenya. East African Agricultural and Forestry Journal. 38(2): 137-140.
3. Berry, F. H.; Lombard, F. F. 1978. Basidiomycetes associated with decay of living oak trees. Broomall. USA. USDA Forest Service Research Paper. 8 p.
4. Bertrand, B.; Nuñez, C.; Sarah, J. L. 2000. Disease complex in coffee involving *Meloidogyne arabicida* and *Fusarium oxysporum*. Plant Pathology. 49(3): 383-388.

5. Bertrand, B.; Ramirez, G.; Topart, P.; Anthony, F. 2002. Resistance of cultivated coffee (*Coffea arabica* and *C. canephora*) trees to corky-root caused by *Meloidogyne arabicida* and *Fusarium oxysporum*, undercontrolled and field conditions. Crop Protection. 21(9): 713-719.
6. Bertrand, B.; Anthony, F. 2008. Genetics of resistance to root-knot nematodes (*Meloidogyne* spp.) and breeding. In: Souza, R. M. (Ed.). Plant-Parasitic Nematodes of Coffee. Brazil. Springer. 165-190.
7. Bertrand, B.; Alpizar, E.; Lara, L.; SantaCreo, R.; Hidalgo, M.; Quijano, J. M.; Montagnon, C.; Georget, F.; Etienne, H. 2011. Performance of *Coffea arabica* F1 hybrids in agroforestry and full-sun cropping systems in comparison with American pure line cultivars. Euphytica. 181(2): 147-158.
8. Bobadilla, L. R.; Cenci, A.; Georget, F.; Bertrand, B.; Camayo, G.; Dechamp, E.; Simpson, J.; Herrera, J. C.; Santoni, S.; Lashermes, P.; Etienne, H. 2013. Highgenetic and epigenetic stability in *Coffea arabica* plants derived fromembryogenic suspensions and secondary embryogenesis as eevealed by AFLP, MSAP and the phenotypic variation rate. PLoS One. 8(2): e56372, http://dx.doi.org/10.1371/journal.pone.0056372.
9. Bonacci, M.; Barros, G. 2019. Genetic diversity and pathogenicity on root seedlings from three soybean cultivars of *Fusarium graminearum* isolated from maize crop residues. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 51(1): 147-160.
10. Cardoso, R. M. L. 1986. A vascular wilt of coffee (*Coffea arabica*) in the state of Parana, Brasil, caused by *Fusarium oxysporum* f. sp. *coffeae*. Fitopatología Brasileira. 11(4): 753-760.
11. Carneiro, R. M. D. G.; Cofcewicz, E. T. 2008. Taxonomy of Coffee-Parasitic Root-Knot Nematodes, *Meloidogyne* spp. In:Souza, R. M. (Ed.). Plant-parasitic nematodes of coffee. Brazil. Springer. p. 87-122.
12. Chiotta, M. L.; Chulze, S.; Barros, G. 2015. Fuentes de inóculo de especies de *Fusarium* potenciales productoras de micotoxinas en el agroecosistema soja. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 47(2): 171-184.
13. Cumagun, C. J. R.; Aguirre, J. A.; Relevante, C. A.; Balatero, C. H. 2010. Pathogenicity and aggressiveness of *Fusarium oxysporum* Schl. in bottle gourd and bitter gourd. Plant Protect. Sci. 46: 51-58.
14. Francl, L. J.; Wheeler, T. A. 1993. Interaction of plant-parasitic nematodes with wilt-inducing fungi. In: Khan, M. W. (Ed.). Nematode Interactions. Springer. Netherlands. 79-103.
15. Freire, E. S.; Campos, V. P.; Pinho, R. S. C.; Oliveira, D. F.; Faira, M. R.; Pohlit, A. M.; Noberto, N. P.; Rezende, E. L.; Pfenning, L. H.; Silva, J. R. C. 2012.Volatile substances produced by *Fusarium oxysporum* from coffee rhizosphere and other microbes affect *Meloidogyne incognita* and *Arthrobotrys conoides*. Journal of Nematology. 44(4): 321-328.
16. García, P. P.; Cid del Prado, V. I.; Zavaleta-Mejía, E.; Téliz, O. D. 1997. La corchosis del cafeto (*Coffea arabica* L.) alternativas de su manejo. Nematropica. 27(2): 111.
17. Haegi, A.; Catalano, V.; Luongo, L.; Vitale, S.; Scotton, M.; Ficcadenti, N.; Belisario, A. 2013. A newly developed real-time PCR assay for detection and quantification of *Fusarium oxysporum* and its use in compatible and incompatible interactions with grafted melon genotypes. Phytopathology. 103(8): 802-810.
18. Haseeb, A.; Sharma, A.; Ahukla, P. K. 2005. Studies on the management of root-knot nematode, *Meloidogyne incognita*-wilt fungus, *Fusarium oxysporum* disease complex of green gram, *Vigna radiata* cv ML-1108. J Zhejiang Univ SCI. 6(8): 736-742.
19. Hernández-Leal, T.; López-Lima, D.; Carrion, G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 48(2): 1-12.
20. Hernández-Martínez, G.; Manson, R.; Contreras-Hernández, A. 2009. Quantitative classification of coffee agroecosystems spanning a range of production intensities in central Veracruz, Mexico. Agriculture, Ecosystems and Environment. 134(1-2): 89-98.

21. Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP). 2005. Clones de *Coffea canephora* como patrones para injertos con tolerancia a la corchosis de la raíz del café. Folleto técnico no. 3. Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación. Available in: http:// www.inifap.gob.mx/Documents/reportes/reporte_ anual2004.pdf (Accessed: April 2016).
22. International Coffee Organization (ICO). 2016. Coffee trade statistics. Available in:http://www.ico.org (Accessed January 2017).
23. Lombard, L.; Crous, P. W. 2012. Phylogeny and taxonomy of the genus *Gliocladiopsis*. Persoonia. 28: 25-33.
24. López, R.; Salazar, L. 1989. *Meloidogyne arabicida* n. sp. (Nemata: Heteroderidae) nativo de Costa Rica. Un nuevo y severo patógeno del cafeto. Turrialba. 39(3): 313-323.
25. López-Lima, D.; Sánchez-Nava, P.; Carrión, G.; Espinosa de los Monteros, A.; Villain, L. 2015. Corky-root symptoms for coffee in central Veracruz are linked to the root-knot nematode *Meloidogyne paranaensis*, a new report for Mexico. European Journal of Plant Pathology. 141(3): 623-629.
26. Michielse, C. B.; Rep, M. 2009. Pathogen profile update: *Fusarium oxysporum.* Molecular Plant Pathology. 10(3): 311-324.
27. Negrón, J. A.; Acosta, N. 1989. The *Fusarium oxysporum* f. sp. *coffeae-Meloidogyne incognita* complex in Bourbon Coffee. Nematropica. 19(2): 161-168.
28. Parke, J. L.; Grau, C. R. 1993. Aphanomyces. In: Singleton, L. L.; Mihail, J. D.; Rush, C. M. (Eds.). Methods for research on soilborne phytopathogenic fungi. USA. APS Press. 27-30.
29. Peverelli, M. C.; Rogers, W. J. 2013. Heat stress effects on crop performance and tools for tolerance breeding. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 45(2): 349-368.
30. Posada, F.; Aime, M. C.; Peterson, S. W.; Rehner, S. A.; Vega, F. E. 2007. Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycological Research. 111(6): 749-758.
31. Reis, A.; Boiteux, L. S. 2007. Outbreak of *Fusarium oxysporum* f. sp. *lycopersici* race 3 in commercial fresh-market tomato fields in Rio de Janeiro State, Brazil. Horticultura Brasileira. 25(3): 451-454.
32. Rodríguez-Molina, M. C.; Medina, I.; Torres-Vila, L. M.; Cuartero, J. 2003. Vascular colonization patterns in susceptible and resistant tomato cultivars inoculated with *Fusarium oxysporum* f.sp. *lycopersici* races 0 and 1. Plant Pathology. 52(2): 199-203.
33. Romero, A.; Carrion, G.; Rico-Gray, V. 2001.Fungal latent pathogens and endophytes from leaves of *Parthenium hysterophorus* (Asteraceae). Fungal Diversity. 7: 81-87.
34. Schoch, C. L.; Seifert, K. A.; Huhndorf, S.; Robert, V.; Spouge, J. L.; Levesque, C. A.; Chen, W. Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS. 109(16): 6241-6246.
35. Sestili, S.; Polverari, A.; Luongo, L.; Ferrarini, A.; Scotton, M.; Hussain, J.; Delledonne, M.; Ficcadenti, N.; Belisario, A. 2011. Distinct colonization patterns and cDNA-AFLP transcriptome profiles in compatible and incompatible interactions between melon and different races of *Fusarium oxysporum* f. sp. *melonis*. BMC Genomics. 12:122.
36. Téliz-Ortíz, D.; Castillo-Ponce, G.; Nieto-Angel, D. 1993. La corchosis del cafeto en México. Revista Mexicana de Fitopatología. 11(1): 5-12.
37. Tian, B. Y.; Cao, Y.; Zhang, K. Q. 2015. Metagenomic insights into communities, functions of endophytes, and their associates with infection by root-knot nematode, *Meloidogyne incognita*, in tomato roots. Scientific reports. 5:17087.
38. van Diepeningen, A. D.; de Hoog, G. S. 2016. Challenges in *Fusarium*, a trans-kingdom pathogen. Mycopathologia. 181(3): 161-163.
39. Vega, F. E.; Posada, F.; Peterson, S. W.; Gianfagna, T. J.; Chaves, F. 2006. *Penicillium* species endophytic in coffee plants and ochratoxin A production. Mycologia. 98(1): 31-42.
40. Vega, F. E.; Simpkins, A.; Aime, M. C.; Posada, F.; Peterson, S. W.; Rehner, S. A.; Infante, F.; Castillo, A.; Arnold, E. 2010. Fungal endophyte diversity in coffee plants from Colombia, Hawaii, Mexico and Puerto Rico. Fungal Ecology. 3(3): 122-138.

41. Villain, L.; Molina, A.; Sierra, S.; Decazy, B.; Sarah, J. L. 2000. Effect of grafting and nematicide treatments on damage by root-lesion nematodes (*Pratylenchus* spp.) to *Coffea arabica* L. in Guatemala. Nematropica. 30(1):87-100.
42. Villain, L.; Sarah, J. L.; Hernández, A.; Bertrand, B.; Anthony, F.; Lashermes, P.; Charmetant, P.; Anzueto, F.; Carneiro R. M. D. G. 2013. Diversity of root-knot nematodes parasitizing coffee in Central America. Nematropica. 43(2):194-206.

Acknowledgements

The authors of this study acknowledge to Greta Hanako Rosas Saito for the coffee root observations under the scanning electron microscope. We also thank Magda Gomez Columna for their technical support in the isolation and maintenance of fungal strains.
This work was supported by the Consejo Nacional de Ciencia y Tecnología (grant number 231215).

Genetic diversity of squash landraces (*Cucurbita maxima*) collected in Andean Valleys of Argentina

Diversidad genética de poblaciones de zapallo (*Cucurbita maxima*) colectadas en los valles andinos de la Argentina

Inés María Lorello [1, 2] , Sandra Claudia García Lampasona [3, 4] , Iris Edith Peralta [2,5]

Originales: *Recepción:* 01/03/2018 - *Aceptación:* 28/07/2018

Abstract

Squash landraces (*Cucurbita maxima*) are maintained by small farmers as a major nutritional food. Twenty seven of these landraces were collected in Argentinian Andean Valleys and morphologically characterized. Genetic diversity was evaluated with microsatellite markers designed for *Cucurbita pepo* and *Cucumis melo* and evaluated for the first time in *C. maxima*. Seven microsatellite primers detected 26 alleles with 3.10 average alleles per locus. The genetic diversity reached an average of 0.26; a Polymorphic Information Content (PIC) of 0.20 and 45.5% of polymorphic *loci*. Higher diversity was found at intra population level. No evidence of lineal correlation between the observed diversity and the geographical distribution of squash landraces was found. Results demonstrate a moderate genetic diversity for all populations, with a wide range of variation in different groups. A subgroup of 10 populations with the highest levels of genetic diversity was considered for maintenance within core collections in the Vegetable Crop Germplasm Bank of Agricultural Research Station (EEA) La Consulta, Mendoza, National Institute of Agricultural Technology (INTA). Anthropogenic and environmental processes, mainly abandonment of cultivated areas and frequent droughts could erode squash landraces diversity. Conservational strategies and new collecting expeditions can be decided based on the genetic diversity found.

Keywords

Microsatellite markers • genetic diversity • germplasm banks • genetic resources. squash landraces • *Cucurbita maxima*

1 Instituto Nacional de Tecnología Agropecuaria. Estación Experimental Agropecuaria. La Consulta.

2 Universidad Nacional de Cuyo. Facultad de Ciencias Agrarias. Cátedra de Botánica Agrícola. Almirante Brown 500 Chacras de Coria. M5528AHB. Mendoza, Argentina. lorello.ines@inta.gob.ar, ilorello@fca.uncu.edu.ar

3 Instituto Nacional de Tecnología Agropecuaria. Estación Experimental Agropecuaria Mendoza.

4 Universidad Nacional de Cuyo. Consejo Nacional de Investigaciones Científicas y Tecnológicas. Instituto de Biología Agrícola de Mendoza. Facultad de Ciencias Agrarias. Mendoza, Argentina.

5 Centro Científico Tecnológico. Consejo Nacional de Investigaciones Científicas y Tecnológicas. Instituto Argentino de Investigaciones de Zonas Áridas. Mendoza, Argentina.

Resumen

En Argentina los pequeños agricultores mantienen poblaciones de zapallo (*Cucurbita maxima*) de gran importancia nutricional. Veintisiete de estas poblaciones fueron recolectadas en los valles andinos y caracterizadas morfológicamente. Marcadores microsatélites diseñados para *Cucurbita pepo* y *Cucumis melo* se aplicaron por primera vez en *C. maxima* para evaluar diversidad genética. Siete cebadores detectaron 26 alelos con 3,10 alelos promedio por *locus*. La diversidad genética alcanzó una media de 0,26; el contenido de información polimórfica (PIC) de 0,20 y el 45,5% de los *loci* resultaron polimórficos. La diversidad a nivel intrapoblacional fue mayor que entre poblaciones. No se encontró correlación lineal entre la diversidad observada y la distribución geográfica poblacional. La diversidad genética fue moderada para el conjunto de poblaciones, con un amplio rango de variación. Un subgrupo de 10 poblaciones con los mayores valores de diversidad genética fue considerado para su mantenimiento dentro del Banco de Germoplasma de la Estación Experimental Agropecuaria (EEA) La Consulta, Instituto Nacional de Tecnología Agropecuaria (INTA). Los procesos antropogénicos y ambientales, principalmente el abandono de áreas cultivadas y las frecuentes sequías, estarían erosionando la diversidad de estos recursos. Sobre la base de los resultados obtenidos se pueden plantear estrategias de conservación y nuevas expediciones de colecta.

Palabras clave

Marcadores microsatélites • diversidad genética • bancos de germoplasma • recursos genéticos • zapallo • *Cucurbita maxima*

Introduction

Squash (*Cucurbita maxima* Duchesne) is a native species of South America traditionally used for its fruit nutritious value, and is cultivated worldwide mostly in temperate zones. *Cucurbita maxima* Duchesne subsp. *andreana* (Naudin) Filov, from humid lowlands of Bolivia and warm temperate zones of Argentina and Uruguay, is considered as the putative ancestor of *C. maxima* (47, 51, 61). Archeological studies indicate that *C. maxima* was brought from Peru to northern Argentina, with evidence of domestication about 1800 years B.C. in Peru and between 500 to 1000 years A.C. in Argentina (38). Squash was domesticated and adapted to different environmental conditions in Andean areas, where diversity of local landraces is found.

The Andean Valleys of Argentina are part of the Peruvian-Bolivian center of origin of different cultivated species, and considered the southern limit of many primitive cultivars and/or crop species used since pre-Columbian times (58). These valuable genetic resources are maintained by local communities using their traditional agriculture, contributing to farmer sustainability. Squash is a fundamental food in their diet, since is highly digestible and provides valuable antioxidant nutrients such as alpha and beta carotenes, precursors of vitamin A (18, 22, 31, 59).

Squash landraces are extremely variable in fruit shape, size and color, characteristics traditionally used for their classification (6, 34).

Cucurbita maxima is a diploid (2n = 2x = 40), decline, allogamous species with a broad genetic base (10). However, conservation of crop diversity is threatened due to environmental and socioeconomic factors, such as frequent droughts and farms abandonment. In this context, landraces preservation is a priority in Argentina. Recuperation of Andean squashes was part of a first systematic effort to preserve the diversity of local crops (2, 3, 41, 43, 56).

Molecular tools are currently widely used for evaluating crop diversity (14, 32, 34, 37). genetic diversity of *C. maxima* collections has been determined with different markers (4, 20, 21, 30, 64) like microsatellites (33, 36, 65, 68).

This study aimed to evaluate 27 populations of *C. maxima* collected in Andean Valleys of Argentina (56), using microsatellites markers (65, 68) for the first time in Argentinian *C. maxima* germplasm. These landraces were previously morphologically characterized (43).

The results obtained are strategic to preserve squash landraces diversity in germplasm banks.

Materials and Methods

Collecting expeditions and morphological characterization

Eight collecting expeditions, between 2005 and 2008, were conducted covering agro-ecological regions of northwestern Argentina (NOA), Cuyo and Patagonia (figure 1, page 296). An exhaustive collection of squash accessions was performed in Andean communities where local farmers maintained their crops in different environments using diverse crop managements (41, 56). Twenty seven accessions of various morphotypes (figure 2, page 297) collected in different localities during the first two expeditions to Valle Fértil (Province of San Juan), and Northwestern Argentina (table 1, page 298), were selected for field evaluation (41, 43), and molecular characterization. During two seasons 49 plant, flower, fruit, and seed traits were measured using morphological traits for the genus *Cucurbita* (27, 43). As a control, *C. maxima* 'Marino FCA' and 'Veronés INTA' were used (43, 56).

DNA extraction

Genomic DNA of five seedlings per accession of the 27 selected landraces (table 1, page 298) was extracted (13). In addition, two cultivars of *C. maxima* 'Marino FCA' and 'Veronés INTA', one of *Cucurbita moschata* Duchesne 'Paquito INTA' and one of *Cucurbita pepo* L. were included.

Amplification and visualization of microsatellite markers

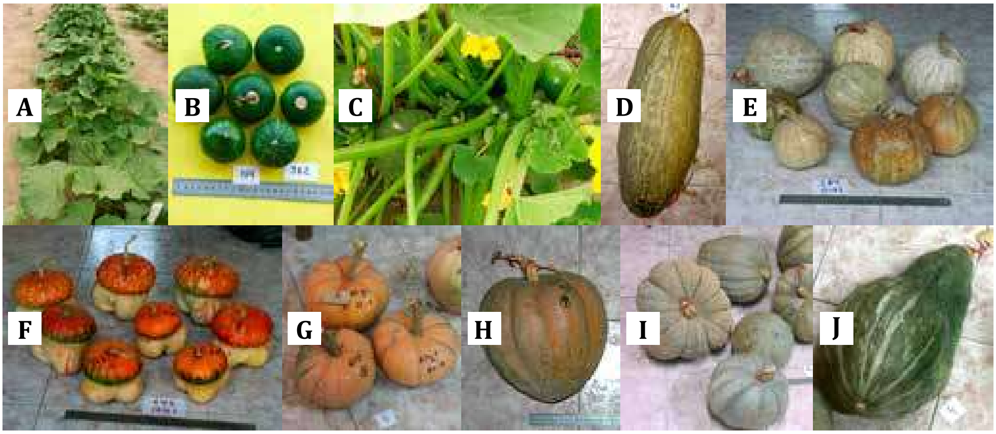
At the time this study was conducted, no reports of microsatellite markers specifi- cally designed for *C. maxima* were available. Therefore markers developed from microsatellites designed for *Cucumis melo* L. (65) and *C. pepo* (68) were used for the first time in *C. maxima*. Two hundred fifty-seven primer pairs designed for *C. melo* were evaluated in five *C. maxima* accessions and two controls of *C. melo* species. Eight primers designed for *C. pepo* and recommended as polymorphic for *C. maxima* (68) were first evaluated in five *C. maxima* accessions and one accessions of *C. pepo* species as control. PCR experiments were performed on a PTC-100 Thermocycler MJ Research Inc. (Bio-Rad, Foster City, California, USA).

Ecoregions are highlighted with different colors: NEA: Northestern Argentina (Chaco, Formosa, Entre Ríos, Corrientes, Misiones); NWA: Northwestern Argentina Jujuy, Salta, La Rioja, Catamarca, Tucumán, Santiago del Estero); Cuyo (San Juan, Mendoza, San Luis); Pampas (Córdoba, Buenos Aires, La Pampa, Santa Fe); Patagonia (Río Negro, Neuquén, Chubut, Santa Cruz, Tierra del Fuego).

Las ecorregiones se destacan con diferentes colores: NEA noreste argentino (Chaco, Formosa, Entre Ríos, Corrientes, Misiones);NOA noroeste argentino (Jujuy, Salta, La Rioja, Catamarca, Tucumán, Santiago del Estero); Cuyo (San Juan, Mendoza, San Luis); Pampa (Córdoba, Buenos Aires, La Pampa, Santa Fe); Patagonia (Río Negro, Neuquén, Chubut, Santa Cruz, Tierra del Fuego).

Figura 1. Geo-referenced collection points of *C. maxima* landraces in Argentina.

Figure 1. Puntos de colecta de poblaciones de *C. maxima* en Argentina georreferenciados.



a, b, c "Zapallito redondo del tronco" (*C. maxima* Duch. var. zapallito (Carrière) Millán) (variety consummed only in Argentina: bushy plants, with fruits growing near the principal stem, edible immature fruit). **d-j** Winter squashes (plants with long trailing vines, edible mature fruit): **d** Elongated oblong. **e** Globular show type. **f** Turban. **g** Flattened. **h** Acorn delicious type. **i** Flattened and/or globular creole gray squash type. **j** Elliptical or oval Hubbard type (27).

a, b, c "Zapallito redondo del tronco" (*C. maxima* Duch. var. zapallito (Carrière) Millán) (variedad consumida solo en Argentina. Desarrolla plantas sin guías, sus frutos crecen cerca del tallo principal y se consumen inmaduros). **d-j** Zapallos de invierno (plantas con guías largas, fruto comestible a la madurez): **d** oblongo alargado. **e** Tipo globular. **f** Turbaniforme **g** Achatado. **h** Tipo delicioso. **i** Zapallo gris criollo aplanado y/o globular**. j** Tipo Hubbard elíptico u oval (27).

Figure 2. Fruit morphotypes found among *C. maxima* landraces.

Figura 2. Morfotipos de frutos encontrados en las poblaciones de *C. maxima*.

The following reaction mixture to 15 µl final volume was formulated: 40ng DNA, 5x buffer with magnesium chloride (final concentration of 1.5mM $MgCl_2$), 0.16 mM dNTPs, 0.03µM of each primer; 1 unit Taq polymerase (Promega, Madison, Wisconsin, USA,). Samples were subjected to the following thermal profile for amplification: four minutes of denaturing at 94°C, 35 cycles of denaturing at 94°C for 45 seconds, 45 seconds of annealing at 48°C and 30 seconds of elongation at 72°C, with a final elongation step of 72°C for five minutes. PCR products were evaluated by electrophoresis on a 6% denaturing polyacrylamide gels (PAGE). Amplification fragments were visualized on a 3130 ABI Genetic Analyzer (Applied Biosystems, Foster City, USA), primers that amplified polymorphic fragments were synthesized attaching a DNA fragment known as M13 (-21) universal tail (62) on the forward primer 5' end. This fragment is complementary to the universal tail M13 (-21) which is labeled with a fluorescent dye and added to the reaction mixture of PCR. Thus, during the polymerase chain reaction, fragments are labeled by incorporating a tail sequence and can be evaluated by capillary electrophoresis and laser detection. The new mixture of PCR included the forward primer synthesized with M13 (-21) tail, reverse primer and tail labeled M13 (-21) with each of the following fluorescent dyes: FAM, JOE, HEX or TAMRA. Mix 15 µl reaction was formulated by the following way: 40ng DNA, 5x buffer, 2mM $MgCl_2$, 0.16mM dNTPs, 0.05µM forward tailed primer, 0,2µM reverse primer, 0.2µM universal M13 tail (-21) marked, 0.5 units Taq polymerase (Promega, Madison, Wisconsin, USA).

Table 1. Squash accessions.

Tabla 1. Entradas evaluadas.

Acc.	Alt.	S Lat.-W Long.	Locality-Department-Province
13 (687)	961	30°29.843-'67°34.514'	Puesto San Marcos. San Agustín del Valle Fértil. San Juan
14 (688)	929	30°31.768-67°33.701'	Usno. San Agustín del Valle Fértil. San Juan
17 (678)	1133	30°19.930'-67°39.299'	Baldes Del Rosario. San Agustín del Valle Fértil. San Juan
19 (694)	653	30°55.544'-67°15.346'	Baldes Del Rosario. San Agustín del Valle Fértil. San Juan
20 (696)	1249	30°13.275'-67°41.736'	Baldecitos. San Agustín del Valle Fértil. San Juan
22 (697)	906	30°33.974'-67°32.288'	Usno. San Agustín del Valle Fértil. San Juan
24 (699)	722	30°47.871'-67°19.641	Agua Cercada. Valle Fértil. San Juan
36 (681)	640	30°55.750'-67°15.54'	Baldes De Astica. San Agustín del Valle Fértil. San Juan
53 (348)	1208	27º56.493'-65º41.887'	La Higuera. Balcosna. Catamarca
62 (361)	1274	27°51.769'-65°45.830'	Balcosna de Afuera. Catamarca
76 (620)	1906	26°43.865'-66°03.179'	Loro Huasi. Santa María. Catamarca
127 (309)	1215	27°43.082'-67°08.234'	Londres. Catamarca
143 (387)	1246	27º39.160'-67º1.358'	Artasa. Belén. Catamarca
161 (673)	1263	27º37.895'-67º01.580'	Belén. Catamarca
173 (2482)	2083	27°09.279'-66°42.368'	Los Nacimientos. Catamarca
185 (676)	2131	26º21.446'-66º01.552'	Pichao. Tucumán
195 (418)	1951	25º59.672'-66º1.686'	San Antonio. Cafayate. Salta
210 (424)	2004	25°59.749'-66°01.681'	San Antonio. Cafayate. Salta
215 (425)	1999	25º59.611'-66º1.498'	San Antonio. Cafayate. Salta
225 (428)	1811	26°05.982'-66°00.953'	Divisadero. Salta
233 (430)	2432	25º22.507'-66º26.134'	Refugios. Luracatao. Salta
244 (431)	2400	25º22.195'-66º25.994'	Refugios. Luracatao. Salta
284 (434)	1663	24°02.022'-65°26.015'	Cabrerías. Luracatao. Salta
324 (440)	2293	23°41.405'-65°27.011'	Chañarcito. Jujuy
367 (441)	2370	23º39.246'-65º25.851'	Hornillos. Jujuy
382 (443)	2761	23°30.892'-65°24.643'	Juella. Tilcara. Jujuy
523 (445)	3169	23º5.324'-65º22.926'	Hornaditas. Humahuaca. Jujuy

Acc. accession number, between brackets number of passport of INTA EEA La Consulta Germplasm Bank; S Lat.-W Long.: Geographical coordinates of collection points; political location.

Acc.: número de entrada, entre paréntesis número de pasaporte, Banco de Germoplasma EEA La Consulta, INTA; S Lat.-W Long.: coordenadas geográficas del punto de colecta; ubicación política.

PCR conditions were the following: 5 minutes of denaturing at 94°C, 40 cycles of five minutes of denaturing at 94°C, 45 seconds of annealing at 48°C and 45 seconds of elongation at 72°C; in the following 11 cycles, the annealing temperature was increased to 53°C, with a final elongation of 10 minutes at 72°C. Table 2 (page 299) lists used primers. The results were analyzed using GeneMapper 4.0 software (Applied Biosystems, Foster City, USA).

Table 2. Microsatellite primers selected.
Tabla 2. Cebadores microsatélites seleccionados.

Name	Expected size (pb)	SSR motif	Forward	Reverse
cm 22	177	(AG)24	5´-TGT AAA ACG ACG GCC AGT CCA AAA CGA CCA AAT GTT CC-3´	5´-ATA CAG ACA CGC CTT CCA CC-3´
cp 24	130-150	(AG)4	5´-TGT AAA ACG ACG GCC AGT GTG CTG CAT GTT GGA TGT CT-3´	5'- GTG ACC ATG GAC AAC ACG TC-3´
cp 25	100-120	(CACC)4	5´-TGT AAA ACG ACG GCC AGT CTC TTC CGA TTC TCC GCT TA-3´	5´-TTC GAA CTT GAG CAA GCA AA-3´
cp 33	190-200	(TC)3(CACC)4	5´-TGT AAA ACG ACG GCC AGT CTC TTC CGA TTC TCC GCT TA-3´	5´- CCG ATC AAG AAC AGC ACA GA -3´
cp 46	160-180	(CACC)4	5´-TGT AAA ACG ACG GCC AGT TCT TCC GAT TCT CCG CTT AG-3´	5´-GCA CAG AAA ACG GGG TAA AA-3´
cp 52	180-195	(CACC)4	5´-TGT AAA ACG ACG GCC AGT TCA CTT CTC CCC TTC TCT GC-3´	5´-TTC GAA CTT GAG CAA GCA AA -3´
cp 56	130-150	(CACC)4	5´-TGT AAA ACG ACG GCC AGT TCC ATT TCC ACT CAT TTT TC-3´	5´-GAT CCA GTT GAA GCG ATT AC-3´

Primer name, expected fragment size expressed in base pairs (bp), microsatellite motif and nucleotide sequence of the forward and reverse primers used for microsatellite amplification. In red: universal fragment sequence M13 (-21) coupled to the forward primer in its 5 'end.

Denominación del cebador, tamaño del fragmento esperado expresado en pares de bases (pb), motivo del microsatélite y secuencia nucleotídica de los cebadores Forward y Reverse utilizados para la amplificación de los fragmentos microsatélites. Se destaca en rojo la secuencia del fragmento universal M13 (-21) anexada al cebador Forward en su extremo 5´.

Data Analysis

Genetic variability from population allele frequencies and genotype was determined. Genetic diversity parameters were estimated for each *locus* and for multiple *loci*: polymorphic *loci* proportion and average genetic diversity, using the formula:

$$D = 1 - \frac{1}{m}\sum_{j=1}^{m}\sum_{i=1}^{l}\rho_{ij}^{2}$$

where
m = number of *loci*
pij = frequency of allele
i = locus *j*

Genetic diversity per *locus* (52), average observed heterozygosity (Ho), unbiased or expected heterozygosity of Nei (He) (53), number of alleles per *locus* and number of effective alleles per *locus* (35) were also calculated. Squash accessions were compared based on the different measures of genetic variability by non-parametric Friedman test (23) under a classification criterion. Population structure was evaluated by calculating allele fixation index *F* that describes the reduction of population heterozygosity:

$$F = \frac{(He - Ho)}{He} = 1 - (Ho / He)$$

Wright's F statistics (70) were measured following the definition of Nei (1973), and the gene flow among populations was estimated from Fst statistic using the formula of Wright (1951). For measuring biological diversity, allelic richness (r) by direct counting and the Shannon-Weaver index (H) (61) were calculated according to the following formula:

$$H = -\sum_{i}^{S} p_i x \log_{2pi}$$

where

S = total number of types of traits studied

pi = measure of relative abundance of each of these types.

Genetic distances between accessions were established by the simple matching index of similarity (66). Similarity was converted to distance by the transformation:

$$\sqrt{1-S}$$

where

S = the similarity coefficient (simple matching)

Analysis of molecular variation (19) was applied to evaluate population structure using squared Euclidean distances. Principal Coordinates Analysis (25) was carried out on the proximity matrix to order populations by similarity. Molecular and morphological data were correlated by Mantel test (45) and the Generalized Procrustes Analysis (15, 26). Percentage of consensus between the two ordinations (molecular and morphological) was calculated as a measure of association between the two groups of markers.

Accessions were grouped according to their morphological characteristics and molecular profiles by cluster analysis, implicating genetic distances of Gower and unweighted pair-group arithmetic average method (UPGMA) algorithm. The InfoGen version 2011 (7) and Genalex 6 (55) softwares were used.

Results

Six from eight designed primers for *C. pepo* were selected. Two hundred fifty-four primer combinations designed for *C. melo* were monomorphic and three polymorphic in *C. maxima*.

Only one primer combination of the three polymorphic ones, amplified consistently according to the conditions detailed in materials and methods. These results demonstrate the difficulty to transfer microsatellite marker between *Cucumis* and *Cucurbita* species.

Seven primer combinations (table 2, page 299) were finally selected to analyze all *C. maxima* accessions and controls (table 1, page 298). Thirty-one alleles with an average of 4.4 alleles per primer were obtained. Twenty six alleles were detected in *C. maxima*, and only five amplified exclusively in *C. moschata* and *C. pepo* accessions used as controls (table 1, page 298). One microsatellite marker (CP24) was monomorphic for all *C. maxima* accessions. However, it gave a differential band for *C. pepo,* becoming useful for discriminating this species (41).

Genetic variability

Genetic diversity estimated for all accessions of *C. maxima* reached an average value of 0.26 (0.007-0.66), Ho average for all seven *loci* was 0.17 (0-0.54), He of 0.23 (0-0.69), *loci* polymorphic percentage of 45.5%, and PIC 0.2 (0.007-0.61). Ho per *locus* was lower than He in all cases. Nevertheless, accessions 17, 53, 76, 127, 143, 195, 215, 225, 233, 244, 382 (table 1, page 298) had allele fixation indices (F) with average negative values over all seven *loci*, indicating a slight excess of heterozygous individuals.

Around eighty percent of the evaluated accessions were homozygous at five *loci* (CP25, CP33, CP46, CP52 and CP56). The difference between populations was given by the presence of certain combinations of fragments instead of being defined by unique fragments.

Forty eight percent of alleles were unique or rare, with frequencies below 5%.

One allele was exclusive for *C. moschata*, four for *C. pepo* and three for *C. maxima*. Moreover, seven alleles resulted rare for *C. maxima* accession set (table 3). Some of these unique alleles allowed adjusting a routine technique in seed lots to discriminate an interspecific commercial hybrid between *C. maxima* and *C. moschata* (40).

Populations 22, 36, 53, 62, 127, 161, 225, 233, 382 and 523 significantly overcame the others in their values of genetic diversity, PIC, Ho and He (figure 3, page 302).

In terms of population structure, a moderate differentiation in population allele frequencies (Fst 0.199) with a small to moderate non-random effect mating within populations (Fis 0.099) was observed.

Molecular analysis of variance (AMOVA) showed that the genetic variability ($p < 0.05$) was large within accessions (82%). Coefficients Phi pop and Phi st (0.18) indicated a moderate differentiation among accessions and individuals from the same population, considering as high values those above 0.25. Regarding accessions diversity and their geographic origin in Argentina (Provinces), only 2.03 % of the variability is explained by this distribution, being this value not significant ($p = 0.18$). The degree of gene flow (Nm) was close to 1 (Nm = 0.88).

Biodiversity

Biodiversity indices showed Catamarca as the Argentinean Province with the highest allelic richness, with 22 of the 26 amplified alleles for *C. maxima*, followed by Salta and San Juan, both with 19 alleles. Catamarca also had the greatest value for the Shannon-Weaver index, surpassing the total mean value, followed by Jujuy and Salta in order of magnitude (table 4). The results found in Catamarca, Salta and Jujuy agree with values obtained in the number of polymorphic *loci*, genetic diversity, He and average number of alleles (figure 4, page 303).

Table 4. Allelic richness (r) and Shannon-Weaver index (Shaw) by province.

Tabla 4. Riqueza alélica (r) e índice de Shannon-Weaver (Shaw) por provincia.

Province	r	ShaW
Total	26	2-61
San Juan	19	2.44
Catamarca	22	2.72
Tucumán	12	2-28
Salta	18	2.45
Jujuy	16	2.54

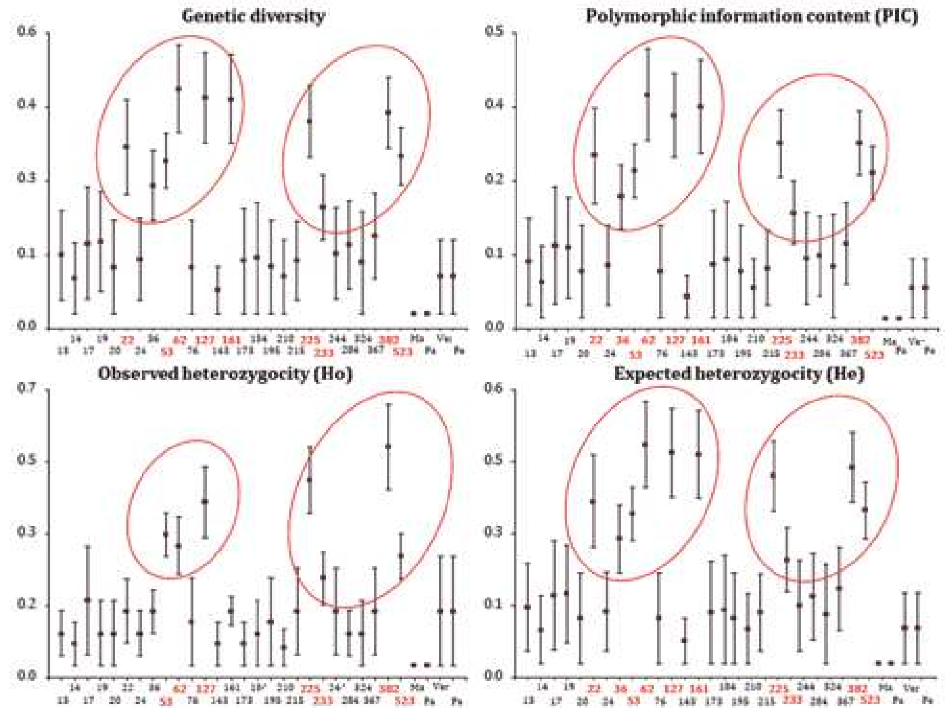
Bootstrap cycles = 1150; confidence 0.95.
Ciclos Bootstrap = 1150; confianza 0,95.

Table 3. Unique or rare alleles found in the different species evaluated.

Tabla 3. Alelos raros o únicos encontrados en las diferentes especies evaluadas.

SSR name	*Cucurbita maxima*	*Cucurbita moschata*	*Cucurbita pepo*
CP24		183 pb (unique)	
CP25	113 pb (rare)		
CP33	192 bp (rare)		200 pb (unique)
CP46	176 bp (rare)		186 bp, 169 bp (unique)
CP52	158 bp (rare)		
CP56	164 bp (rare) 171 pb (unique, acc. 22)		
CM22	170 bp (rare) 168 pb (rare) 184 bp (unique, acc. 184) 162 pb (unique, acc. 215)		171 bp (unique)

Acc.: accession number. / Acc.: número de entrada.



Numbers in the abscissa indicate *C. maxima* accessions (table 1, page 298); accessions used as controls: Ma 'Marino FCA' and Ve 'Veronés INTA' *C. maxima*, Pa: 'Paquito INTA' *C. moschata*, Pe: 'Pepo angola' *C. pepo*.

En la abscisa se indican las poblaciones de *C. maxima* (tabla 1, pág. 298) y las variedades utilizadas como controles: Ma 'Marino FCA' y Ve 'Veronés INTA' *C. maxima*, Pa: 'Paquito INTA' *C. moschata*, Pe: 'Pepo angola' *C. pepo*.

Figure 3. Average population profiles based on: Genetic Diversity, PIC, Ho, He and their corresponding standard errors.

Figura 3. Perfiles poblacionales promedio y sus errores estándar basados en los parámetros de Diversidad Genética, PIC, Ho, He.

Genetic distances

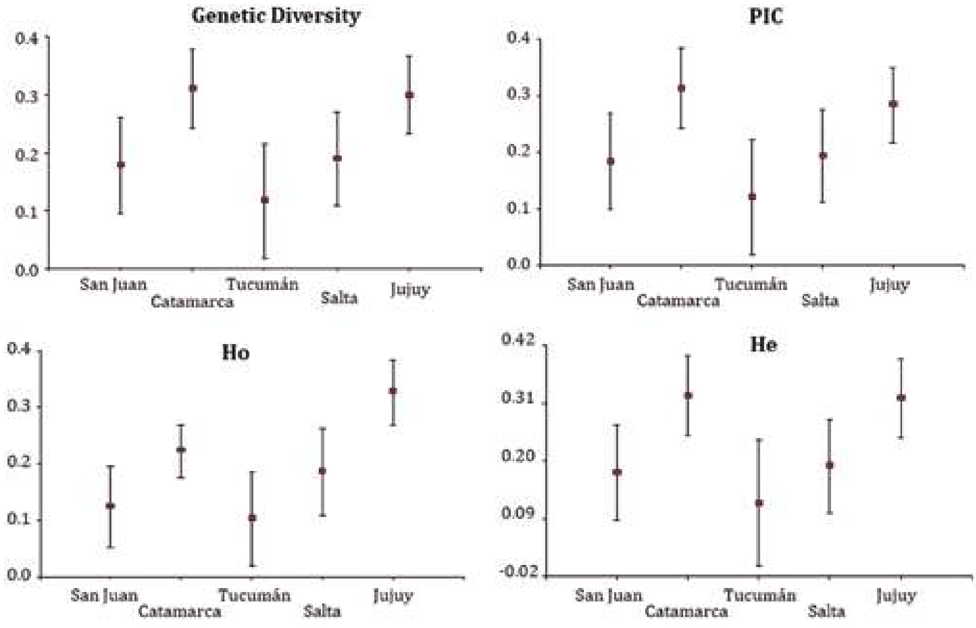
Genetic distances among populations reached values ranging from 0.18 to 0.82, with an average of 0.50. In many cases, genetic distances between *C. maxima* populations were greater than the average distance between *C. maxima* and *C. moschata* species, used as control.

A subset of squash populations were dissimilar to the majority of the accessions and exceeded the average distance for all populations evaluated. This special subgroup included accessions 22, 36, 53, 62, 127, 161, 225, 233, 382 and 523 (figure 3).

Cluster analysis

The first three coordinates in Principal Coordinates Analysis (PCoA) explained 56% of observed variability (figure 5, page 304) and produced four accessions clusters.

First coordinate grouped accessions based on genetic diversity parameters: group number 1 (G1) included accessions 62, 127 and 161 from Catamarca, which recorded the highest number of alleles (21), greater number of effective alleles (2.04), genetic diversity (0.43) and He (0.48).



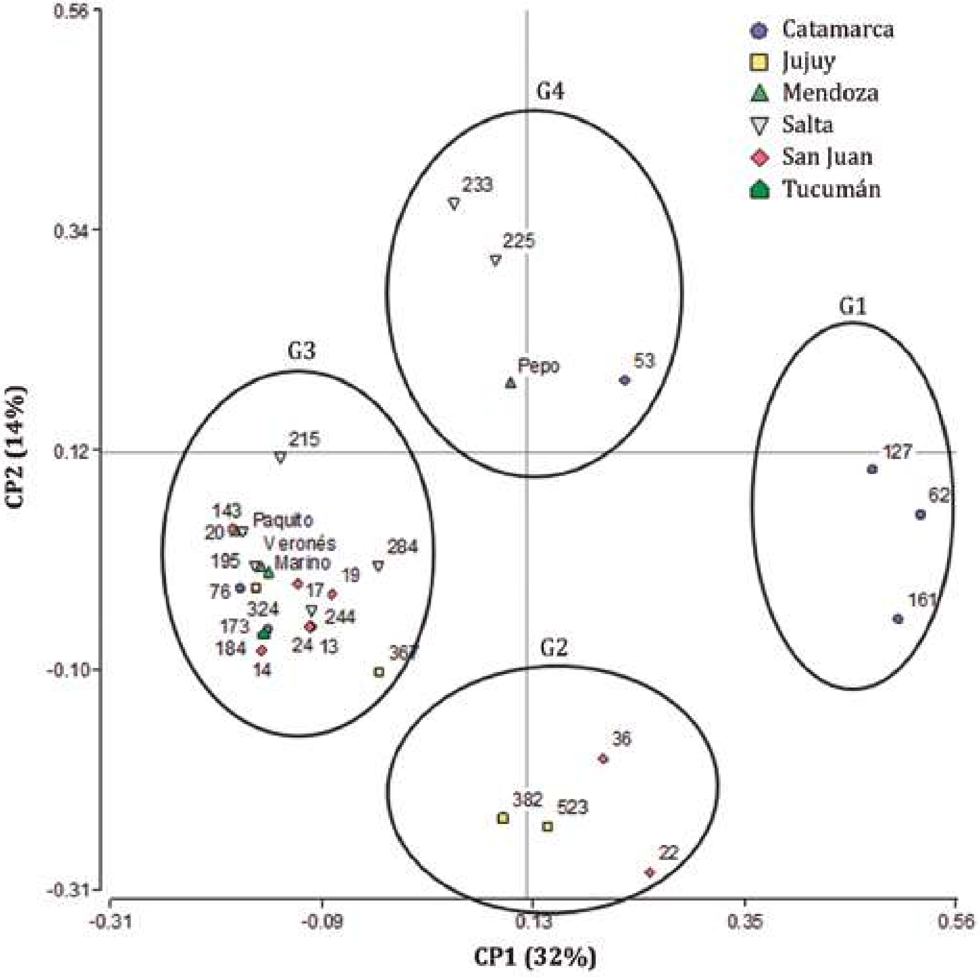
Province names in the abscissa, indices values in ordinates.

Nombre de las provincias en las abscisas, valores de los índices en las ordenadas.

Figure 4. Average provincial profiles based on genetic diversity, PIC, Ho, He, and their corresponding standard errors.

Figura 4. Gráfico de perfiles promedio provinciales, con sus errores estándar, de diversidad genética, PIC, Ho, He.

A core group (G2) with intermediate characteristics of diversity included accessions 22, 36, 523, 382; with 15 alleles and average genetic diversity values of 0.3, Ho of 0.31, He of 0.34, an average number of 2.1 alleles and 1.56 effective alleles. The third group (G3) was the least diverse including the majority of the accessions and controls of *C. maxima* and *C. moschata* species. This group had 10 alleles and a lower value of polymorphic *loci* of 0.22 % *versus* 0.86 % found in the previous groups. The genetic diversity of G3 was 0.1, Ho of 0.09, He of 0.11, average number of alleles was 1.45 and 1.27 effective alleles. The second coordinate ordered the depending on the number of shared alleles. Accessions 233, 225, 53 and *C. pepo* accession formed group 4 (G4), differing from the previous clusters and sharing five of the eight alleles present in *C. pepo.* However, this last control presented lower diversity values than the rest of the accessions of G4. The third coordinate separated accessions 17, 62, 173 and 184 for sharing a rare heterozygous genotype for the CM22 marker with two alleles of 168 and 185 bp (figure 5, page 304).



Marino FCA and Veronés INTA (C. maxima), Paquito INTA (C. moschata) and Pepo (C. pepo) showing the first two coordinates of the Principal Coordinates Analysis (ACoorP) and four groups (G1-G4). Numbers indicate accessions according to table 1 (page 298); icons indicate Argentinean Provinces.

Marino FCA y Veronés INTA (C. maxima), Paquito INTA (C. moschata) y Pepo (C. pepo) según las dos primeras coordenadas del Análisis de Coordenadas Principales (ACoorP) y cuatro grupos (G1-G4). Los números en el gráfico indican las poblaciones de acuerdo a la tabla 1 (pág. 298); los íconos indican la provincia de procedencia.

Figure 5. Scatter plot of 27 populations of *C. maxima* and 4 controls.

Figura 5. Gráfico de dispersión de 27 poblaciones de *C. maxima* y 4 testigos.

Generalized Procrustes Analysis, based on previous morphological data (41, 42, 43, 56) and molecular information, explained 78.7% of the variability in the first two axes. Ordering consensus between molecular and morphological markers was moderate (73.65%), in addition 14 of the 27 populations showed a consensus order below means (73.65%). Both, molecular and morpho- logical markers discriminated against the two controls belonging to *C. maxima* from the set of populations: Marino FCA differed to a greater extent at morphological level and Veronés INTA at molecular level.

A cluster analysis was also performed using morphological and molecular data. Interestingly, four clusters of accessions were also generated, mainly explained by fruit traits, growth habit, and type of consumption (figure 6, page 306).

Molecular genetic variability was taken in consideration in second place.

Cluster 1 included accessions from *C. maxima* var. *zapallito* "zapallito redondo del tronco", with an average genetic diversity of 18%, 40% of polymorphic *loci* and 10.75 average alleles per accession.

Cluster 2 included entries with mixtures of commercial types; genetic diversity reached 7%, 14.3% polymorphic *loci* and eight average alleles per accession.

Cluster 3 included accessions with long vines and winter fruits type with a genetic diversity average of 11.3 %, 26 % polymorphic *loci* and 10.41 alleles per accession.

Cluster 4 included those entries with larger winter fruits and greatest genetic variability; genetic diversity averaged 35.7%, 85.75% of the *loci* were polymorphic and amplified 17 alleles per accession on average.

Discussion

The Argentinean squashes landraces from different Andean environments are characterized for the first time by molecular markers and now preserved in the Vegetable Crop Germplasm Bank of EEA La Consulta, Mendoza, which belongs to the National Network of Germplasm Collections, INTA. An initial screening of microsatellite from *C. pepo* and *C. melo* allowed the selection of proper markers to use in *C. maxima* diversity analysis. A technique for generating amplification products and detecting useful markers for conservation of C. maxima genetic resources in germ- plasm banks, was developed. These results will facilitate future work in molecular studies.

The transferability of microsatellite markers between *C. maxima* and *C. melo* was very low, but one selected microsatellite effectively segregated *C. moschata* accession, and was useful in interspecific hybrid detection for breeding programs (39).

Most, diversity values were similar to the ranges found in other *Cucurbitaceae* studies (8, 20, 60, 67), and lower than those obtained by Lv *et al*. 2012, Mashilo *et al*. (2016) and Kong *et al*. 2014.

Meanwhile, Hamrick and Godt (1996) suggested a polymorphic *loci* ratio of 40% and an expected heterozygosity of 0.168 for *Cucurbitaceae*, similar to those found in the Argentinean accessions evaluated.

High frequency of homozygous genotypes for *loci* CP25, CP33, CP46, CP52 and CP56 suggests a tendency of allele fixation by mating between relatives. The small size of *C. maxima* populations grown in orchards of Andean farmers could favor inbreeding.

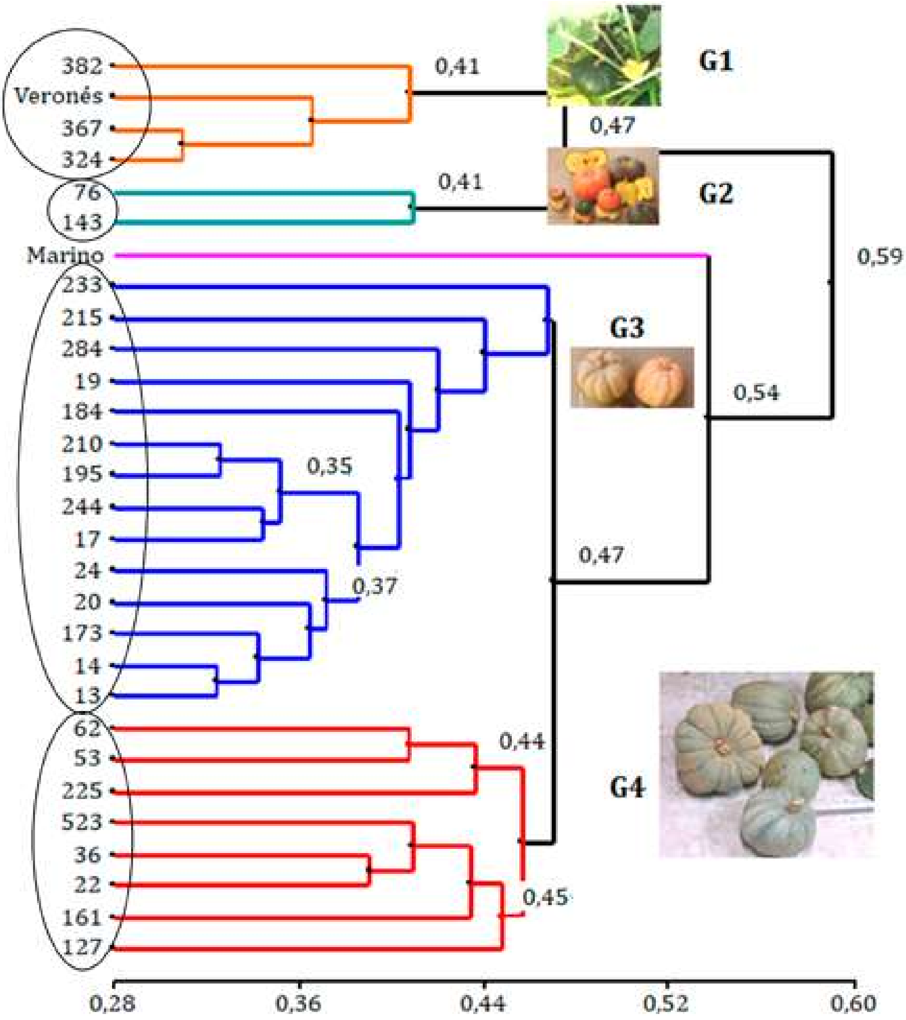


Figure 6. Cluster analysis based on morphological and molecular traits of 27 *C. maxima* accessions and two controls: 'Marino FCA' and 'Veronés INTA' *C. maxima*.

Figura 6. Análisis de conglomerados de 27 entradas de *C. maxima* y dos testigos de la misma especie: Marino FCA y Veronés INTA, utilizando caracteres morfológicos y moleculares.

Rare alleles found are of great interest for the improvement and preservation of biodiversity given that they match with genetically diverse entries. Moreover, the presence of unique alleles allowed differentiating related species such as *C. maxima*, *C. moschata* and *C. pepo*. These rare alleles may also be useful to assess gene flow, pollen, and seed contamination, and to facilitate identification of duplicates and unique accessions in gene banks. Species-specific alleles for Cucurbita species were also observed by Ferriol *et al.* (2003) and Kazminska *et al.* 2017.

Genetic differentiation among entries and among individuals within the same accession was moderate (Phi st and Phi pob 0.18), coinciding with the results obtained by Andrés (1990), Cerón González *et al*. (2010), and Decker-Walters *et al*. (1990) within the genus *Cucurbita*. The degree of gene flow, close to one, indicates that genetic drift acts independently and may generate population differentiation. Migration rate and genetic drift act in equivalent magnitudes. Genetic drift is favored by the gradual reduction in the number and size of *C. maxima* cultivated populations as well as by anthropic selection. On the other hand, gene flow acts as a force that maintains genetic cohesion between populations (17, 57). Different results were found by Mashilo *et al*. (2016) in *Lagenaria siceraria* (Molina) Standl. with low differentiation levels justified by the high levels of genetic flow.

Observed variability was concentrated at intra population level (82%), coinciding with previous studies in related species as *Citrullus lanatus* L. (49) and *C. moschata* (9). Reproductive system of *C. maxima* propitiates intra population diversity because of its flower protandry, high fertility, monoecious structure and entomophily pollination. Moreover, *Cucurbitaceae* natural seed dispersal is carried out by animals that consume their fruits and disperse them with their feces. Farmer's management includes diversification strategies for food production supporting the observed intra population diversity observed. These strategies include cultivating diverse *C. maxima* varieties and other cucurbit species simultaneously inside the orchard without reproductive barriers, and selecting cultivars for their different qualities for consumption, conservation features, and resistance to pests, drought and diseases.

Closest populations generally tend to be similar as geographical proximity favors gene flow. However, a consistent pattern for this statement, is not observed. There is no linear spatial structuring of the observed genetic diversity and no evidence of isolation by distance, meaning that population genetic differentiation, is not explained by geographic distance. These results agree with those obtained by Montes-Hernández and Eguiarte (2002), who pointed out the high potential for pollen dispersion of *Cucurbita* genus despite having specific pollinators.

In addition, the farmer's tradition of seed exchange, deeply anchored in Andean culture, contributes to generate genetic diversity in family orchards. Thus, geographical isolation of farmers in some high Andean valleys of north-western Argentina, mainly in Salta and Jujuy, seems to lack the effect of promoting greater differentiation among accessions found in different areas. The results differ from those of Nanoumé *et al.* (2013), who studied watermelon landraces from Mali founding positive correlation between genetic distances and geographical ones, indicating "that seed exchange has not been so widely used that it can overcome local adaptation".

Biodiversity indices point Catamarca and Jujuy as the Provinces with the greatest genetic diversity for cultivated squashes. These results are consistent with Vavílov (1931), who postulate the region of the tropical Andes, including NW Argentina, as the center of origin of *C. maxima*. Higher genetic diversity at molecular level in NWA region was also evident at the morphological level (41, 43); particularly in Calchaquíes valleys region (Catamarca, Jujuy) where probably some factors allow greater genetic differentiation. Environmental conditions where temperate climate prevails, with an average altitude of 1200 m and access to irrigation, may promote the establishment of larger orchards where the effects of genetic drift are attenuated.

Values of biological diversity observed for 27 accessions of *Cucurbita maxima* were higher than those found by Ceron González *et al*. (2010) for *Cucurbita argyrosperma* Huber, *Cucurbita ficifolia* Bouché, *C. moschata,* and *C. pepo*. Indices of genetic and biological diversity, in general, had a wide range of variation among entries. Most accessions showed an excess of homozygotes relative to what would be expected if populations were in Hardy-Weinberg equilibrium, revealing loss of genetic variability. This phenomenon favors fertility decline, and lower adaptative capacity.

However, a subset of ten entries (22, 36, 53, 62, 127, 161, 225, 233, 382 and 523) showed a slight excess of heterozygotes. These entries had significantly higher diversity indices, showed rare and unique alleles and constituted unique gene pools. These features place them in an advantageous position against environmental changes that exert selective pressure on *C. maxima*. Also, these entries result interesting for suggesting progenitors for hybrid varieties, and inferring evolutionary and crop management phenomena.

Genetic distances were similar to those found by Ferriol *et al*. (2003) and higher than the ones in Baranek *et al*. (2000) and Gong *et al.* (2013) for *C. maxima*. They were also higher than the ones of Ntuli *et al*. (2015) for *C. pepo*, Kong *et al*. (2014) for *C. maxima* and *C. moschata*, and Wu *et al*. (2011) for *C. moschata*.

In many cases, genetic distances between populations of *C. maxima* was greater than the average distance between *C. maxima* and the control (*C. moschata* species). These results may indicate the presence of interspecific gene flow between *C. maxima* and *C. moschata* due to the tradition of cultivating different different, altogether in orchards. Decker-Walters *et al*. (1990) mentioned the natural formation of interspecific hybrids between *C. maxima* and *C. moschata*. Therefore the existence of these hybrids within the set of studied populations, is feasible.

No clear association was found between molecular and morphological markers. This lack of correlation corroborates that the used molecular markers, were designed from genomic libraries enriched in microsatellites (65, 68). Markers obtained by this method are generally found in unexpressed genomic regions.

Generalized Procrustes Analysis allowed a wide interpretation of the relationships between accessions and to get a better description of the genetic diversity (12). Molecular markers discriminated populations of *C. maxima* better than morphological ones. There is a tendency that the most vigorous and larger fruit populations express higher genetic diversity. However, the population number 382 resulted to be one of the most diverse of the collection, even though it belongs to *C. maxima* var. *zapallito* ("zapallito redondo del tronco"). This result may be due to the significantly lower number of populations of this commercial type, representing 3 out of the 27 populations evaluated.

Molecular diversity was lower than morphological diversity. Despite of the great range of morphotypes and agro-ecological adaptation of *C. maxima* native populations, its genetic base is not as wide as expected. These results agree with other native plant species of South America, such as bean (*Phaseolus vulgaris* L.) (5), peanut (*Arachis hypogea* L.) (28), and maize (*Zea mays* L.), where many races with different morphological characteristics are observed, but are genetically closely related (11). Results indicate that the Andean populations of *C. maxima* derived from one or a few wild populations of *C. maxima* subsp*. andreana*, possibly domesticated in the humid lowlands of Bolivia and in warm temperate areas of South America (61). The morphological diversity observed would be the result of species adaptation to Andean heterogeneous ecological environments, and anthropic selection.

Conclusion

Microsatellite markers revealed moderate genetic diversity among 27 *C. maxima* landraces from different Andean environments. Genetic diversity, both between (18%) as within populations (82%) was found. A subset of ten entries showed significantly higher diversity indices, constituiting unique gene pools.

This evaluation will allow the incorporation of *C. maxima* populations in breeding programs; facilitate its management in the Vegetable Crop Germplasm Bank of INTA EEA La Consulta; enable broadening the species genetic base, raise core collections, and aid in planning future collecting expeditions. Moreover, it will enable to establishing *in situ* and *ex situ* conservation strategies and promote its use by the Andean communities.

References

1. Andrés, T. C. 1990. Biosystematics, theories on the origin and breeding potential of *Cucurbita ficifolia*. In: Bates D.M. (ed) Biology and Utilization of the Cucurbitaceae. Edited by D.M. Bates. Comstock Publishing Associates. Ithaca. New York. 102-119 p.
2. Asprelli, P. D.; Lorello, I. M.; Occhiuto, P. N.; Togno L. S.; Makuch M. A.; García Lampasona, S. C.; Peralta, I. E. 2012. First collection of landrace vegetable crops cultivated in Valle Fértil, Argentina. Agriscientia. 29: 41-50.
3. Asprelli, P. D.; Occhiuto, P. N.; Makuch M. A.; Lorello, I. M.; Togno L. S.; García Lampasona, S. C.; Peralta I. E. 2011. Recolección de germoplasma criollo de especies cultivadas y su distribución en regiones andinas de Argentina. Horticultura Argentina. 30(71).
4. Athanasios, L.; Tsivelikas, A. L.; Koutita, O.; Anastasiadou, A.; Skaracis, G. N.; Traka-Mavrona, E.; Koutsika-Sotiriou, M. 2009. Description and analysis of genetic diversity among squash accessions. Braz Arch Biol Technol. 52: 271-283. DOI: 10.1590/S1516-89132009000200003.
5. Balarezo, J. C.; Camarena Mayta, F.; Baudoin, J. P.; Huaringa Joaquin, A.; Blas Sevillano, R. 2009. Evaluación agromorfológica y caracterización molecular de la ñuña (*Phaseolus vulgaris* L.). Idesia (Chile). 27: 29-40. DOI: 10.4067/S0718-34292009000100005.

6. Balkaya, A.; Özbakir, M.; Kurtar E. S. 2010. The phenotypic diversity and fruit characterization of winter squash (*Cucurbita maxima*) populations from the Black Sea Region of Turkey. African Journal of Biotechnology. 9 (2): 152-162. Available in://www.academicjournals.org/AJB.
7. Balzarini, M. G.; Di Rienzo, J. A. 2011. InfoGen versión 2011. FCA, Universidad Nacional de Córdoba. Argentina. Available in: http://www.info-gen.com.ar. [accessed 28 October 2015].
8. Baranek, M.; Stift, G.; Vollmann, J.; Lelley, T. 2000. Genetic diversity within and between the species *Cucurbita pepo, C. moschata* and *C. maxima* as revealed by RAPD markers. Cucurbit Genet Coop Rpt. 23: 73-77.
9. Barboza, N.; Albertazzi, F. J.; Sibaja-Cordero, J. A.; Mora-Umaña, F.; Astorga, C.; Ramírez, P. 2012. Analysis of genetic diversity of *Cucurbita moschata* (D.) germplasm accessions from Mesoamerica revealed by PCR SSCP and chloroplast sequence data. Scientia Hort. 134:60-71. doi:10.1016/j.scienta.2011.10.028.
10. Benítez Burraco, A. 2005. Avances recientes en biotecnología vegetal e ingeniería genética de plantas. Reverté S. A. Barcelona.
11. Blas, R.; Ribaut, J.; Warburton, M.; Chura, J.; Sevilla, R. 2002. Análisis molecular de razas de maíz peruano con marcadores AFLP y microsatélites. In: Simposium: El mejoramiento genético de las plantas en el Perú. Sociedad Peruana Genética. 3: 241-250.
12. Bramardi, S. J.; Bernet, G. P.; Asíns, M. J.; Carbonell, E. A. 2005. Simultaneous agronomic and molecular characterization of genotypes via the generalised procrustes analysis: an application to cucumber. Crop Sci 45: 1603-1609. DOI: 10.2135/cropsci2004.0633.
13. Brown, R. N.; Myers, J. R.; Hutton, M.; Miller, P. 1998. A simple protocol for isolating DNA from fresh Cucurbita leaves. Cucurbit Genet Coop Rpt. 21: 46-47.
14. Carloni, E.; López Colomba, E.; Ribotta, A.; Quiroga, M.; Tommasino, E.; Griffa, S.; Grunberg, K. 2018. Analysis of genetic variability *in vitro* regenerated buffelgrass plants through ISSR molecular markers. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 1-13.
15. Cerón González, L.; Legaria Solano, J. P.; Villanueva Verduzco, C.; Sahagún Castellanos, J. 2010. Diversidad genética en cuatro especies mexicanas de calabaza (*Cucurbita spp.*). Rev Fitotec Mex. 33: 189-196.
16 Decker-Walters, D. S.; Walters, T. W.; Poluszny, U.; Kevan, P. G. 1990. Genealogy and gene flow among annual domesticated species of Cucurbita. Can J Bot. 68: 782-789.
17. Ellstrand, N. C. 2014. Is gene flow the most important evolutionary force in plants? American Journal of Botany. 101(5): 737–753. DOI: 10.3732/ajb.1400024. http://www.amjbot.org/ © 2014 Botanical Society of America.
18. Esteras, C.; Gómez P.; Monforte A. J.; Blanca J.; Vicente-Dólera N.; Roig C.; Nuez F.; Picó, B. 2012. High-throughput SNP genotyping in *Cucurbita pepo* for map construction and quantitative trait loci mapping. BMC Genomics. 13:80. Available from http://www.biomedcentral.com/1471-2164/13/80. [accessed 13 November 2017].
19. Excoffier, L.; Smouse, P.; Quattro, J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics. 131: 479-491.
20. Ferriol, M.; Picó, B.; Nuez, F. 2003. Genetic diversity of some accessions of *Cucurbita maxima* from Spain using RAPD and SBAP markers. Genet Resources Crop Evolution. 50: 227-238.
21. Ferriol, M.; Picó, B.; Nuez, F. 2004. Morphological and molecular diversity of a collection of *Cucurbita maxima* landraces. J Amer Soc Hort Sci. 129: 60-69.
22. Ferriol, M.; Picó, B. 2008. Pumpkin and winter squash. In Handbook of Plant Breeding Vegetables I Part 4. Volume 1. Edited by: Prohens J, Nuez, F. Springer. 317-349.
23. Friedman, M. 1937. The use of ranks to avoid the assumption of normality implicit in the analysis of variance. J Amer Stat Assn. 32: 675-701.
24. Gong, L.; Paris, H. S.; Stift, G.; Pachner, M.; Vollmann, J.; Lelley, T. 2013. Genetic relationships and evolution in Cucurbita as viewed with simple sequence repeat polymorphisms: the centrality of *C. okeechobeensis.* Genet Resour Crop Evol. 60:1531-1546. DOI: 10.1007/s10722-012-9940-5.

25. Gower, J. C. 1967. Multivariate analysis and multidimensional geometry. Statistician. 17: 13-28.
26. Gower, J. C. 1975. Generalized procrustes analysis. Psychometrika. 40: 33-51.
27. Guzmán, L.; Ávila, G.; Céspedes, M. 2001. Lista de Descriptores de Cucurbita. En Catálogo de recursos genéticos bolivianos de: Amaranthus, Capsicum, Cucurbitaceae, Lupinus y Phaseolus: conservados en el banco de germoplasma del Centro de Investigaciones Fitoecogenéticas de Pairumani. Edited by Centro de Investigaciones Fitoecogenéticas de Pairumani (ed). Catálogo de recursos genéticos bolivianos de: Amaranthus, Capsicum, Cucurbitaceae, Lupinus y Phaseolus: conservados en el banco de germoplasma del Centro de Investigaciones Fitoecogenéticas de Pairumani. Cochabamba. 69-79 p.
28. Halward, T.; Stalker, T.; Larue, E.; Kochert, G. 1992. Use of single primer DNA amplifications in genetic studies of peanut (*Arachis hypogea* L.). Plant Mol Biol. 18: 315-325.
29. Hamrick, J. L.; Godt, M. J. W. 1996. Conservation genetics of endemic plant species. In Conservation genetics case histories from nature. Edited by J. C. Avise J. C. and J. L. Hamrick J. L. (eds) Conservation genetics case histories from nature. Chapman & Hall, New York. 281-304 p.
30. Heikal, A. H.; Abdel-Razzak, H. S.; Hafez, E. E. 2008. Assessment of genetic relationships among and within Cucurbita species using RAPD and ISSR markers. J Appl Sci Res. 4(5): 515-525.
31. Jaeger de Carvalho, L. M.; Barros Gomes, P.; de Oliveira Godoy, R. L.; Pacheco S.; Fernandes do Monte, P. H.; Viana de Carvalho, J. L.; Regini Nutti, M.; Lima Neves A. C.; Rodrigues Alves Vieira, A. C.; Rabelo Ramalho Ramos, S. 2012. Total carotenoid content, α-carotene and β-carotene, of landrace pumpkins (*Cucurbita moschata* Duch): A preliminary study. Food Research International. 47: 337-340.
32. Kalia, R. K.; Rai, M. K.; Kalia, S.; Singh, R.; Dhawan, A. K. 2011. Microsatellite markers: an overview of the recent progress in plants. Euphytica. 177: 309-334. DOI: 10.1007/s10681-010-0286-9.
33. Kazmińska, K.; Sobieszek, K.; Targońska, M.; Korzeniewska, A.; Niemirowicz-Szczytt, K.; Bartoszewski, G. 2016. Genetic diversity analysis of winter squash (Cucurbita maxima Duchesne) accessions using SSR markers. In Cucurbitaceae 2016, XIth Eucarpia Meeting on Cucurbit Genetics & Breeding, Warsaw. Poland. Edited by Kozik, E. U.; Paris, H. S. p. 210-213.
34. Kazmińska K.; Sobieszek, K.; Targońska-Karasek, M.; Korzeniewska, A.; Niemirowicz-Szczytt, K.; Bartoszewski, G. 2017. Genetic diversity assessment of a winter squash and pumpkin (*Cucurbita maxima* Duchesne) germplasm collection based on genomic Cucurbita-conserved SSR markers. Scientia Horticulturae 219 (2017) 37-44. Available in: http://dx.doi.org/10.1016/j.scienta. 2017.02.035.
35. Kimura, M.; Crow, J. F. 1964. The number of alleles that can be maintained in a finite population. Genetics. 49: 725-738.
36. Kong, Q.; Chen, J.; Liu, Y.; Ma, Y.; Liu, P.; Wu, S.; Huang, Y.; Bie, Z. 2014. Genetic diversity of Cucurbita rootstock germplasm as assessed using simple sequence repeat markers. Scientia Horticulturae 175: 150-155. Available in http://www.sciencedirect.com/science/article/pii/S0304423814003252?via%3Dihub. [accessed 10 December 2017].
37. Kozub, P. C.; Barboza, K.; Cavagnaro, J. B.; Cavagnaro, P. F. 2018. Development and characterization of SSR markers for *Trichloris crinita* using sequence data from related grass species. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 1-16.
38. Lira, R. 1995. Estudios taxonómicos y ecogeográficos de las Cucurbitaceae latinoamericanas de importancia económica: Cucurbita, Sechium, Sicana y Cyclanthera. In Systematic and Ecogeographic Studies on Crop Gene Pools N° 9. International Plant Genetic Resources Institute. Rome. 1-115 p.
39. Lorello, I. M.; Leite, T.; García Lampasona, S.; Salles Cortopassi Buso, G. 2009. Transferencia de Marcadores microsatélites desarrollados en *Cucumis melo* para su utilización en *Cucurbita maxima* y *Cucurbita moschata*. VII Simposio Nacional de Biotecnología REDBIO-Argentina y II Congreso Internacional de Biotecnología Vegetal. Rosario. 138 p.

40. Lorello, I. M.; García Lampasona, S.; Della Gáspera, P. G. 2010. Identificación molecular del híbrido interespecífico Aconcagua INTA, obtenido del cruzamiento entre las líneas LC1 (*Cucurbita maxima*) y LC2 (*Cucurbita moschata*). XIV Congreso Latinoamericano de Genética ALAG 2010. Viña del Mar. 23 p.
41. Lorello, I. M. 2012. Recolección, conservación y caracterización morfológica y molecular de poblaciones "criollas" de zapallo (*Cucurbita maxima*) colectadas en los valles andinos de la Argentina. Phd thesis, Dissertation Abstracts 98. Programa de Doctorado en Biología (PROBIOL). Universidad Nacional de Cuyo. Mendoza.
42. Lorello, I. M.; García Lampasona S. C.; Peralta I. E. 2013. Caracterización de zapallos criollos (*Cucurbita maxima* Duch.), colectados en Valle Fértil, San Juan, y en el Noroeste argentino. XXXVI Congreso Argentino de Horticultura ASAHO y II Congreso Internacional de Plásticos Agrícolas. Tucumán. 298 p.
43. Lorello, I. M.; García Lampasona, S. C.; Makuch M. A.; Peralta I. E. 2016. Caracterización morfo-agronómica de poblaciones de zapallo criollo (*Cucurbita maxima* Duch.) colectadas en los valles andinos de la Argentina. Agriscientia. 33 (1): 46-59. Available in http://www.scielo.org.ar/scielo.php?script=sci_arttext&pid=S1668-298X2016000100005. [accessed 14 November 2017].
44. Lv, J.; Qi, J.; Shi, Q.; Shen, D.; Zhang, S.; Shao, G.; Li, H.; Sun, Z.; Weng, Y.; Shang, Y.; Gu, X.; Li, X.; Zhu, X.; Zhang, J.; van Treuren, R.; van Dooijeweert, W.; Zhang, Z.; Huang, S. 2012. Genetic Diversity and Population Structure of Cucumber (*Cucumis sativus* L.). PLoS ONE 7(10): e46919. DOI:10.1371/journal.pone.0046919.
45. Mantel, N. A. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209-220.
46. Mashilo, J.; Shimelis, H.; Odindo, A.; Amelework, B. 2016. Genetic diversity of South African bottle gourd [*Lagenaria siceraria* (Molina) Standl.] Landraces revealed by simple sequence repeat markers. Hort Science 51(2): 120-126.
47. Millán, R. 1945. Variaciones del zapallito amargo *Cucurbita andreana* y el origen de *Cucurbita maxima*. Revista Argentina de Agronomía. 12: 86-93.
48. Montes-Hernández, S.; Eguiarte, L. E. 2002. Genetic structure and indirect estimates of gene flow in three taxa of Cucurbita (*Cucurbitaceae*) in western Mexico. Amer J Bot. 89: 1156-1163.
49. Mujaju, C.; Sehic, J.; Werlemark, G.; Garkava-Gustavsson, L.; Fatih, M.; Nybom, H. 2010. Genetic diversity in watermelon (*Citrullus lanatus*) landraces from Zimbabwe revealed by RAPD and SSR markers. Hereditas. 147: 142-153.
50. Nantoume, A. D.; Andersen, S. B.; Jensen, B. D. 2013. Genetic differentiation of watermelon landrace types in Mali revealed by microsatellite (SSR) markers. Genet Resour Crop Evol (2013) 60: 2129-2141. DOI: 10.1007/s10722-013-9980-5.
51. Nee, M. 1990. The domestication of Cucurbita (Cucurbitaceae). Economic Bot. 44: 56-68.
52. Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA. 70 (12, part I): 3321-3323.
53. Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89: 583-590.
54. Ntuli, N. R.; Tongoona, P. B.; Zobolo, A. M. 2015. Genetic diversity in *Cucurbita pepo* landraces revealed by RAPD and SSR markers. Scientia Hort. 189: 192-200. DOI:10.1016/j.scienta. 2015.03.020.
55. Peakall, R.; Smouse, P. E. 2006. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes. 6:288-295. DOI: 10.1111/j.1471-8286.2005.01155.x.
56. Peralta, I. E.; Makuch, M.; García Lampasona, S.; Occhiuto, P. N.; Asprelli, P. D.; Lorello, I. M.; Togno, L. 2008. Catálogo de poblaciones criollas de pimiento, tomate y zapallo colectadas en valles andinos de la Argentina. Instituto Nacional de Tecnología Agropecuaria (Editors). Mendoza, Argentina.
57. Piñero, D.; Barahona, A.; Eguiarte, L.; Rocha Olivares, A.; Salas Lizana, R. 2008. La variabilidad genética de las especies: aspectos conceptuales y sus aplicaciones y perspectivas en México. In Capital natural de México. vol. I: Conocimiento actual de la biodiversidad. Conabio. México. 415-424 p.

58. Popenoe, H.; King, S. R.; León, J.; Kalinowski, L. S. 1990. Lost Crops of the Incas. Natl Academy Press, Washington.
59. Rahman, A. H. M. M.; Anisuzzaman, M.; Ahmed, F.; Rafiul Islam, A. K. M.; Naderuzzaman, A. T. M. 2008. Study of nutritive value and medicinal uses of cultivated Cucurbits. J Appl Sci Re. 4(5): 555-558.
60. Restrepo, J. A.; Franco, A.; Vallejo, C. 2008. Caracterización molecular de introducciones colombianas de zapallo *Cucurbita moschata*. Acta Agronómica. 57(1): 9-17.
61. Sanjur, O. I.; Piperno, D. R.: Thomas, C. A.; Wessel-Beaver, L. 2002. Phylogenetic relationships among domesticated and wild species of Cucurbita (Cucurbitaceae) inferred from a mitochondrial gene: implications for crop plant evolution and areas of origin. Proc Natl Acad Sci USA. 99(1): 535-540. DOI: 10.1073/pnas.012577299.
62. Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. A poor man's approach to genotyping for research and high-throughput diagnostics. Nature Biotechnol. 18: 233-234. DOI:10.1038/72708.
63. Shannon, C. E.; Weaver, W. 1949. The mathematical theory of communication. Univ IL Press Urbana.
64. Sikdar, B.; Bhattacharya, M.; Mukherjee, A.; Banerjee, A.; Ghosh, E.; Ghosh, B.; Roy, S. C. 2010. Genetic diversity in important members of Cucurbitaceae using isozyme, RAPD and ISSR markers. Biologia Plantarum 54 (1): 135-140.
65. Silva Ritschel, P.; De Lima Lins, T. C.; Tristan, R. L.; Salles Cortopassi Buso, G.; Buso, J. A.; Ferreira, M. E. 2004. Development of microsatellite markers from an enriched genomic library for genetic analysis of melon (*Cucumis melo* L.) BMC Plant Biol. 4:9. DOI:10.1186/1471-2229-4-9.
66. Sokal, R. R.; Michener, C. D. 1958. A statistical method for evaluating systematic relationships. Univ Ks Scientific Bul. 38: 1409-1438.
67. Staub, J. E.; López-Sesé, A. I.; Fanourakis, N. 2004. Diversity among melon landraces (*Cucumis melo* L.) from Greece and their genetic relationships with other melon germplasm of diverse origins. Euphytica. 136:151-166. DOI: 10.1023/B:EUPH.0000030667.63614.bd.
68. Stift, G.; Zraidi, A.; Lelley, T. 2004. Development and characterization of microsatellite markers (SSR) in Cucurbita species. Cucurbit Genet Coop Rpt. 27: 61-65.
69. Vavílov, N. I. 1931. The problem of the origin of the world's agriculture in the light of the latest investigations. In: Science at the Crossroads: Papers Presented to the International Congress of the History of Science and technology, London. 97-106 p. Available in:// www.marxists.org/subject/science/essays/vavilov.htm#a1 [Accessed 4 March 2012].
70. Wright, S. 1951. The genetical structure of populations. Ann Eugenics. 15: 323-354.
71. Wu, J.; Chang, Z.; Wu, Q.; Zhan, H.; Xie, S. 2011. Molecular diversity of Chinese *Cucurbita moschata* germplasm collections detected by AFLP markers. Scientia Horticulturae 128: 7-13. https://doi.org/10.1016/j.scienta.2010.12.006.

Acknowledgments

We thank Rafael Bottero for elaborating the map used in this work and Cecilia Bruno for statistical advice.

We also thank Gláucia Salles Cortopassi Buso who kindly provided some primer sequences, Pedro Della Gaspera for sharing his huge knowledge about squash culture and María Virginia Sánchez Puerta for their contributions to the manuscript.

The following projects financed this work: Proyecto de Investigación Científica y Tecnológica Orientado (PICT-O 2002 N ° 08-12903) Recuperación, Evaluación y Aprovechamiento Social del Germoplasma "Criollo" de Hortalizas en Regiones Andinas. Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT), Secretaría de Ciencia, Tecnología e innovación Productiva (SECTYP), UNCuyo and INTA. Filogeografía e implementación de un sistema de monitoreo molecular de la identidad genética de cultivos andinos (INTA-AEBIO 2443 (EPPR), 2006-2008).

This paper is part of Inés María Lorello Ph D. thesis in Biological Sciences, UNCuyo. August 2012.

First record of *Feltiella curtistylus* Gagné (Diptera: Cecidomyiidae) in Argentina

Primer registro de *Feltiella curtistylus* Gagné (Diptera: Cecidomyiidae) en Argentina

Claudia Fernanda Funes, Lorena Inés Escobar, Braian Eduardo Palavecino, Daniel Santiago Kirschbaum

Originales: *Recepción:* 03/07/2018 - *Aceptación:* 23/10/2018 *Nota científica*

Abstract

The first records of *Feltiella curtistylus* Gagné (Diptera: Cecidomyiidae) in Argentina are reported. Larvae from this species were captured in strawberry (*Fragaria* x *ananassa* Duch.) leaflets heavily infested with *Tetranychus urticae* Koch (Acari: Tetranichidae) in Famaillá, province of Tucumán, Argentina (27°03'S, 65°25'W). In 2015 (June, September and October), 16 larvae were collected; in 2016 (October, November and December), 23 larvae; and in 2017 (September, October and November), 49 larvae. Since *F. curtistylus* was found associated to a mite of agricultural importance in strawberry crops. This predatory gall midge has a potential for being considered a biological control agent.

Keywords
predatory gall midge • *Tetranychus urticae* • strawberry • biological control

Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Famaillá. Ruta Prov. 301, Km 32. (4132) Famaillá, Tucumán. Argentina. kirschbaum.daniel@inta.gob.ar

Resumen

Se presenta los primeros registros de *Feltiella curtistylus* Gagné (Diptera: Cecidomyiidae) en Argentina. Las larvas de esta especie fueron capturadas en folíolos de frutilla *(Fragaria* x *ananassa* Duch.) fuertemente infestados con *Tetranychus urticae* Koch (Acari: Tetranichidae) en Famaillá, provincia de Tucumán, Argentina (27°03'S, 65°25'O). En 2015 (junio, septiembre y octubre) se recolectaron 16 larvas; en 2016 (octubre, noviembre y diciembre), 23 larvas; y en 2017 (septiembre, octubre y noviembre), 49 larvas. Dado que *F. curtistylus* se encontró asociado a un ácaro de importancia agrícola en los cultivos de frutilla, este mosquito depredador tiene un potencial para ser considerado un agente de control biológico.

Palabras clave

mosquito depredador • *Tetranychus urticae* • frutilla • control biológico

The genus *Feltiella* (Diptera: Cecidomyiidae), represented by 10 species, is cosmopolitan and its larvae almost exclusive predators of red spider mites (Acari: Tetranychidae) (5, 6).

Argentina cultivates ≈1300 ha of strawberries (*Fragaria* x *ananassa* Duch.) and produces ≈45500 t/ year, with the strawberry industry being very important from both, social and economic points of view (9). Surveys of the arthropod fauna associated with strawberry crops in Argentina, indicated that the most abundant species is *Tetranychus urticae* Koch (Acari: Tetranychidae) (10), an important strawberry pest that causes growth retardation and decreased production and quality (3).

The larval stage of *Feltiella*, as described for *F. acarisuga* (Vallot), the most studied species of this genus, feeds on all of the developmental stages of spider mites, from eggs to adults (1). During three annual strawberry (*Fragaria* x *ananassa* Duch.) production seasons (2015, 2016 and 2017), strawberry leaflets infested with two-spotted spider mites (*T. urticae*) were collected, taken to the lab and observed by light microscopy at INTA's Estación Experimental Agropecuaria Famaillá (Tucumán province, Argentina; 27°0'S, 65°2'W, 363 m altitude). Many of those leaflets presented Diptera larvae feeding on eggs, protonymphal and adult stages of two-spotted spider mites (figure 1, page 316). In 2015 (June, September and October), 16 larvae were collected; in 2016 (October, November and December), 23 larvae; and in 2017 (September, October and November), 49 larvae. Mean monthly temperatures in which gall midges larvae were collected are presented in table 1 (page 316).

A total of 58 leaflets with 83 larvae were placed in Petri dishes with the bottom covered by smooth plaster base, and kept humid until adults emerged. Adult specimens (males and females) were preserved in 70° alcohol and sent to Raymond J. Gagné, Washington DC, USA for identification. They were identified as *Feltiella curtistylus* Gagné (table 2, page 217), which is the first record of this species in Argentina. Some of the collected specimens were deposited in INTA Famaillá and Smithsonian Institution (USNM), Washington, DC, USA collections.

Figure 1. *Feltiella curtistylus* larva feeding from two-spotted spider mites on the abaxial underside of a strawberry leaf.

Figura 1. Larva de *Feltiella curtistylus* se alimenta de arañuelas roja en la parte abaxial de una hoja de frutilla.

Table 1. Temperatures (mean, mean minimum and mean maximum) of months in which *F. curtistylus* larvae were collected from strawberry leaves in the 2015-17 period.

Tabla 1. Temperaturas (media, media mínima y media máxima) de los meses en que se recolectaron larvas de *F. curtistylus* de hojas de frutilla en el período 2015-2017.

	Temperature (°C)		
Month	**Mean**	**Mean min.**	**Mean max.**
2015			
Jun	13.8	8.7	19.9
Sep	17.2	10.8	24.1
Oct	18.3	12.9	24.1
2016			
Oct	20.0	14.0	25.9
Nov	21.7	15.9	28.1
Dic	24.5	18.8	31.4
2017			
Sep	17.2	10.1	24.5
Oct	20.3	14.2	27.8
Nov	23.7	17.0	30.7

Table 2. Morphology of *F. curtistylus*. Adapted from Gagné (1984).
Tabla 2. Morfología de *F. curtistylus.* Adaptado de Gagné (1984).

Adult	
Body	Body without conspicuous markings.
Head	Eyes large, about 8 facets long at vertex; facets circular, slightly farther apart at midheight of eye than elsewhere. Posvertical peak present. Labrum shorter than labellae, triangular. Hypoproct as short as labrum, lined with long setulae laterally. Labella short, broad, hemispherical in lateral view. Palpus 4 segmented. Male antenna: flagellomeres binodal, tricircumfilar; flagellomere 3 with internode and neck slightly shorter than preceding node, circumfilar loops regular, not quite reaching next distal node or circumfilum, and setulae sparse on basal node beyond circumfilum and between circumfila 2 and 3.
Thorax	Wing length, 1.1- 1.2 mm; R5 almost straight, joining C a little before wing apex; Rs not evident. Anepisternum bare. Anepimeron with vertical row of setae. Foretarsal claws toothed, other bare. Empodia as long as claws.
Male abdomen	Tergites 1-6 short, with single sparse, entire row of caudal setae, a few lateral setae, 2 basal, widely spaced trichoid sensilla, and scattered, sparse scales. Tergite 7 with 1-2 caudal setae laterally, 0-1 lateral setae, 2 trichoid sensilla basally, and O scales. Tergite 8 bare, weakly sclerotized. Sternites 2-7 as short as tergites, with single row of caudal setae and a horizontal row at midlength, 2 closely approximated trichoid sensilla, and no scales. Sternite 8 with both horizontal rows of setae approximated, the trichoid sensilla farther apart than on preceding sternites.
Female abdomen	Tergites 1-8 and sternites 2-8 as for male with the following differences: tergite 7 with complete caudal row of setae, 1-2 lateral setae, and a few scales; tergite 8 only with 2 trichoid sensilla, unsclerotized. Sternite 7 with double row of caudal setae and the 2 horizontal rows not closely approximated; sternite 8 unsclerotized, with only 2 basal trichoid sensilla. Tergite 9 and sternite 9 evenly covered with setae of equal length. Cerci large, evenly covered with setae that decrease in length from base of cercid to apex, with 2 ventrocaudal basiconic setae. Hypoproct short, with 2 caudal setae.
Larva (third instar)	
Mead capsule short, cupulate, antennae and cephalic apodemes longer than head. Only anterior end of spatula visible on specimens studied. Integument mostly smooth; spicules only on pseudopods and venter of terminal segment. Collar papillae not visible. Ventral thoracic papillae not visible. Posteroventral papillae of abdomen on short lobes but setae barely longer than wide. The 2 pseudopods of thoracic segments 2 and 3 and the 3 pseudopods of abdominal segments 1-7 short. Dorsal and pleural papillae on short lobes, setae of approximately equal length, more or less blunt-toothed, pleural setae slightly more pointed than dorsals. Terminal segment with 6 dorsal papillae, all on short lobes, innermost pair with shortest setae, 2 outermost pairs longer, of equal length, curved. Anus dorsal, anal papillae not evident.	
Pupa (unknown)	

Thus far, in South America, *F. curtistylus* was known only from Petrolina (9°23'34"S, 40°30'28"W), state of Pernambuco, Brazil (4), associated with *Tetranychus evansi* (6), one of the main tomato pests in Brazil's Northeastern Region (8). Specimens of this cecidomyiid were also captured in mosquito traps in Vaca Key, Florida, USA (24°43'1.2"N, 81°4'22.8"W) (7). From 10 species of *Feltiella* worldwide known, only *F. insularis* Felt has been found in Argentina, more precisely in La Plata city horticultural belt (Buenos Aires province), associated with *T. urticae* in tomato crops (2).

This is the first record of *F. curtistylus* in Argentina, and the first time this predator has been found associated with *T. urticae*. Its adaptive capacity is noteworthy, since it was found in different environments/ climates, separated by thousands of kilometers, within the American continent (table 3 and figure 2, page 318).

Table 3. Dispersion of *Feltiella curtistylus* Gagné in the American continent: characteristics of the sites where the insect has been reported, including the present record.

Tabla 3. Dispersión de *Feltiella curtistylus* Gagné en el continente americano: características de los sitios donde se ha reportado el insecto, incluido el registro actual.

Location	Country	Koppen-Geiger climatic classification	Climate	Average temperature (°C)	Annual rain (mm)
Famaillá	Argentina	Cfa	hot and warm	19.9	965
Petrolina	Brazil	BSh	local stepe	24.8	435
Florida Keys	USA	Aw	tropical	25.2	1004

Figure 2. Locations where *F. curtistylus* has been found in the American continent.

Figura 2. Ubicaciones donde se ha encontrado *F. curtistylus* en el continente americano.

This finding should be complemented with bioecological studies of *F. curtistylus* to determine its role in the strawberry agroecosystem, since it would compete for its prey with other predators regularly found in strawberry crops in Argentina, such as *Phytoseiulus longipes* Evans and *Neoseiulus californicus* (Mc Gregor) (Acari: Phytoseiidae), *Orius* sp. (Hemiptera: Anthocoridae) and *Crysoperla externa* (Hagen) (Neuroptera: Chrysopidae), among others (10). *F. curtistylus* could also be considered a potential biological control tool for other strawberry spider mites such as *T. cinnabarinus* (Boisduval), a serious strawberry pest in Chile (11). Predation bioessays should be carried out to determine the efficacy of *F. curtistylus* as spider mite predator, and to analyze its potential inclusion in IPM programs.

References

1. Abe, J.; Ganaha-Kikumura, T.; Yukawa, J. 2011. Morphological features, distribution, prey mites and life history traits of *Feltiella acarisuga* (Vallot) (Diptera: Cecidomyiidae) in Japan. Appl Entomol Zool. 46: 271-279.
2. Cédola, C. 2002. Primera cita para Argentina de *Feltiella insularis* (Cecidomyiidae), díptero predador de ácaros tetraníquidos. Rev Soc Entomol Argent. 61: 45-46.
3. Correa, M. V.; Reguilón, C.; Kirschbaum, D. S. 2013. Bioensayos de depredación de *Chrysoperla argentina* (Insecta: Neuroptera) sobre la arañuela roja (Acari: Tetranychidae) en cultivo de frutilla bajo condiciones de laboratorio (Tucumán). Horticultura Argentina. 32: 76.
4. Gagné, R. J. 1984. Five new species of Neotropical Cecidomyiidae (Diptera) associated with cacao flowers, killing the buds of Clusiaceae, or preying on mites. Brenesia 22: 123-138.
5. Gagné, R. J. 1995. Revision of tetranychid (Acarina) mite predators of the genus *Feltiella* (Diptera: Cecidomyiidae). Ann Entomol Soc Am. 88: 16-30.
6. Gagné, R. J.; Jaschhof, M. 2017. A Catalog of the Cecidomyiidae (Diptera) of the World. 4º Edition. Digital. 762 p. Available in: https://www.ars.usda.gov/ARSUserFiles/80420580/Gagne_2017_World_Cat_4th_ed.pdf
7. Hribar, L. J.; Plakidas, J. D. 2011. Collection records for some gall midges from the Florida Keys (Diptera: Cecidomyiidae). Florida Scientist .74: 38-42.
8. Humber, R. A.; Moraes, G. J.; Dos Santos, J. M. 1981. Natural infection of *Tetranychus evansi* (Acarina: Tetranychidae) by a *Triplosporium* sp. (Zygomycetes: Entomophthorales) in northeastern Brazil. Entomophaga. 26: 421-425.
9. Kirschbaum, D. S.; Vicente, C. E.; Cano-Torres, M. A.; Gambardella-Casanova, M.; Veizaga-Pinto, F. K.; Correa-Antunes, L. E. 2017. Strawberry in South America: from the Caribbean to Patagonia. Acta Horticulturae. 1156: 947-955.
10. Lemme, M. C.; Jaime de Herrero, A. P.; Kirschbaum, D. S.; Nasca, A. J. 1996. Artrópodos asociados al cultivo de la frutilla, *Fragaria* × *ananassa*, en Tucumán, Argentina. Vedalia 3: 51-52.
11. Tello-Mercado, V.; Derosas-Arriagada, M. 2014. Estudio preliminar de los efectos letales y subletales de extractos etanólicos de cuatro especies xerófitas del altiplano chileno contra *Tetranychus cinnabarinus* (Acarina: Tetranychidae). Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 46(2): 135-148.

Acknowledgements

We thank to Dr. Raymond J. Gagné (Systematic Entomology Laboratory-ARS-USDA and, Smithsonian Institution, Washington DC, USA) for identification of specimens and critical review of the manuscript.

Financial support was provided by Instituto Nacional de Tecnología Agropecuaria (projects PNHFA1106073, TUSGO1231101 and CIAC940162) and Fondo Nacional de Ciencia y Tecnología-Agencia Nacional de Promoción Científica y Tecnológica (FONCyT-ANPCyT, Argentina), through grant PICT-2013-0604.

Incidence, prevalence and persistence of bovine venereal diseases in La Pampa (Argentina): estimations for the period 2007 - 2020

Incidencia, prevalencia y persistencia de enfermedades venéreas de los bovinos en La Pampa (Argentina): estimaciones para el período 2007 - 2020

Leonardo L. Molina [1,2], Antón García [3], Elena Angón [3*], Ricardo Moralejo [1], Javier Caballero-Villalobos [3], José Perea [3]

Originales: *Recepción:* 12/12/2017 - *Aceptación:* 14/08/2018

Abstract

The venereal diseases Bovine Trichomoniasis (BT) and Bovine Genital Campylobacteriosis (BGC) cause economic losses in endemic areas, such as the province of La Pampa in Argentina, where bovine production is typically extensive. This study used data compiled from 2007 to 2013 by the Official Program for the Control and Eradication (PCE) of venereal diseases, to determine the prevalence, incidence and persistence of BT and BGC and to provide projections up to 2020. Fourteen univariate models were used to adjust each time series. The prevalence and incidence of both diseases significantly decreased during the studied period, while the persistence has remained constant. The prevalence of BT has diminished from 7.48% in 2007 to 3.03% in 2013, while the prevalence of BGC has diminished from 9.36% to 3.15%. The incidences have been reduced to an annual average of 0.60 for BT and 0.67 for BGC. Although the estimation models are not able to accurately predict the future epidemiologic rates of BT and BGC in La Pampa, projections show a significant decreasing trend of the prevalence and incidence of BT and BGC. The persistence of BGC is expected to remain close to the 2007-2013 average, while the persistence of BT did not adjust to any of the 14 models used. These results indicate that PCE has been effective in reducing the infection of disease-free herds. However, in order to reduce the ratio of persistent herds, other preventive and management measures should be considered.

Keywords
Bovine • bovine genital campylobacteriosis • monitoring venereal diseases • bovine trichomoniasis • modelling

1 National University of La Pampa. School of Veterinary Medicine. Calle 5 Esq. 116, 6360. General Pico. La Pampa. Argentina. leonardoluismolina@gmail.com
2 National Service of Health and Agro-Food Quality (SENASA). Centro Regional La Pampa-San Luis. Corrientes 80. Santa Rosa 6300. Argentina.
3 University of Cordoba. School of Veterinary. Department of Animal Production. Crta. Madrid-Cádiz km. 396-a. 14071.Córdoba, Spain. *Corresponding author: E-mail address: eangon@uco.es (E. Angón)

Resumen

Las enfermedades venéreas Tricomoniasis bovina (BT) y Campilobacteriosis genital bovina (BGC) causan pérdidas económicas en áreas endémicas, como en la provincia de La Pampa en Argentina, donde la producción bovina es típicamente de carácter extensivo. Este estudio ha utilizado datos compilados de 2007 a 2013 por el Programa Oficial para el Control y la Erradicación (PCE) de enfermedades venéreas, con el objetivo de determinar la prevalencia, incidencia y persistencia de BT y BGC y proporcionar proyecciones hasta 2020. Catorce modelos univariantes fueron utilizados para ajustar cada serie temporal. La prevalencia e incidencia de ambas enfermedades han disminuido significativamente durante el período estudiado, mientras que la persistencia se ha mantenido constante. La prevalencia de BT ha disminuido del 7,48% en 2007 al 3,03% en 2013, mientras que la prevalencia de BGC ha disminuido del 9,36% al 3,15%. Las incidencias se han reducido a un promedio anual de 0,60 para BT y 0,67 para BGC. Aunque los modelos de estimación no pueden predecir con precisión las tasas epidemiológicas futuras de BT y BGC en La Pampa, las proyecciones muestran una tendencia decreciente significativa de la prevalencia e incidencia de BT y BGC. Se espera que la persistencia de BGC se mantenga cerca del promedio de 2007-2013, mientras que la persistencia de BT no se ajustó a ninguno de los 14 modelos utilizados. Estos resultados indican que PCE ha sido eficaz para reducir la infección de rebaños libres de enfermedades. Sin embargo, para reducir la proporción de rebaños persistentes, se deben considerar otras medidas preventivas y de manejo.

Palabras clave

Bovino • campilobacteriosis genital bovina • monitoreo de enfermedades venéreas • tricomoniasis bovina • modelización

Introduction

Bovine Trychomoniosis (BT) and Bovine Genital Campylobacteriosis (BGC) are venereal diseases of economic importance, characterized by infertility, embryonic death, abortions, irregular reproductive cycles and long culling intervals (6). BGC infected herds can reduce fertility rates up to 20% and increase abortion rate up to 10% (16). Additionally, sterility may occur in about 11% of infected heifers (24). BT has also been associated to low weights at birth and reductions of more than 50% in the weaning rate (4, 35). In the USA infection produced by BT is estimated to generate economic losses of over 650 million dollars (48).

BT is caused by *Tritrichomonas foetus* and BGC by *Campylobacter fetus veneralis* (11, 45). Both diseases are transmitted during coitus, being the bulls asymptomatic carriers. When they reach 3 or 4 years of age they remain as permanently infected reservoirs, while cows are usually recovered after a period of 6-12 months (1, 8, 22). Furthermore, there is neither treatment nor vaccination effective enough for these diseases (5, 7, 52).

Both diseases are distributed worldwide, although they tend to be endemic in areas where bovine production is typically extensive and based on natural breeding, such as the province of La Pampa in Argentina (15, 31).

The economic importance of the bovine sector and the concern for the low reproductive efficiency led to the implementation in 2006 of a Provincial Program for the Control and Eradication (PCE) of BT and BGC. The inclusion in PCE is compulsory for all herds, and positive animals must be removed from the herd within 120 days. However, animals can be medically treated as long as negativity is certified through three post-treatment negative tests (29).

Data generated by PCE provides an opportunity to determine epidemiological indicators for BT and BGC. Furthermore, in Argentina no epidemiological indicators are generated at a national level. In these situations where there is no existing information about occurrence at a national level, the estimations and projections represent and essential tool in order to understand the health requirements and, consequently, to establish prevention and control measures (30, 53). However, aspects such as resistance to diseases, reproductive characteristics or adaptation to difficult environments will always be of value and should be target of greater scientific and informative efforts (34).

Different estimation methods have been used in order to predict occurrence rate, including decomposition methods, ARIMA models, Bayesian models or linear regression (20, 26, 42, 46, 57). The advantages and applicability conditions are specific of each model of analysis and depend on the type of data that constitute the time series (10).

The present study aims to determine population epidemiological indicators for BT and BGC in the province of La Pampa (Argentina) for the period 2007 – 2013, and to provide projections for the incidence, prevalence and persistence of both diseases until 2020.

Material and methods

Study area and population

The study area was the province of La Pampa in Argentina, which includes approximately 6% of the total bovine population of the country (43). La Pampa is located in the geographic center of Argentina and covers an area of around 143.440 km^2 (approximately about 5.2% of Argentina). Farm production in La Pampa is extensive and involves two main production systems: herds that produce calves for fattening establishments and herds where breeding, rearing and fattening are carried out on the same premises (full-cycle herds).

The study population consists in all herds (from 2,000 to 6,000) annually tested under PCE from January, 1st to December, 31rd 2013. PCE regulation in La Pampa requires BT and BGC testing of all non-virgin bulls in the herd in order to authorize the movement of cattle to another herd, feedlot or slaughterhouses (43). Therefore, the study population corresponds to all the existing herds in La Pampa between 2007 and 2013, except the few herds with no animal movements during this period.

All non-virgin bulls in La Pampa are tested twice a year as part of PCE. The methodology for sample collection and diagnosis is thoroughly described by Molina *et al.* (29). A bull is classified as negative if the results obtained in two consecutive tests are negative, and positive if at least one test yielded positive results (34). Herds with at least one positive bull were classified as positive.

Data

This study used annual data gathered and reported by PCE from January, 1st 2007 to December, 31rd 2013. Annual prevalence, incidence and persistence of BT and BGC were analyzed.

Prevalence is defined as the ratio of positive herds to the total tested herds. Incidence is defined as the ratio of new positive herds to the total tested herds. Persistence is defined as the ratio of positive herds in the year *n* that were also positive in the year *n* – 1 to the total tested herds.

Estimation methods

In order to characterize the behavior of each time series, different models have been built and evaluated using prevalence, incidence and persistence as dependent variables (*Y*) and time as the independent variable (*X*). Overall, 14 models have been evaluated for each time series: random walk, random walk with drift, constant mean model, lineal trend model, quadratic trend model, exponential growth trend model, S-curve model, simple exponential smoothing, Brown's lineal exponential smoothing, Holt's exponential smoothing, quadratic exponential smoothing, ARIMA (1,0,0) and ARIMA (1,0,1).

Coefficients for each model have been estimated using least square method and contrasted by t-tests (2, 13). Adjustments were determined by the root mean square error (RMSE), the mean absolute error (MAE) and the mean absolute percentage error (MAPE). Adequacy was contrasted using white noise tests to check if the residuals were independent and normally distributed (14). It is possible that several models could be identified for each time series, so it is necessary to choose an optimum model. This optimum model was determined based on Akaike information criterion (AIC) and on Schwartz Bayesian criterion (SBC) (19). All models were retrospectively validated by comparing the means of the obtained estimates and those observed during the period 2007–2013 (45).

All statistical analysis was performed with a significance level of alpha ≤ 0.05 and using the software SPSS v.15.0.

Results

Table 1 shows the prevalence of BT and BGC registered during the period 2007 – 2013 in La Pampa. An average 9.51% of the sampled herds showed at least one bull positive to BT or BGC. Herds infected with either of the two diseases have been reduced from 14.18% in 2007 to 5.57% in 2013, involving an annual average decrease of 0.78%. Herds with positive bulls to both diseases averaged 1.60%. Co-infection has been reduced by an annual average of 0.18%, from 2.66% in 2007 to 0.60% in 2013.

Table 1. Herd-level prevalence of BT and BGC during the period 2007–2013 in La Pampa (Argentina).

Tabla 1. Prevalencia a nivel de rebaño de BT y BGC durante el período 2007-2013 en La Pampa (Argentina).

Year	Herds	BT	BGC	BT or BGC	BT and BGC
2007	3,610	270 (7.48 %)	338 (9.36 %)	512 (14.18 %)	96 (2.66 %)
2008	4,105	418 (10.18 %)	421 (10.26 %)	679 (16.54 %)	160 (3.90 %)
2009	2,352	95 (4.04 %)	127 (5.40 %)	190 (8.08 %)	32 (1.36 %)
2010	4,078	140 (3.43 %)	250 (6.13 %)	346 (8.48 %)	44 (1.08 %)
2011	5,167	168 (3.25 %)	283 (5.48 %)	397 (7.68 %)	54 (1.05 %)
2012	5,588	135 (2.42 %)	232 (4.15 %)	337 (6.03 %)	30 (0.54 %)
2013	5,777	175 (3.03 %)	182 (3.15 %)	322 (5.57 %)	35 (0.61 %)

Prevalence of BT and BGC has been reduced by an annual average of 0.44% and 0.51%, respectively. Prevalence of BT has decreased from 7.48% in 2007 to 3.03% in 2013, while the prevalence of BGC has diminished from 9.36% to 3.15%.

The average persistence of BT and BGC was 26.78% and 18.98%, respectively. While the persistence of BT has been reduced from 53.03% in 2007 to 39.13% in 2013, the persistence of BGC has slightly increased from 25.93% to 26.47% in 2013 (table 2).

The incidence of both diseases has been reduced by an annual average of 0.61% for BT and 0.67% form BGC.

The projections reveal a reduction of the rates of prevalence and incidence of BT and BGC (table 3).

Results exposed in table 4 (page 325) indicate that the expected changes are statistically significant. The prevalence of BT showed the best adjustment with the S-curve model. An annual reduction of BT prevalence by 0.25% is expected, reaching 0.54 % (0.10–2.07 95% CI) in 2020.

Table 2. Persistence and incidence of BT and BGC during the period 2007-2013 in La Pampa (Argentina).

Tabla 2. Persistencia e incidencia de BT y BGC durante el período 2007-2013 en La Pampa (Argentina).

Year	BT Persistence	BGC Persistence	BT Incidence	BGC Incidence
2007	-	-	270 (7.48 %)	338 (9.36 %)
2008	143 (53.03 %)	88 (25.93 %)	275 (6.94 %)	333 (8.30 %)
2009	24 (5.84 %)	27 (6.51 %)	71 (3.03 %)	100 (4.28 %)
2010	13 (13.33 %)	26 (20.59 %)	127 (3.13 %)	224 (5.52 %)
2011	37 (26.42 %)	52 (20.65 %)	131 (2.55 %)	231 (4.52 %)
2012	39 (22.95 %)	39 (13.75 %)	96 (1.74 %)	193 (3.48 %)
2013	53 (39.13 %)	61 (26.47 %)	122 (2.13 %)	121 (2.11 %)

Table 3. Estimation models with the best adjustment for epidemiological indicators of BT and BGC in La Pampa (2007-2013).

Tabla 3. Modelos de estimación con el mejor ajuste para indicadores epidemiológicos de BT y BGC en La Pampa (2007-2013).

Variable	Model	RMSE	MAE	MAPE	AIC	SBC
Prevalence BT	$Y = e^{(\beta_1 + \beta_2/t)}$	1.846	1.076	20.441	1.798	1.782
Prevalence BGC	$Y = e^{(\beta_1 + \beta_2 \cdot t)}$	1.189	0.769	11.540	0.917	0.902
Prevalence BT or BGC	$Y = e^{(\beta_1 + \beta_2/t)}$	2.146	1.184	10.853	2.099	2.083
Prevalence BT and BGC	$Y = e^{(\beta_1 + \beta_2 \cdot t)}$	0.762	0.430	22.834	0.030	0.014
Persistence BT	$Y = \beta_1 + \beta_2 \cdot t$	13.742	10.245	42.171	5.352	5.937
Persistence BGC	$Y = \beta_1$	4.780	3.675	19.361	3.414	3.407
Incidence BT	$Y = e^{(\beta_1 + \beta_2/t)}$	0.964	0.642	17.548	0.499	0.483
Incidence BGC	$Y = e^{(\beta_1 + \beta_2/t)}$	0.984	0.667	15.202	0.539	0.523

RSME: the root mean square error; MAE: the mean absolute error; MAPE: the mean absolute percentage error; AIC: Akaike information criterion; SBC: Schwartz Bayesian criterion.

RSME: error cuadrático medio; MAE: error absoluto medio; MAPE: error absoluto medio (%); AIC: criterio de información de Akaike; SBC: criterio bayesiano de Schwartz.

Table 4. Coefficients of the estimation models of epidemiological indicators of BT and BGC with the best adjustment in La Pampa (2007-2013).

Tabla 4. Coeficientes de los modelos de estimación de indicadores epidemiológicos de BT y BGC con el mejor ajuste en La Pampa (2007-2013).

Variable	Model	Constant				Slope			
		β_1	E.E.	t	P	β_2	E.E.	t	P
Prevalence BT	$Y = e^{(\beta_1 + \beta_2/t)}$	-415.152	111.637	-3.71	0.011	837,364	224,390	3.73	0.012
Prevalence BGC	$Y = e^{(\beta_1 + \beta_2 \cdot t)}$	365.193	62.409	5.85	0.002	-0.180	0.031	-5.82	0.002
Prevalence BT or BGC	$Y = e^{(\beta_1 + \beta_2/t)}$	-347.606	67.329	-5.16	0.003	703,063	135,332	5.19	0.003
Prevalence BT and BGC	$Y = e^{(\beta_1 + \beta_2 \cdot t)}$	619.812	117.838	5.25	0.003	-0.308	0.058	-5.25	0.003
Persistence BT	$Y = \beta_1 + \beta_2 . t$	4384.31	-2.165	0.61	0.569	-2.165	3.524	-0.61	0.572
Persistence BGC	$Y = \beta_1$	22.005	1.951	11.27	0.000	-	-	-	-
Incidence BT	$Y = e^{(\beta_1 + \beta_2/t)}$	-480.657	91.747	-5.234	0.003	969,026	184,504	5.25	0.003
Incidence BGC	$Y = e^{(\beta_1 + \beta_2/t)}$	-440.106	77.656	-5.67	0.002	887,775	156,088	5.69	0.002

The prevalence of BGC was adjusted with the exponential growth trend model and shows an expected annual decrease of 0.35%, reaching 0.94% (0.38–1.93 95% CI) in 2020.

The prevalence of BT and BGC was adjusted with the S-curve model. Herds infected by either one disease are expected to be reduced by an annual average of 0.52%, reaching 1.56% (0.58-3.18 95% CI) in in 2020 (figure 1, page 326). Co-infection by BT and BGC was adjusted with the exponential growth trend model and shows an expected annual decrease of 0.06% (figure 2, page 326).

The incidences of BT and BGC were adjusted with the S-curve model and showed and expected annual decrease of 0.19% and 0.28%, respectively.

The persistence of BT was adjusted with the lineal trend model, although it was not statistically significant (table 4). The best adjustment for persistence of BGC was with the constant mean model, what suggests that persistence will remain constant at about 22% during the period 2013-2020.

Discussion

The accumulated data in La Pampa during 7 years have been used to determine the population epidemiological indicators of BT and BGC in the province. The initial prevalence of both diseases was generally lower than those reported in other endemic areas in Asia, Australia, North America, South America and South Africa (3, 4, 12, 21, 22, 24, 33, 41, 56). A prevalence of 37% for BGC was reported in Uruguay, while no herds were found to be positive to BT (39). In Buenos Aires (Argentina) a prevalence of 1.5% was reported for BGC and of 19.4% for BT (40). In the east of La Pampa, a prevalence of 11.1% was found for BT and of 7.0% for BGC (49). Most of the reported data of prevalence comes from studies conducted with few herds and limited conditions, so it is possible that they do not accurately reflect the actual situation. For instance, in South Africa, the occurrence of BGC seems to be vastly underestimated (31).

The detection of BT and BGC in La Pampa has been based on the collection of two consecutive preputial smegma samples and on cultural methods (29).

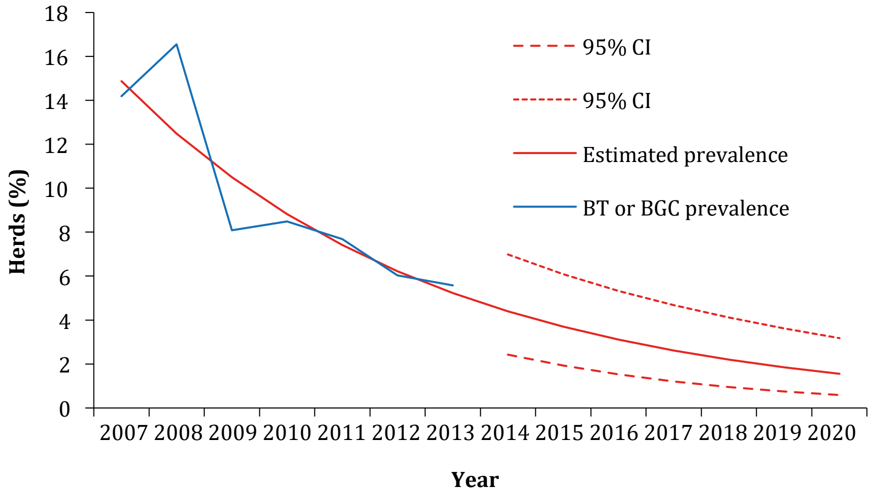


Figure 1. Adjustment and prediction of the prevalence of BT or BGC in La Pampa with the S-curve model.

Figura 1. Ajuste y predicción de la prevalencia de BT o BGC en La Pampa con el modelo de curva en S.

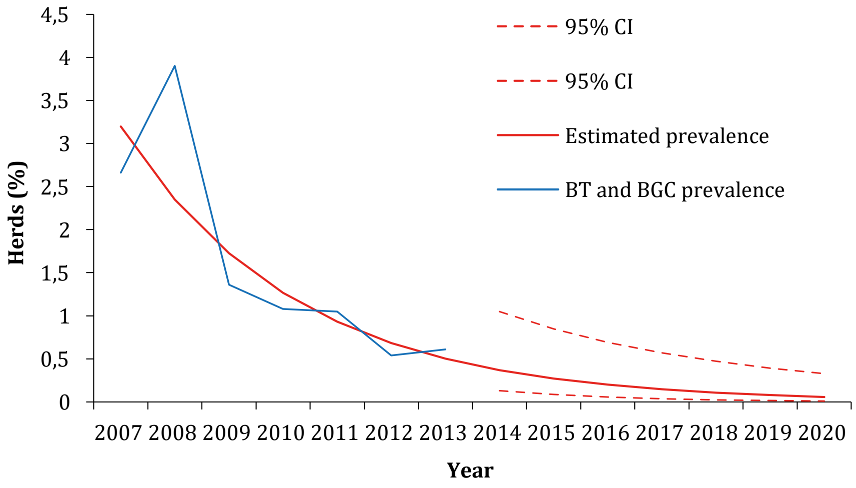


Figure 2. Adjustment and prediction of the prevalence of BT and BGC in La Pampa with the exponential growth trend model.

Figura 2. Ajuste y predicción de la prevalencia de BT y BGC en La Pampa con el modelo de tendencia de crecimiento exponencial.

There are, however, different factors that could negatively affect the accuracy of the diagnoses, being some of them easily upgradable. For instance, sample collection is not always effective due to the intermittent presence of pathogenic agents in the foreskin of bulls (17, 27). Furthermore, the frequency and the sampling intervals affect the effectiveness of the diagnosis techniques (32, 37). Although none of them is 100% sensible, PCR based techniques represent an improvement compared to those obtained by bacterial culture (25, 50). According to Yao *et al.* (2013), an unpredictable and often ignored factor is the delay in the delivery of samples to the diagnostic laboratories after sample collection. Another factor is the existence of positive cows that are currently not detected as they are not included in PCE. In order to improve accuracy in diagnosis, it would be highly recommended to consider these factors and to analyze the relationship between the sample collection and the situation of the herd.

Prevalence of BT and BGC reached their highest peak in 2008. Ever since, they have decreased, proving effective control measures. In Wyoming (USA), a similar to PCE control plan, managed to reduce the herd-level prevalence of BT to 1.29% in nine years (55). Besides, the estimation models show a decreasing trend, and both prevalences are expected to continue diminishing in the future, although the models are not capable of accurately predicting the levels of prevalence. This is partly due to the fact that only data from 2007 to 2013 is available. The decrease of the prevalence of BT and BGC is mainly due to a reduction of new infections, although the proportion of persistent herds has remained broadly stable. Although they cannot precisely predict the levels of incidence and persistence, estimation models suggest that in the future, incidence will continue to decrease while persistence will remain constant.

PCE has been especially useful to reduce the infection of disease-free herds. This is explained by the reposition with bulls certified as negative to both diseases. However, PCE has not managed to significantly reduce the rate of persistent herds. On one side, the disposal of positive bulls might not be enough to eliminate the disease from the flock, resulting also necessary to remove breeding cows or to establish a period of reproductive rest during 6-12 months to facilitate recovery (22, 38). On the other hand, it could be possible to eliminate the disease replacing positive bulls, although effective measures to prevent new infections are not simultaneously taken. In this sense, in the areas of higher BT and BGC risk in La Pampa, the exchange of bulls between farmers and pasture sharing, are very common (29).

According to Yao *et al.* (2013), in order to eradicate the disease, it is not enough to detect and remove the positive bulls, but also replacing them with disease-free bulls. In addition to PCE, other preventive and herd management measures should be considered. BT and BGC share in La Pampa some of their main risk factors (15, 18, 23, 27). Besides, there is some spatial correlation between the risk of BT and BGC (29). Thus, the development of integrated actions focused on the common features of BT and BGC should enhance the effectiveness and efficiency of the intervention methods (9).

Taking into consideration the common features of both diseases and the productive conditions in La Pampa, the priority actions should focus on the improvement of the reproductive control and keeping the herd in a closed cycle. Practices as seasonal breeding or rectal examination are not usual in the province of La Pampa and yet could greatly improve reproductive efficiency and control of venereal diseases. On one side, they allow the early identification of reproductive failures and the discard of non-breeding cows. On the other hand, testing bulls before breeding and after a period of sexual repose, reduces the probability of false negatives due to low concentration of microorganisms, and avoids positive bulls to encounter other cows in the herd (28). Finally, keep the herd in a closed cycle prevents contact with animals from other herds with an unknown sanitary status. In this sense, it is important to keep wiring and perimeter fences in good conditions, avoiding the exchange of bulls and the sharing of pastures (18, 23). According to Yao *et al.* (2013) eradication of BT and BGC is only possible through the replacement of natural breeding by artificial insemination. However, although artificial insemination generally involves a considerable reduction of the rates of occurrence or even eradication, there are typically extensive areas free of BT and BGC where artificial insemination is not in practice, such as the in Spanish grasslands (27, 51).

Conclusions

Univariate analysis was an effective tool for modeling the historical and future prevalence, incidence and persistence of *T. foetus* and *C. fetus* infections in La Pampa (Argentina). Prevalence and incidence of both diseases have significantly decreased during the studied period, while the persistence has remained constant. The estimation models show projections with a significant decreasing trend of the prevalence and incidence of BT and BGC, indicating that PCE has been effective to reduce the infection of disease-free herds. However, in order to reduce the ratio of persistent herds, other preventive and management measures should be considered.

References

1. Anderson, M. L. 2007. Infectious causes of bovine abortion during mid-to late-gestation. Theriogenology. 68: 474-486.
2. Angón, E.; García A., Perea, J.; Acero, R.; Toro-Mújica, P.; Pacheco, H.; González, A. 2013. Eficiencia técnica y viabilidad de los sistemas de pastoreo de vacunos de leche en la Pampa. Argentina. Agrociencia. 47: 443-456.
3. Bawa, E. K.; Adekeye, J. O.; Oyedipe, E. O.; Umoh, J. U. 1991. Prevalence of bovine campylobacteriosis in indigenous cattle of three states in Nigeria. Tropical Animal Health and Production. 23:157-160.
4. BonDurant, R. H.; Anderson, M. L.; Blanchard, P.; Hird, D.; Danaye-Elmi, C.; Palmer, C.; Sischo, W. M.; Suther, D.; Utterback, W.; Weigler, B. J. 1990. Prevalence of trichomoniasis among California beef herds. Journal of the American Veterinary Medical Association. 10: 1590-1593.
5. BonDurant, R. H. 2005. Venereal diseases of cattle: natural history, diagnosis, and the role of vaccines in their control. Veterinary Clinics of North America: Food Animal Practice. 21: 383-408.

6. Campero, C. M.; Rodriguez Dubra, C.; Bolondi, A.; Cacciato, C.; Cobo, E.; Perez, S.; Odeon, A.; Cipolla, A.; BonDurant, R. H. 2003. Two-step (culture and PCR) diagnostic approach for differentiation of non-T. foetus trichomonads from genitalia of virgin beef bulls in Argentina. Veterinary Parasitology. 112: 167-175.
7. Cobo, E. R.; Morsella, C.; Cano, D.; Cipolla, A.; Campero, C. M. 2004. Immunization in heifers with dual vaccines containing Tritrichomonas foetus and Campylobacter fetus antigens using systemic and mucosal routes. Theriogenology. 62: 1367-1382.
8. Corbeil, L. B.; Campero, C. M.; Rhyan, J. C.; BonDurant, R. H. 2003. Vaccines against sexually transmitted diseases. Reproductive Biology and Endocrinology. 1: 118.
9. Cowie, C. E.; Marreos, N.; Gortázar, C.; Jaroso, R.; White, P. C. L.; Balseiro, A. 2014. Shared risk factors for multiple livestock diseases: A case study of bovine tuberculosis and brucellosis. Research in Veterinary Science. 97: 491-497.
10. Diggle, P. 1990. Time Series: A Biostatistical Introduction. Oxford University Press. Oxford. UK.
11. Eaglesome, M. D.; Garcia, M. M. 1992. Microbial agents associated with bovine genital tract infections and semen. Part 1. Brucella abortus, Leptospira, Campylobacter fetus and Tritrichomonas foetus. Veterinary Bulletin. 62: 743-775.
12. Erasmus, J. A.; De Wet, J. A. L.; Van der Merwe, H. E.; Pienaar, G. C. J. 1989. Bovine trichomoniasis in the north western Cape Province, western Transvaal and the Orange Free State. Journal of the South African Veterinary Association. 60: 51-52.
13. Faruk, D. Ö. 2010. A hybrid neural network and ARIMA model for water quality time series prediction. Engineering Applications of Artificial Intelligence. 23(4): 586-94.
14. Fong, P. W.; Li, W. K. 2003. On time series with randomized unit root and randomized seasonal unit root. Computational statistics & data analysis. 43(3): 369-95.
15. Gay, J. M.; Ebel, E. D.; Kearley, W. P. 1996. Commingled grazing as a risk factor for trichomonosis in beef herds. Journal of the American Veterinary Medical Association. 209: 643-646.
16. Hum, S. 1987. Bovine abortion due to Campylobacter fetus. Australian Veterinary Journal. 64: 319-320.
17. Irons, P. C.; Henton, M. M.; Bertschinger, H. J. 2002. Collection of preputial material by scraping and aspiration for the diagnosis of Tritrichomonas foetus in bulls. The Journal of the South African Veterinary Association. 73: 66–69.
18. Jiménez, D. F.; Perez, A. M.; Carpenter, T. E.; Martinez, A. 2011. Factors associated with infection by Campylobacter fetus in beef herds in the Province of Buenos Aires, Argentina. Preventive Veterinary Medicine. 101: 157-162.
19. Koehler, A. B.; Murphree, E. S. 1988. A comparison of the Akaike and Schwarz criteria for selecting model order. Applied Statistics. 37: 187-95.
20. Luz, P. M.; Mendes, B. V. M.; Codeao, C. T.; Struchiner, C. J.; Galvani, A. P. 2008. Time series analysis of dengue incidence in Rio de Janeiro, Brazil. American Journal of Tropical Medicine Hygiene. 79: 933-939.
21. Mai, H. M.; Irons, P. C.; Kabir, J.; Thompson, P. N. 2013. Prevalence of bovine genital campylobacteriosis and trichomonosis of bulls in northern Nigeria. Acta Veterinaria Scandinavica. 55: 56.
22. Mancebo, O. A.; Russo, A. M.; Carabajal, L. L.; Monzon, C. M. 1995. Persistence of Tritrichomonas foetus in naturally infected cows and heifers in Argentina. Veterinary Parasitology. 59: 7-11.
23. Mardones, F. O.; Perez, A. M.; Martínez, A.; Carpenter, T. E. 2008. Risk factors associated with Tritrichomonas foetus infection in beef herds in the Province of Buenos Aires. Argentine. Vet. Parasitol. 153: 231-237.
24. McCool, C. J; Townsend, M. P.; Wolfe, S. G.; Simpson, M. A.; Olm, T. C.; Jayawardhana, G. A.; Carney, J. V. 1988. Prevalence of bovine venereal disease in the Victoria River district of the northern territory: Likely economic effects and practical control measures. Australian Veterinay Journal. 65: 153-156.
25. McMillen, L.; Lew, A. E. 2006. Improved detection of Tritrichomonas foetus in bovine diagnostic specimens using a novel probe-based real time PCR assay. Veterinary Parasitology. 141: 204-215.

26. Medina, D. C.; Findley, S. E.; Doumbia, S. 2008. State–space forecasting of Schistosoma haematobium time-series in Niono, Mali', PLoS Neglected Tropical Diseases. 2: 1-12.
27. Mendoza Ibarra, J. A.; Pedraza Díaz, S.; García Peña, F. J.; Rojo Montejo, S.; Ruíz Santa Quiteria, J. A.; San Miguel Ibañez, E.; Navarro Lozano, V.; Ortega Mora, L. M.; Osoro, K.; Collantes Fernandez, E. 2011. High prevalence of Tritrichomonas foetus infection in Asturiana de la Montaña beef cattle kept in extensive conditions in Northern Spain. The Veterinary Journal. 193(1): 146-51.
28. Michi, A. N.; Favetto, P. H.; Kastelic, J.; Cobo, E. R. 2016. A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health. Theriogenology. 85: 781-791.
29. Molina, L.; Perea, J.; Meglia, G.; Angon, E.; Garcia, A. 2013. Spatial and temporal epidemiology of bovine trichomoniasis and bovine genital campylobacteriosis in La Pampa province (Argentina). Preventive Veterinary Medicine. 110: 388-94.
30. Moller, B.; Fekjaer, H.; Hakulinen, T.; Tryggvadottir, L.; Storm, H. H.; Talback, M.; Haldoresen, T. 2002. Prediction of cancer incidence in the Nordic countries up to the year 2020. European Journal of Cancer Prevention. 11: Suppl 1: S1-96.
31. Mshelia, G. D.; Amin, J. D.; Woldehiwet, Z.; Murray, R. D.; Egwu, G. O. 2010. Epidemiology of bovine venereal campylobacteriosis: Geographic distribution and recent advances in molecular diagnostic techniques. Reproduction in Domestic Animals. 45: 221-230.
32. Mukhufhi, N.; Irons, P. C.; Michel, A.; Peta, F. 2003. Evaluation of a PCR test for the diagnosis of Tritrichomonas foetus infection in bulls: effects of sample collection method, storage and transport medium on the test. Theriogenology. 60: 1269-1278.
33. Pefanis, S. M.; Herr, S.; Venter, C. G.; Kruger, L. P.; Queiroga, C. C.; Amaral, L. 1988. Trichomoniasis and campylobacteriosis in bulls in the Republic of Transkei. Journal of the South African Veterinary Association 59: 139-140.
34. Perea, J.; Barba, C.; Luque, M.; González, A.; Angón, E.; García, A. 2018. Conocimiento científico y políticas de conservación: interrelaciones en las razas ganaderas autóctonas españolas en peligro de extinción. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 171-184.
35. Perez, A.; Cobo, E.; Martínez, A.; Campero, C.; Späth, E. 2006. Bayesian estimation of Tritrichomonas foetus diagnostic test sensitivity and specificity in range beef bulls. Veterinary Parasitology. 142: 159-162.
36. Rae, D. O. 1989. Impact of trichomoniasis on the cow-calf producer's profitability. Journal of the American Veterinary Medical Association. 194: 771-775.
37. Rae, D. O.; Chenoweth, P. J.; Genho, P. C.; McIntosh, A. D.; Crosby, C. E.; Moore, S. A. 1999. Prevalence of Tritrichomonas fetus in a bull population and effect on production in a large cow-calf enterprise. Journal of the American Veterinary Medical Association. 214: 1051-1055.
38. Rae, D. O.; Crews, J. E.; Greiner, E. C.; Donovan, G. A. 2004. Epidemiology of Tritrichomonas foetus in beef bull populations in Florida. Theriogenology 61: 605-618.
39. Repiso, M. V.; Gil, A.; Bañales, P.; D'Anatro, N.; Fernández, L.; Guarino, H.; Herrera, B.; Núñez, A.; Olivera, M.; Osawa, T.; Silva, M. 2005. Prevalencia de las principales enfermedades infecciosas que afectan el comportamiento reproductivo en la ganadería de carne y caracterización de los establecimientos de cría del Uruguay. Veterinaria. 40(157): 5-28.
40. Rojas, M.; Vázquez, P.; Verdier, M.; Campero, C. 2011. Evolución y distribución de las enfermedades de transmisión sexual en bovinos del partido de Rauch, prov. Buenos Aires, años 2001-2009. Revista Veterinaria Argentina. 27(273): 1-14.
41. Ryley, D. E.; Wagner B.; Polley, L. T.; Krieger, J. N. 1995. PCR-Based study of conserved and variable DNA sequences of Tritrichomonas foetus isolated from Saskatchewan, Canada. Journal of Clinical Microbiology. 33: 1308-1313.
42. Sebastiani, P.; Mandl, K. D.; Szolovits, P.; Kohane, I. S.; Ramoni, M. F. A. 2006. Bayesian dynamic model for influenza surveillance'. Statistics in Medicine. 25: 1803-1825.

43. SENASA. Res. 358/2008. Se reconoce el “Programa de control y erradicación de las enfermedades venéreas en bovinos de la Provincia de La Pampa” [on line]. Available from: http://www.senasa.gov.ar/contenido.php?to=n&in=1334&ino=1334&io=7938 (Accessed 12.07.17).
44. SENASA, 2017. Indicadores ganaderos. Existencias bovinas por categoría y departamento 2017. http://www.senasa.gob.ar/cadena-animal/bovinos-y-bubalinos/informacion/informes-y-estadisticas (Accessed 13/07/2017).
45. Skirrow, S. Z.; BonDurant, R. H. 1988. Bovine trichomoniasis. Veterinary Bulletin. 58: 591-603.
46. Soebiyanto, R. P.; Adimi, F.; Kiang, R. K. 2010. Modeling and predicting seasonal influenza transmission in warm regions using climatological parameters. PLoS One 5, e9450.
47. Souza, D. L. B.; Bernal, M. M. 2012. Incidencia, prevalencia y mortalidad del cáncer renal en España: estimaciones y proyecciones para el periodo 1998-2022. Actas Urológicas Españolas. 36(9): 521-526.
48. Speer, C. A.; White, M. W. 1991. Bovine trichomoniasis. Large Animal Veterinary. 46: 18-20.
49. Suárez, V. H.; Miranda, A. O.; Arenas, S. M.; Schmidt, E. E.; Lambert, J.; Schieda, A.; Felice, G.; Imas, D.; Sola, E.; Pepa, H.; Bugnone, V.; Calandri, H.; Lordi, L. V. 2008. Prevalencia de patologías e incidencia de la sanidad en los sistemas bovinos en el este de la provincia de La Pampa, Argentina. Revista Veterinaria Argentina. 25: 258-280.
50. Szonyi, B.; Srinath, I.; Schwartz, A.; Clavijo, A.; Ivanek, R. 2012. Spatio temporal epidemiology of Tritrichomonas foetus infection in Texas bulls based on state-wide diagnostic laboratory data. Veterinary Parasitology. 186: 450–455.
51. Taylor, M. A.; Marshall, R. N.; Stack, M. 1994. Morphological differentiation of Tritrichomonas foetus from other protozoa of the bovine reproductive tract. British Veterinary Journal. 150: 73-80.
52. Villarroel, A.; Carpenter, T. E.; BonDurant, R. H. 2004. Development of a simulation model to evaluate the effect of vaccination against Tritrichomonas foetus on reproductive efficiency in beef herds. American Journal of Veterinary Research. 65: 770-775.
53. Wangdi, K.; Singhasivanon, P.; Silawan, T.; Lawpoolsri, S.; White, N. J.; Kaewkungwal, J. 2010. Development of temporal modelling for forecasting and prediction of malaria infections using time-series and ARIMAX analyses: a case study in endemic districts of Bhutan. Malaria Journal. 9: 251.
54. Yao, C. 2013. Diagnosis of Tritrichomonas foetus-infected bulls, an ultimate approach to eradicate bovine trichomoniasis in US cattle? Journal of Medical Microbiology. 62: 1-9.
55. Yao, C.; Bardsley, K. D.; Litzman, E. A.; Hall, M. L.; Davidson, M. R. 2011. Tritrichomonas foetus infection in beef bull populations in Wyoming. Journal of Bacteriology and Parasitology. 2: 117.
56. Yang, N.; Cui, X.; Qian, W.; Yu, S.; Liu, Q. 2012. Survey of nine abortifacient infectious agents in aborted bovine fetuses from dairy farms in Beijing, China, by PCR. Acta Veterinaria Hungarica. 60: 83-92.
57. Zhang, X.; Zhang, T.; Pei, J.; Liu, Y.; Li, X.; Medrano-Gracia, P. 2016. Time Series Modelling of Syphilis Incidence in China from 2005 to 2012. PLoS ONE 11(2): e0149401. doi:10.1371/journal.pone.0149401.

Milk production in dairy cows supplemented with herbal choline and methionine

Producción de leche en vacas suplementadas con colina y metionina herbales

German David Mendoza [1], Mario Francisco Oviedo [2], Juan Manuel Pinos [3], Héctor Aarón Lee-Rangel [2], Anayeli Vázquez [2], Rogelio Flores [4], Francisco Pérez [4], Alejandro Roque [2], Oswaldo Cifuentes [2]

Originales: *Recepción:* 29/11/2017 - Aceptación: 17/10/2018

Abstract

The objective of this study was to evaluate the supplementation of herbal choline and methionine to dairy cows on milk production and milk composition during 60 days of lactation, while also identifying the volatile compounds of the herbal methionine and choline. Fourteen compounds were identified for herbal methionine and fifteen in herbal choline including aromas, alcohols, aldehydes and phenolics, some with nutraceutical properties. Twenty-multiparous Holstein cows (body condition score, BCS = 3.1 ± 0.15; mean ± SE) were fed a basal diet (16.3% CP, 1.6% DP and 1.71. Mcal/kg ME). Seven days after calving, cows were randomly assigned to treatments, which consisted of control basal diet or an oral dose of herbal choline (15 g/d) plus herbal methionine (10 g/d). The experiment lasted 60 days with measurements of milk production and composition every 7 days. Supplementation with herbal choline plus herbal methionine improved ($P < 0.05$) milk production (32.96 *vs.* 34.03 kg/d) and 4% FCM (28.23 *vs.* 29.91 kg/d). Protein content decreased ($P < 0.05$) on supplemented cows (29.9 *vs.* 31.7 g/kg). However, no effects on the remaining composition (fat, lactose, total solids and non-fatty solids) was found. Milk production can be improved by supplementing cows with the evaluated herbal sources of choline and methionine.

Keywords
volatile compounds • herbal • dairy production • choline • methionine

1 Universidad Autónoma Metropolitana. Unidad Xochimilco. 04960 México
2 Universidad Autónoma de San Luis Potosí. Facultad de Agronomia y Veterinaria. San Luis Potosí. 78321. México. hector.lee@uaslp.mx
3 Universidad veracruzana. Facultad de Medicina Veterinaria y Zootecnia. Veracruz. 91710. Mexico.
4 Universidad Autónoma del Estado de México. Centro Universitario UAEM Temascaltepec. Temascaltepec 51300. México.
5 Universidad Autónoma de San Luis Potosí. Centro de Biociencias.

Resumen

El objetivo de este experimento fue evaluar la suplementación con metionina y colina herbales en ganado lechero sobre la producción, cambios en composición de leche durante 60 días de lactancia, e identificar los compuestos fitoquímicos volátiles en metionina y colina herbales. Se identificaron 14 compuestos para metionina y quince para colina herbales entre los que se incluyen aromáticos, alcoholes, aldehídos, fenoles, algunos con propiedades nutracéuticas. Se usaron 20 vacas multíparas raza Holstein (condición corporal CC=3,1 ± 0,15) alimentadas con una dieta basal (16,3% PC, 6% RDP y 2,08 Mcal/kg EM). Siete días después del parto fueron asignadas a uno de los tratamientos que consistían en dieta testigo y la dieta testigo con dosis oral de colina (15 g/d) más metionina (10 g/d) herbales. El experimento tuvo una duración de 60 días, se registró la producción de leche y se caracterizó su composición. La suplementación de colina herbal con metionina herbal mejoró la producción (P<0.05) de leche (32,96 *vs.* 34,03 kg/d) y 4% FCM (28,23 *vs.* 29,91 kg/d), el contenido de proteína disminuyó (P<0,05) (29,9 *vs.* 31,7 g/kg) pero no afectó la composición. Se mejora la producción de leche al suplementar vacas con fuentes naturales de colina y metionina.

Palabras clave
compuestos volátiles • herbal • producción de leche • colina • metionina

Introduction

Herbal additives may improve animal health and production. However, many phytochemical action mechanisms are unknown. In this sense, to correctly identify these phytochemicals and their appropriate doses in order to safely use them is important (15). Many standardized herbal products used in animal feed have not been fully characterized yet.

The use of electronic nose based on different sensor technologies has been suggested as a rapid detection of quality -related volatile compounds for various food products (16, 40) facilitating the identification of nutra- ceutical properties in those products.

The use of ruminally protected choline (RPC) has demonstrated that choline is a limiting nutrient for milk production in dairy cattle (20, 36). Evaluations of herbal products in lambs indicate that some of these products have ruminally protected choline (18) that could be an alternative for dairy cattle. Methionine has been recognized as one of the limiting amino acids for milk production in dairy cows (33) and even when the ruminally protected form is available and has been evaluated (24) the benefit-cost ratio can make its inclusion difficult in some units.

During the transition from pregnancy to lactation, dairy cows present a period of negative energy and protein balance as a result of an increased metabolic demand from the mammary gland (11). Since methyl donors are required for the synthesis of key compounds such as phosphatidylcholine and carnitine in tissues (37), a negative methyl donor balance also may be an important challenge for the transition dairy cow.

Due to extensive microbial degradation in the rumen, dietary availability of key methyl donors is limited (17). Thus, the possibility of improving milk production in dairy cows by increasing the duodenal flow of choline and methionine with herbal products should be evaluated. Some authors report that evaluations for 30 to 90 days are valid to find responses in dairy cows (4, 9, 25, 36, 37). Therefore, the objective of this study was to evaluate the supplementation whith herbal methionine and choline on dairy cows on milk production and milk composition during 60 days of lactation.

Material and methods

Evaluations of volatile organic compounds (VOCs) in herbal choline and methionine by flash gas chromatography electronic nose

The flash gas chromatography electronic nose (FGC- E-Nose) model Heracles II, equipped with an automatic injection unit HS100 (AlphaMOS®, Tolouse, France), was used to detect the VOCs of the herbal choline and methionine.

The Heracles II was equipped with two columns working in parallel mode: a non-polar column (DB-5: 5% phenyl- 95% dimethylpolysiloxane) and DB-1701 (14% cyanopropylphnyl- 86% dimethylpoly-siloxane). The injector was maintained at a constant temperature of 200°C. The samples of the feed plant additives were placed in 20 mL magnetically sealed vials with a plug and without any treatment or extraction solvent. The vials were placed in the Heracles II auto-sampler, which was placed in a shaker oven and shaken at 500 rpm for 900 seconds at 40°C. Next, 1 mL sample was taken from the headspace in the electronic nose. Samples were analyzed in triplicate. A single chromatogram was created by joining two columns of overlapping chromatograms, helping to reduce identification errors. The identifications were made using the Kovats index with a C6-C16 standard (29, 35). The GC subjected the samples to a temperature program separating the volatile organic compounds and maintaining a constant flow of hydrogen of 1 mL/min. Then the samples were brought to a temperature of 50°C for 30 s, before increasing it 10°C / s until it reached 280°C. Separate species were detected by the electronic nose software using multi-variable statistical analysis (Alpha Soft® by Alpha MOS®).

Chemometrics

In this study, a first explorative step was carried out using peak areas that were automatically calculated by the software Alpha Soft® which uses raw data from the abundance of metabolites to construct a multivariate model using Principal Component Analysis (PCA). The PCA uses orthogonal transformation to convert a set of observations by the different compounds of possibly correlated variables into values of linearly uncorrelated variables. This analysis guaranteed independence if the group of data jointly normally distributed. The PCA is a chemometric procedure that rotates the original space to another one and its vectors resultants are the principal components (PC) oriented along directions containing the maximum explained variance (29).

Productive phase

The experiment was conducted at the experimental station of the UASLP (22°11' N, 100°56' O, 1850 m above sea level) with a mean temperature of 17.5°C. Twenty multiparous dairy Holstein cows (body condition score, BCS = 3.1 ± 0.15;

mean ± SE), were fed a basal diet (16.3% CP, 6 RDP and 2.08 Mcal/kg ME) of oat hay, alfalfa hay, rolled corn and concentrate (65% forage, 35% concentrate).

Seven days after parturition, the cows were randomly assigned to one of the two treatments (N=10), which consisted in a control group and an oral dose of herbal choline (15 g/d) plus herbal methionine (10 g/d).

To prepare the doses of herbal products, a mixture of 250 g of molasses and 1250 g of corn flour was prepared and stored in the refrigerator at 3°C. Later, a total of 50 g of this mixture was mixed with the daily dose of herbal choline and herbal methionine for each animal, preparing a mixture and individually feeding to ensure its consumption.

The cows received the herbal mixture individually in the milking parlor at 6:00 hour. During the rest of the day, cows had access to a yard with water *ad libitum*.

The herbal products used were OptiMethionine and BioCholine (Technofeed, Mexico, Nuproxa Switzerland, Indian Herbs) supplied individually for 60 days. Milk production was recorded daily and its composition, (fat, protein, lactose, total solids and non-fatty solids) characterized every 7 days using morning and afternoon samples which were mixed, homogenized in a water bath for 1 min (40°C) until the temperature reached 29°C and analyzed with a Lactoscan Ultrasonic milk analyzer (Milkotronic®, Bulgaria). 4% fat corrected milk (FCM) of each cow was calculated as follows: FCM = [(0.4 kg milk) + (0.15 kg milk fat %)].

The yield of energy corrected milk (ECM) was calculated by the formula proposed by DeFrain *et al.* (2006): ECM = [(0.327 kg milk) + (12.95 kg fat) + (7.2 kg protein)]. Body condition score was assessed twice on d 1 and 56 using the scale of 1 to 5, in increments of 0.25 according whit Edmonson *et al.* (1998), at the time of enrollment.

Feed analysis

Samples of feed were composited every 15 days to analyze dry matter and total nitrogen according to the AOAC (1999) (table 1). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses were carried out according to Van Soest *et al.* (1991).

Table 1. Experimental diet and chemical composition.

Tabla 1. Dieta experimental y composición química.

Item	%
Alfalfa hay	53.45
Oat hay	11.29
Corn rolled	8.06
Concentrate[a]	24.20
Minerals and vitamins[b]	3.0
Chemical composition	
Dry matter (%)	89.73
Crude protein (%)	16.30
Rumen degradable protein (%)[b]	6.0
FDN (%)	34.54
FDA (%)	26.15
ED (Mcal/kg)[c]	2.08

[a] Nu-3® Ganado Lechero18% Línea Campestre: DM 88%, CP 18%, EE 2%, CF 15%, Ash 11.5% and TND 41.5%.

[b] Mineral Premix: Ca 5.6g, vitamin A 500,000 IU, vitamin D 150,000 IU and vitamin E 1000 IU.

[c] Estimated according to the NRC (2001).

[a] Nu-3® Ganado Lechero18% Línea Campestre: MS 88%, PC 18%, EE 2%, FC 15%, Ceniza 11,5% y NDT 41,5%

[b] Premezcla Mineral: Ca 5,6g, vitamina A 500,000 UI, vitamina D 150,000 UI y vitamina E 1000 UI.

[c] Estimado de acuerdo con el NRC (2001).

Statistical analyses

Data were analyzed as a completely randomized design (42). Data were analyzed with the JMP7 software (39) using the General Linear Model.

Results

The Kovat's indices database allowed identification of 14 major relevance compounds in herbal methionine and 15 in herbal choline (table 2 and table 3, page 337) including aromas, alcohols, aldehydes, and phenolics, some with nutraceutical properties.

Figure 1 (page 337), shows that, according whit de PCA, and regarding composition, herbal choline and methionine are totally different (99.84%).

Milk production was significantly increased (3.2%, $P<0.01$). 4% fat corrected milk and yield of energy corrected milk decreased ($P<0.02$). Protein and fat contents decreased. However, no effects on the other milk components were detected (table 2).

Discussion

The electronic nose was used for rapid qualitative detection and discrimination of herbal choline and methionine while a gas chromatography mass spectrometer with headspace analyzer (GCMS-HS) was used for aroma profiling.

The Principal component analysis (PCA) allowed the visualization of the resemblance and difference among the products. OptiMethionine and BioCholine samples were separated in PC1 which described 57.8% of the peak variations (figure 1, page 337).

Table 2. Tentative identification of volatile compounds of herbal methionine from the electronic nose profile.

Tabla 2. Identificación tentativa de los compuestos volátiles de metionina herbal derivados del cromatógrafo de nariz electrónica.

Retention Time, m	Compound	Relative Area	Relative height
Polar Column			
36.81	2-methylbutanoic acid	1.24	1.49
41.38	Alpha-phellandrene	3.9	5.29
45.24	Undecane	12.92	9.37
47.15	[Z]-3-hexenyl isobutyrate	11.33	7.98
58.14	Methyl undecanoate	2.87	3.36
No Polar Column			
15.28	Trimethylamine	3.31	2.35
17.8	Diethyl ether	3.3	3.45
23.35	2,2,4-trimethylpentane	2.59	2.20
32.26	[E]-2-penten-1-ol	1.43	1.65
36.34	2-methylbutanoic acid	3.78	4.57
53.31	Alpha-ionone	23.22	18.43
60.36	Delta-decalactone	2.60	2.48

Table 3. Tentative identification of volatile compounds of herbal choline from the electronic nose profile.

Tabla 3. Identificación tentativa de los compuestos volátiles de colina herbal derivados del cromatógrafo de nariz electrónica.

Retention Time, m	Compound	Realtive Area	Relative height
Polar Column			
18.93	Diethyl ether	4.44	4.61
43.19	[Z]-2-octenal	5.48	6.18
44.53	P-Cresol	2.55	3.27
47.51	4-ethylphenol	33.07	34.64
53.59	4-vinylguaiacol	5.81	7.27
56.14	Trans-2-undecenal	3.97	3.20
No Polar Column			
15.26	Trimethylamine	2.49	2.02
17.27	Diethyl ether	1.50	1.58
21.62	1-propanol	2.69	2.40
24.25	Isopropyl acetate	3.82	3.60
34.90	Beta pinene	4.56	6.91
48.53	Methylnonanedione	5.38	8.10
51.45	4-vinylguaiacol	3.87	3.21
55.38	P-menthadienhydroperoxide	36.91	27.90

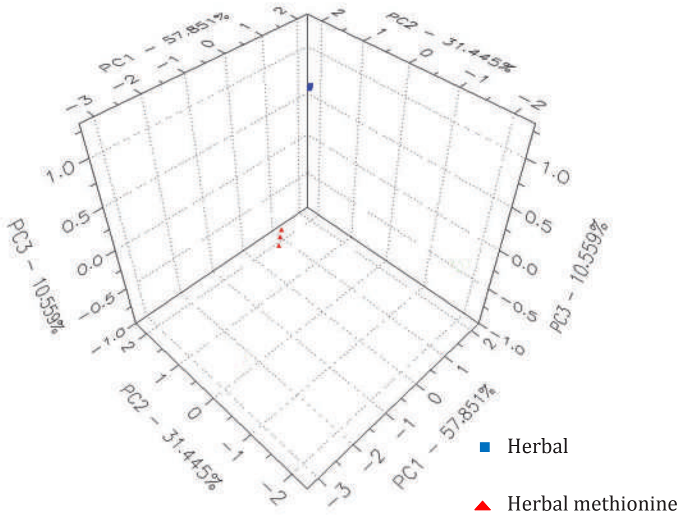


Figure 1. PCA model built with the electronic nose data related to the herbal methionine and herbal choline.

Figura 1. PCA con datos obtenidos del cromatógrafo de nariz electrónica para colina y metionina herbales.

The fact that the herbal products are positive in one axis and negative on the other, is because the metabolites are from different plants with different aromatic profiles, for example BioCholine is elaborated with plans such as *Trachyspermum ammi, Azadirachta indica* and *Andrographis paniculata* whereas OptiMethionine *Trigonella foenumgraceum* and *Allium sativum.*

Milk production, 4% fat corrected milk and yield of energy corrected milk were increased (*P*<0.01). The benefits of supplementing choline and methionine protected from rumen degradation have been reported by several authors including meta-analyses (34, 37). However, none evaluated herbal sources. Several authors have reported that 12 to 15 grams per day of RPC, increase milk production on 7 to 8% (20, 24) representing about 2 kg/d of milk, similar to that observed in this experiment.

Although some experiments have used doses from 25 to 60 g of different RPC sources based on choline chloride (30, 38, 41) or even higher doses (6, 13), Pinotti *et al*. (2005) concluded that the best responses for improved milk production are obtained when 12-20 g/day of RPC are provided which is similar to the dose used with the herbal product.

Regarding ruminally protected methionine (RPM), Lara *et al.* (2006) found that milk production was increased up to 14% above the control with 16 g per day, that later decreased. Similar rresponses were observed in first-calf heifers with doses from 14 to 16 g/d (5).

In both studies, the concentration of milk protein was increased by RPM. Zhou *et al.* (2016b) observed an increment of 9% in milk production and increasing milk protein content with an estimated dose of 14 g/d. Some experiments have supplemented the combination of Met and choline in dairy cattle rations. Zhou *et al.* (2016a) did not find RPC - RPM interactions in any measured variable. Sun *et al.* (2016) reported that both nutrients improved milk production (5.04%) and increased milk fat and protein. In contrast, Soltan *et al.* (2012) observed that milk production increased with both nutrients in a greater magnitude (14%) than with RPC (11%) or RPM (6%) separately, without changes in milk composition. Milk protein content was decreased (P<0.02) by the herbal supplementation however, no effects on the other milk components were detected (table 4, page 339). After methionine supplementation, an average increase of 3.8 kg/d in milk production in the first 30 d of lactation was detected by Zhou *et al.* (2016b). Considering that Met has been identified as one of the 2 most limiting AA for lactating cows (33) and that a greater DMI would increase daily protein intake, the milk yield response when supplementing Met to achieve a Lys: Met close to the suggested optimum, was as expected. The supplementation with rumen-protected methionine improved milk protein synthesis (48), while high percentages of total solids in the choline+methionine group was not associated with milk protein and milk fat percentage in those cows. The supplementation of methionine and/or choline has been evaluated in terms of the contribution to the improved performance and immuno-metabolic status in dairy cows by Zhou *et al.* (2016b) who recognized the predominate effects of Met.

Table 4. Effect of herbal choline and methionine supplementation on milk production and composition of Holstein cows.

Tabla 4. Efecto de la suplementación con colina y metionina herbales en la producción y composición de leche de vacas Holstein.

	Control	Choline + Methionine	SEM	*P*-value
Milk yield (kg/d)	32.96 [b]	34.03 [a]	0.2394	0.01
4% FCM (kg/d)	28.23 [b]	29.91 [a]	0.2321	0.01
ECM (kg/day)	.34 [a]	31.56 [b]	0.2456	0.01
Body Score Condition				
Day 1	3.13	3.08	0.044	0.44
Day 56	2.77	2.69	0.057	0.31
Milk composition (g/kg)				
Fat	33.9	28.3	0.2562	0.51
Protein	31.7 [a]	29.9 [b]	0.039	0.02
NFS	66.0	72.5	0.396	0.28
Lactose	46.1	48.0	0.087	0.17
Total Solids	176.0	180.7	0.423	0.45

NFS: non-fat solids; SEM: standard error of the mean.

NFS: sólidos no grasos; SEM: error estándar de la media.

In contrast, Sun *et al.* (2016) show a correlated response in the blood antioxidant status and in the immune response (plasma interleukin 2, concentration and tCD4+/CD8+ T lymphocyte ratio) in postpartum cows. Other studies have demonstrated the antioxidant capacity of choline *per se* (43, 49) while the supplementation of RPM and RPC has also improved reproductive performance in dairy cows (4).

Considering health risks during the peripartal period (4, 49), methionine and choline should be supplemented around parturition. Methionine supplementation has led to a lower incidence of ketosis (49), besides improving lactation performance. It has also demonstrated greater pre- and postpartum DMI, milk fat yield, and milk protein yield during the peripartal period. However, this has not been consistent with choline supplementation (48).

The herbal choline and methionine containing bioactive compounds (table 2, page 336 and table 3, page 337) may help protect dairy cattle against diseases, maintaining animal health during the critical peripartal period.

Reviewing the properties of the main compounds found in Biocholine and Optimethionine, it may be speculated that herbal products have nutraceutical properties. Andrade *et al.* (2016) reported high cytotoxicity effects of p-menthane and its derivatives against human tumor cells evaluated in mice experimental sarcoma tumors. Hüe *et al.* (2015) reported that the main constitutes of p-menthane are thymol (30.5%) and γ-terpinene (33.0%) with nutraceutical properties.

The 4-vinylguaiacol has demonstrated antioxidant properties in cultured hepatocytes (14), while the β-pine, part of the essential oils of different *Pinus* species, has anti-inflammatory and cytotoxic activity.

The β-pinene is a potential agents for anticancer and anti-inflammatory drugs (7) and it is possible that the aldehyde β-pines monoterpene conformation is responsible for the ruminal protection of the biocholine given that β-pinene has bacteriostatic and bactericidal effects (46).

Lin *et al.* (2013) reported that α-phellandrene promoted immune responses in a murine model, stimulating macrophage proliferation and promoting cell function *in vivo*.

The Undecane has been found as part of the structure of limonoids with cytotoxic activities and anti-inflammatory activity studied in the root barks of *Walsura robusta* (14). The Trans-2-Undecenal, also found in *Citrullus vulgaris* (23) and *Curcuma amada,* is an aldehyde recognized for the characteristic mango aroma (31) that we hypothesized contributed to the ruminal protection of BioCholine. Other compounds in *Mango ginger* have been recognized with some anti-breast cancer effects (27).

Some compounds such as alcohols (table 2, page 336; table 3, page 337) may not be beneficial given that various *in vitro* studies indicate that ethanol may reduce ruminal microbial activity (19). Ethanol was detected in OptiMethionine; nevertheless, mixed ruminal microbes can convert ethanol to carboxylic acids, acetic, butyric and hexanoic (47). Ethanol can be found in the rumen in minor concentrations with barley additions. There are no reports of its enrolment in metabolic diseases in cows (8).

The 1-propanol has antiseptic and disinfectant properties (44) and could be metabolized by ruminal microbes as another alcohol. The 2 Pentanol is an aromatic compound reported in fresh bananas and other foods (22, 32). The amounts of alcohols found in the herbal products should not be a concern, as demonstrated in cow performance.

Conclusion

The herbal choline and methionine contain numerous volatile phytochemical compounds. Milk production can be improved by supplementing cows with the evaluated herbal sources, considering a reduction in the milk protein content but without altering other milk components.

References

1. Andrade, L. N.; Lima, T. C.; Amaral, R. G.; Do, Ó.; Pessoa, C.; De Moraes Filho, M. O.; Soares, B. M.; Do Nascimento, L. G.; Carvalho A. A. C.; De Sousa, D. P. 2015. Evaluation of the cytotoxicity of structurally correlated p-menthane derivatives. Molecules. 20(7): 13264-13280.
2. Andrade, L. N.; Amaral, R. G.; Dória, G. A. A.; Fonseca, C. S.; Da Silva, T. K. M.; Júnior, R. L. C.; Thomazzi, S. M.; Nascimento, L. G. D.; Carvalho, A. A.; De Sousa, D. P. 2016. *In vivo* anti-tumor activity and toxicological evaluations of perillaldehyde 8,9-epoxide, a derivative of perillyl alcohol. International Journal of Molecular Science. 17(1): 32, 2-11.
3. AOAC (Association of Official Analytical Chemists). 1999. Methods of Analysis. Washington D.C. USA.
4. Ardalan, M.; Rezayazdi, K.; Dehghan-Banadaky, M. 2010. Effect of rumen-protected choline and methionine on physiological and metabolic disorders and reproductive indices of dairy cows. Journal of Animal Physiology and Animal Nutrition. 94: 259-265.
5. Ayala, J.; Mendoza, G. D.; Pinos, J. M.; Lara, A.; Sánchez, M. T. 2010. Effects of fat and rumen-protected methionine supplementation on production response of first-lactation cows. Acta Agriculturae Scandinavica. Section A, Animal Science. 60: 159-165.

6. Banevičius, E.; Einorytė, S.; Falkauskas, R. 2016. Choline chloride possible impact on cow´s productivity. Veterinarija ir Zootechnika. 73: 95.
7. Basholli-Salihu, M.; Roswitha, S.; Avni, H.; Dafina, M.; Helmut, V.; Behxhet, M.; Monika, M. 2017. Phytochemical composition, anti-inflammatory activity and cytotoxic effects of essential oils from three *Pinus spp.* Pharmaceutical Biology. 55: 1553-1560.
8. Burim, A.; Qendrim, Z.; Fozia, S. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. Metabolomics*.* 6: 583-594.
9. Davidson, S.; Hopkins, B. A.; Odle, J.; Brownie, C.; Fellner, V.; Whitlow, L. W. 2008. Supplementing limited methionine diets with rumen-protected methionine, betaine, and choline in early lactation Holstein cows. Journal of Dairy Science. 91: 1552-1559.
10. DeFrain, J. M.; Hippen, A. R.; Kalscheur, K. F.; Schingoethe, D. J. 2006. Feeding lactose to increase ruminal butyrate and the metabolic status of transition dairy cows. Journal of Dairy Science*.* 89: 267-276.
11. Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: The final frontier? Journal of Dairy Science. 82: 2259-2273.
12. Edmonson, A. J.; Lean, I. J.; Weaver, D. L.; Farver, T.; Webster, G. 1998. A body condition-scoring chart for Holstein dairy cows. Journal of Dairy Science*.* 72: 68-78.
13. Elek, P.; Newbold, J. R.; Gaal, T.; Wagner, L.; Husveth, F. 2008. Effects of rumen-protected choline supplementation on milk production and choline supply of periparturient dairy cows. Animal. 2: 1595-1601.
14. Esatbeyoglu, T.; Ulbrich, K.; Rehberg, C.; Rohn, S.; Rimbach, G. 2015. Thermal stability, antioxidant, and anti-inflammatory activity of curcumin and its degradation product 4-vinyl guaiacol. Food and Function. 6: 887-893.
15. Frankič, T.; Voljč, M.; Salobir, J.; Rezar, V. 2009. Use of herbs and spices and their extracts in animal nutrition. Acta agriculturae Slovenica. 94: 95-102.
16. Gardner, J. W.; Bartlett, P. N. 1999. Electronic noses. Principles and applications. Oxford University Press: Oxford. England.
17. Girard, C. L.; Matte J. J. 2005. Folic acid and vitamin B12 requirements of dairy cows: A concept to be revised. Livestock Production Science. 98: 123-133.
18. Godinez-Cruz, J.; Cifuentes-López, O.; Cayetano, J.; Lee-Rangel, H. A.; Mendoza, G.; Vázquez, A.; Roque, A. 2015. Effect of choline inclusion on lamb performance and meat characteristics. Journal of Animal Science. 93 (Suppl. 3) 766 (Abstr).
19. Gomes, M. F. F.; Ítavo, C. C.; Ítavo, L. C.; Leal, C. R.; Silva, J. A.; Heimbach, N. S.; Leal, E. S. 2017. *In vitro* fermentation characteristics of ruminant diets using ethanol extract of brown propolis as a nutritional additive. Revista Brasileira Zootecnia. 46(7): 599-605.
20. Hartwell, J. R.; Cecava, M. J.; Donkin, S. S. 2000. Impact of dietary rumen undegradable protein and rumen-protected choline on intake, peripartum liver triacylglyceride, plasma metabolites and milk production in transition dairy cows. Journal Dairy Science. 83: 2907-2917.
21. Hüe, T.; Cauquil, L.; Fokou, J. B. H.; Dongmo, P. M. J.; Bakarnga-Via, I.; Menut, C. 2015. Acaricidal activity of five essential oils of *Ocimum* species on *Rhipicephalus (Boophilus) microplus* larvae. Parasitoogyl Research. 114(1): 91-99.
22. Jordan, M. J.; Tandon, K. S.; Philip, E.; Goodner, K. L. 2001. Aromatic profile of aqueous banana essence and banana fruit by Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography-Olfactometry (GC-O). Journal Agricultural Food Chemistry*.* 49(10): 4813-4817.
23. Kemp, T. R. 1975. Identification of some volatile compounds from *Citrullus vulgaris*. Phytochemistry. 14(12): 2637-2638.
24. Lara, A.; Mendoza, G. D.; Landois, L. A.; Bárcena, R.; Sánchez, T.; Rojo, R.; Ayala, J.; Vega, S. 2006. Milk production in Holstein cows supplemented with different levels of ruminally protected methionine. Livestock Science. 105: 105-108.

25. Leiva, T.; Cooke, R.; Brandao, A. 2015. Efects of rumen protected choline supplementation on metabolic and performance responses of transition dairy cows. Journal of Animal Science. 93: 1896-1904.
26. Li, Y.; Li, S.; Meng, X.; Gan, R. Y.; Zhang, J. J.; Li, H. B. 2017. Dietary natural products for prevention and treatment of breast cancer. Nutrients. doi: 10.3390/nu9070728.
27. Lima, F. S.; Sá Filho, M. F.; Greco, L. F.; Santos, J. E. P. 2012. Effects of feeding rumen-protected choline on incidence of diseases and reproduction of dairy cows. Veterinary Journal. 193: 140-145.
28. Lin, J. J.; Lin, J. H.; Hsu, S. C.; Weng, S. W.; Huang, Y. P.; Tang, N. Y.; Lin, J. G.; Chung, J. G. 2013. Alpha-phellandrene promotes immune responses in normal mice through enhancing macrophage phagocytosis and natural killer cell activities. *In Vivo.* 27(6): 809-14.
29. Melucci, D. A.; Bendini, F.; Tesini, S.; Barbieri, A.; Zappi, S.; Vichi, T.; Toschi, G. 2016. Rapid direct analysis to discriminate geographic origin of extra virgin olive oils by flash gas chromatography electronic nose and chemometrics. Food chemistry. 204: 263-273.
30. Munafo, J. P.; Didzbalis, J.; Schnell, R. J.; Schieberle, P.; Steinhaus, M. 2014. Characterization of the major aroma-active compounds in mango (*Mangifera indica L.*) cultivars haden white alfonso, praya sowoy, royal special, and malindi by application of a comparative aroma extract dilution analysis. 2014, Journal Agricultural of Food Chemistry. 62(20): 4544-4551.
31. Nogueira, J. M. F.; Fernandes, P. J. P.; Nascimento, A. M. D. 2003. Composition of volatiles of banana cultivars from Madeira Island. Phytochemical Analysis. 14: 87-90.
32. NRC. 2001. Nutrient Requirements of Dairy Cattle. National Research Council. The National Academies Press, Washington. DC. USA.
33. Patton, R. A. 2015. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: A meta-analysis. Journal of Dairy Science. 93: 2105-2118.
34. Peng, Q.; Tian, R.; Chen, F.; Li, B.; Gao, H. 2015. Discrimination of producing area of Chinese Tongshan kaoliang spirit using electronic nose sensing characteristics combined with the chemometrics methods. Food chemistry. 178: 301-305.
35. Piepenbrink, M. S.; Overton, T. R. 2003. Liver metabolism and production of cows fed increasing amounts of rumen-protected choline during the periparturient period. Journal of Dairy Science. 86: 1722-1733.
36. Pinotti, L.; Baldi, A.; Dell'Orto V. 2002. Comparative mammalian choline metabolism with emphasis on the high-yielding dairy cow. Nutrition Research Reviwes. 15: 315-332.
37. Pinotti, L.; Campagnoli, A.; Dell'Orto, V.; Baldi, A. 2005. Choline: Is there a need in the lactating dairy cow? Livestock Production Science. 98: 149-152.
38. Sales, J.; Homolka, P.; Koukolova, V. 2010. Effect of dietary rumen-protected choline on milk production of dairy cows: A meta-analysis. Journal of Dairy Science. 93: 3746-3754.
39. Sall, J.; Lehman, A.; Stephens, M.; Creighton, L. 2012. JMP® Start Statistics: A Guide to Statistics and Data Analysis. Cary. NC. USA: SAS Institute In.
40. Soltan, M. A.; Mujalli, A. M.; Mandour, M. A.; El-Shinway Abeer, M. 2012. Effect of dietary rumen protected methionine and/or choline supplementation on rumen fermentation characteristics and productive performance of early lactating cows. Pakistan Journal Nutrition. 11: 221-230.
41. Steel, G. D. R.; Torrie, J. H.; Dickey, D. A. 1997. Principles and Procedures of Statistics. A Biometrical Approach. New York: McGraw-Hill.
42. Sun, F.; Cao, Y.; Cai, C.; Li, S.; Yu, C.; Yao, J. 2016. Regulation of nutritional metabolism in transition dairy cows: Energy homeostasis and health in response to post-ruminal choline and methionine. PLoS One. 11(8), e0160659.
43. Tattanahalli, L.; Nagabhushan. 1980. 1-Aryl-2-acylamido-3-fluoro-1-propanols, methods for their use as antibacterial agents and compositions useful therefore. Patent US4235892.
44. Uribe, S.; Ramirez, J.; Peña, A. 1985. Effects of beta-pinene on yeast membrane functions. Journal of Bacteriology. 161: 1195-1200.

45. Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. 1991. Methods for dietary fibre, neutral detergent fibre, and nonstarch carbohydrates in relation to animal nutrition. Journal of Dairy Science. 74: 3583-3597.
46. Weimer, P. J.; Kohn, R. A. 2016. Impacts of ruminal microorganisms on the production of fuels: how can we intercede from the outside?. Applied Microbiology and Biotechnology. 100: 3389-3398.
47. Zhou, Z.; Bulgari, O.; Vailati-Riboni, M.; Trevisi, E.; Ballou, M. A.; Cardoso, F. C.; Luchini, D. N.; Loor, J. J. 2016a. Rumen-protected methionine compared with rumen-protected choline improves immunometabolic status in dairy cows during the peripartal period. Journal of Dairy Science. 99: 8956-8969.
48. Zhou, Z.; Vailati-Riboni, M.; Trevisi, E.; Drackley, J. K.; Luchini, D. N.; Loor, J. 2016b. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. Journal of Dairy Science. 99: 8716-8732.
49. Zhu, J.; Wu, Y.; Tang, Q.; Leng, Y.; Cai, W. 2010. The effects of choline on hepatic lipid metabolism, mitochondrial function and antioxidative status in human hepatic C3A cells exposed to excessive energy substrates. Nutrients. 6: 2552-2571.

Acknowledgement

Authors thank financial support to FAI-UASLP C18-FAI-05-34.34.
The authors acknowledge Nuproxa México for donating the herbal product and Eng. Martin Castillo for technical assistance.

Effect of protein source on *in situ* digestibility of sugarcane silage-based diets

Efecto de la fuente de proteína en la digestibilidad *in situ* de dietas a base de ensilado de caña de azúcar

José Andrés Reyes-Gutiérrez [1], Oziel Dante Montañez-Valdez [1*],
Cándido Enrique Guerra-Medina[1, 2] Alejandro Ley de Coss [3]

Originales: *Recepción:* 15/02/2018 - *Aceptación:* 17/12/2018

Abstract

The objective of this study was to evaluate the effect of the protein source in sugarcane silage-based diets on the ruminal pH and *in situ* dry matter digestibility (DMD). The treatments were: 1)- 60% sugarcane silage + 15% soybean meal (SBM); 2)- 60% sugarcane silage + 15% fish meal (FM); 3)- 55% sugarcane silage + 20% canola meal (CM); and T4)- 50% sugarcane silage + 30% coconut meal (CCM). *In situ* DMD was determined by the nylon bag technique using four cows equipped with ruminal cannula. Five grams of each experimental diet were weighted in nylon bags and incubated for 8, 12, 24, 48, 72 and 96 h. Dry matter digestibility for SBM, CM, and CCM showed higher values compared to FM. A similar pH among treatments was recorded; however, at 4 h decreases in SBM and FM were observed. Sugarcane silage in integral diets with the different protein sources used in this study, did not modify ruminal pH but showed lower DMD when fish meal was the protein source.

Keywords
bacterial inoculum • ruminal kinetic • tropical forages

1 University of Guadalajara/University Center of the South. Animal Nutrition Research Group. Ave. Enrique Arreola Silva 883. Ciudad Guzmán. 49000. Jalisco. México. * montanez77@hotmail.com
2 National Institute of Forestry, Agriculture and Livestock Research/Rosario Izapa Experimental Campus. Tuxtla Chico. Chiapas. México.
3 Autonomous University of Chiapas. Faculty of Agronomic Sciences. Campus V Villaflores. 30460. Chiapas. México.

Resumen

El objetivo de este estudio fue evaluar el efecto de la fuente proteica de dietas a base de ensilado de caña de azúcar sobre el pH ruminal y la digestibilidad *in situ* de la materia seca (DMD). Los tratamientos fueron: 1)- 60% de ensilado de caña de azúcar + 15% harina de soya (SBM); 2)- 60% ensilado de caña de azúcar + 15% harina de pescado (FM); 3)- 55% ensilado de caña de azúcar + 20% harina de canola (CM); y 4)- 50% de ensilado de caña de azúcar + 30% de harina de coco (CCM). La DMD se determinó mediante la técnica de bolsa de nylon utilizando cuatro vacas equipadas con cánula ruminal. Se pesaron cinco gramos de cada dieta experimental y se incubaron en bolsas de nylon por 8, 12, 24, 48, 72 y 96 h. La digestibilidad de la materia seca para SBM, CM y CCM mostraron los valores más altos en comparación con FM. No hubo cambios en el pH ruminal en los tratamientos, pero, a las 4 h disminuyó en SBM y FM. El ensilado de caña de azúcar en dietas integrales con las diferentes fuentes de proteínas no modifica el pH ruminal, pero reduce la DMD cuando la harina de pescado es la fuente de proteína.

Palabras clave

inóculo bacteriano • dinámica ruminal • forrajes tropicales

Introduction

In the tropics, grasses are the main source of food for livestock; however, during the drought season, growth and quality of forages is low, affecting animals productivity. Therefore to evaluate alternatives for forage replacement during that period, turns necessary.

Sugarcane is a crop produced in more than 100 countries worldwide, and its biomass production exceeds that of any other forage, making it a good animal feed strategy for sustainable agricultural development in many countries (2).

Sugarcane and particularly sugarcane silage can be an important forage given that it keeps its quality for long periods. However, silage causes losses of up to 30% of dry matter (DM), and concentration of the cell walls components, reducing the *in vitro* digestibility of DM (6).

Furthermore, silage has high levels of lactic acid and residual carbohydrates, which can potentially inhibit, by pH lowering, microorganisms that spoil the silage, such as yeasts and molds (16). In recent years, there has been increased interest in the use of additives in sugarcane silage, with the objective of inhibiting yeast growth that promote alcoholic fermentation (6). Furthermore, these products have a high protein value and absorbing characteristics that could improve the nutritive value and the fermentation profile by correcting the low protein values of sugarcane and reducing effluent losses. However, strategies have been developed to improve feed intake and reduce sugarcane's nutritional deficiencies by using other ingredients in the ration, allowing sugarcane to be an important fraction of the diet (17).

Studies have demonstrated that diets containing sugarcane and proteic ingredients improve animal performance, take advantage of the high concentration of fermentable carbohydrates, and improve ruminal function (10), but the lack of information on digestibility parameters and ruminal variables of sugarcane silage combined with common protein ingredients has created the need to conduct studies on sugarcane ruminal degradation.

Objective

Provide useful information about the effect of different protein sources on the ruminal digestibility parameters and its effect on rumen pH fluctuations of ensiled sugarcane based diets.

Materials and methods

This study was carried out at Zapotlán El Grande, Jalisco, Mexico, with geographic coordinates of 19°27'13" North latitude and meridians 103°27'57" West longitude, with an altitude of 1,520 m. The biomass of one hectare of sugarcane-variety CP 72-2086, which was approximately 13 months old, second cut-was used in this experiment.

The forage was harvested by hand and chopped in a stationary chopper adjusted for a theoretical cut length of 2.5 cm.

Total biomass was separated into five parts to make the same number of silages. Ensiling was initiated simultaneously in mini silos with 1% bacterial inoculum and 1% additive.

The inoculum consisted of 10.0% molasses, 1.0% commercial yogurt (LALA®; containing: *Lactobacillus plantarum*, *L. bulgaricus*, *L. casei*, *L. acidofilus*, and *L. bifidus*), 5.0% chicken manure, 0.5% urea, and 83.0% water; the additive was formulated with 1.0% urea, 0.1% ammonium sulfate, and 0.25% phosphorus.

The silo was opened after 40 days of storage. The treatments were: 1. 60% sugarcane silage + 15% soybean meal (SBM); 2. 60% sugarcane silage + 15% fish meal (FM); 3. 55% sugarcane silage + 20% canola meal (CM); and 4. 50% sugarcane silage + 30% coconut meal (CCM).

The diets consisted of sugarcane silage with the different protein sources mixed with alfalfa hay, ground corn, ground sorghum, and sugarcane molasses. The Rations were fed in two sessions (AM and PM) to ensure greater cellulolytic activity of rumen microflora. *Ad libitum* fresh clean water was provided. The experimental diets and analyzed composition of the diets are shown in table 1 (page 347).

Samples of the diets were dried in a circulating air oven at 60°C for 24 h and then milled in a hammer mill equipped with a 2-mm sieve for further analysis.

Total DM was determined using a circulating air oven (100°C for 24 h). Crude protein (CP) was determined by Kjeldahl, ash (A) and organic matter (OM) was calculated by difference using the technique described by the AOAC (2007).

Fiber fractions (NDF and ADF) were determined using alpha amylase without a correction, as specified by Van Soest *et al.* (1991).

Table 1. Ingredients and chemical composition of the experimental diets (%).

Tabla 1. Ingredientes y composición química de las dietas experimentales (%).

	Treatments[1]			
	SBM	**FM**	**CM**	**CCM**
Ingredients proportion	%			
Sugarcane silage	60	60	55	50
Soybean meal	15	-	-	-
Fish meal	-	15	-	-
Canola meal	-	-	20	-
Coconut meal	-	-	-	30
Alfalfa hay	10	10	10	10
Ground corn	5	5	5	2.5
Ground sorghum	5	5	5	2.5
Cane molasses	5	5	5	5
Chemical composition				
Dry matter	49.30 ^{a}	45.50 ^{b}	50.45 ^{a}	53.00 ^{a}
Organic matter	93.80 ^{a}	85.64 ^{b}	93.15 ^{a}	92.86 ^{a}
Crude protein	19.39 ^{a}	21.18 ^{a}	21.06 ^{a}	19.32 ^{a}
Acid detergent fiber	11.77 ^{c}	11.46 ^{c}	17.59 ^{b}	26.98 ^{a}
Neutral detergent fiber	24.31 ^{cd}	27.76 ^{c}	33.89 ^{b}	49.50 ^{a}
Hemicellulose	12.54 ^{c}	16.30 ^{b}	16.30 ^{b}	22.52 ^{a}
Ash	6.20 ^{b}	14.36 ^{a}	6.85 ^{b}	7.14 ^{b}

^{a,b,c} Different letters in the same row indicate differences of $P<0.05$. [1]SBM: sugarcane silage + soybean meal; FM: sugarcane silage + fish meal; CM: sugarcane silage + canola meal; CCM: sugarcane silage + coconut meal.

^{a,b,c} Diferentes letras en la misma fila indica diferencias de $P<0,05$. [1]SBM: ensilado de caña de azúcar + harina de soya; FM: ensilado de caña de azúcar + harina de pescado; CM: ensilado de caña de azúcar + harina de canola; CCM: ensilado de caña de azúcar + harina coco.

In situ digestibility (DMD) was determined using four 4-year-old Holstein cows (625 ± 63 kg) equipped with permanent rumen cannula with a core diameter of 10 cm (Bar Diamond Lane, Parma, ID, USA). Cows were randomly assigned to a 4 × 4 Latin square and they were housed in individual pens. The statistical model was:

$$\mathbf{Y_{ijk} = \mu + H_i + C_j + T_k + \varepsilon_{ijk}}$$

where:

Y_{ijk} = the response variable

μ = the general mean

H_i = the effect of the i^{th} period (row)

C_j = the effect of j^{th} animal (column)

T_k = the effect of k^{th} treatment (diet)

ε_{ijk} = the experimental error

Each period was 15 d, 10 for adaptation to diets and 5 to collect samples. DMD was determined after Vanzant *et al*. (1998).

Nylon bags were used (10 x 15 cm, pore size 40-60 μm) with 5 g of sample. Each sample was incubated in rumen for 8, 12, 24, 36, 48, 72, and 96 h. Additionally, at each time point, blanks secured with nylon thread to a piece of string (length: 30 cm; weight: 150 g) were added and left suspended in the rumen.

Subsequently, the bags were removed from the rumen according to the incubation times along with the zero hour and washed with running water at low pressure until the water came out just as clear.

Nextly, the bags were dried in a circulating air oven (48 h at 60°C). Ruminal fluid samples were taken from the ruminal cannula at two-h intervals for 12 h, and one was taken one h before daytime feeding (-1, 0, 2, 4, 6, 8, 10 and 12).

Ruminal fluid pH was measured using a portable potentiometer (Model PC18, México) immediately after the rumen fluid was collected. The DMD for the experimental material from each incubation time, was calculated by the weight loss of the samples in bags during ruminal incubation using the model described by Ørskov and McDonald (1979) and modified by McDonald (1981):

$$\mathbf{P = a + b\ (1 - e^{-ct})}$$

where:
a = the washing loss or soluble (%)
b = the insoluble, but potentially digestible fraction (%)
P = the degradation of DM (%)
a + b = potential degradability (%)
c = the fractional degradation rate (h^{-1})
t = the time (h)

Ruminal turnover constants (k) at 1, 5, and 10 % h^{-1} were used to model effective degradation (ED;12): ED = a + (b*c) / (c+k). Data from DMD and chemical composition were analyzed using PROC GLM and the ruminal pH with PROC MIXED using the statistical package SAS Version 8.0 (19).

Results and discussion

Dry matter and OM content were higher in soybean, canola, and coconut meal, and lower in FM. This last treatment also showed the greatest A concentration.

CP was similar among treatments, but ADF and NDF were higher for CM and CCM. Hemicellulose concentration was lower in SBM, whereas the greatest concentration was found in CCM. FM and CM resulted similar (table 1, page 347).

Differences were found in DMD ($P<0.05$) due to the protein supplements of complete diets.

The SBM showed the greatest values while FM had the lowest values. Starting at 12h of incubation, DMD results were more than 50% in all treatments. However, during the following hours, DMD values for FM were the lowest (table 2, page 349).

The effective degradability was higher for treatment with SBM, at all times, while FM had the lowest values of the experimental diets ($P < 0.05$).

Ruminal degradability parameters were similar for soluble fraction of DM (a) across all treatments ($P > 0.05$). Only FM showed lower values for the rest of the parameters ($P < 0.05$). Sugarcane silage without additives is characterized by high DM losses (12). Forage-based diets supplemented with protein sources have better amino acid composition and improved nutrient digestibility compared with non-supplemented diets (6, 12).

The improvement in digestibility is due to the greater availability of nutrients required by bacteria for growth and other activities in the rumen. In this study, FM showed the lowest DMD coefficients wich is expectable since fish meal has lower rumen degradable protein (RDP) content (3, 4).

However, rumen undegradable protein is necessary to provide essential amino acids to the animal, given that the amount of digested and absorbed protein in the small intestine is an important factor for growth. For this reason, supplementation with rumen undegradable protein provides limiting amino acids, such as lysine and methionine, to the animal.

Table 2. Effect of the protein source on *in situ* dry matter and ruminal degradability parameters of the experimental diets (%).

Tabla 2. Efecto de la fuente de proteína en la digestibilidad *in situ* de la materia seca y parámetros de degradabilidad ruminal de las dietas experimentales (%).

Incubation time, h	Treatments[1]				SEM[2]
	SBM	FM	CM	CMM	
	%				
96	86.57 a	72.14 c	83.10 b	79.87 b	0.45
72	85.83 a	67.22 c	80.32 ab	77.31 b	0.39
48	84.70 a	64.73 c	79.45 ab	76.34 b	0.43
36	78.80 a	61.15 c	75.76 ab	73.66 b	0.62
24	68.42 a	50.71 b	64.58 a	64.77 a	1.05
12	63.22 a	50.38 c	58.26 b	62.75 ab	1.11
8	54.41 a	47.37 b	52.96 a	50.86 ab	0.97
DM degradability parameters					
Soluble (*a*)	41.94 a	42.35 a	40.92 a	40.98 a	1.12
Potentially digestible (*b*)	46.10 a	36.70 c	43.06 a	38.45 b	1.09
Potential degradability (*a*+*b*)	88.11 a	79.05 d	83.97 c	79.43 b	1.16
Constant of degradation (*c*)	0.043 a	0.017 b	0.041a	0.049 a	0.003
Effective degradability modeled at the fractional passage rate (h^{-1})					
0.01	79.40 a	65.60 c	75.40 b	73.00 b	0.86
0.05	63.30 a	51.70 c	60.20 a	60.10 a	1.02
0.10	55.90 a	47.70 c	53.30 a	53.70 a	1.00

[a,b,c,d] Different letters in the same row indicate differences of P<0.05. [1] SBM: sugarcane silage + soybean meal; FM: sugarcane silage + fish meal; CM: sugarcane silage + canola meal; CCM: sugarcane silage + coconut meal. [2] Standard error of the mean.

[a,b,c,d] Diferentes letras en la misma fila indica diferencias de P<0,05. [1] SBM: ensilado de caña de azúcar + harina de soya; FM: ensilado de caña de azúcar + harina de pescado; CM: ensilado de caña de azúcar + harina de canola; CMM: ensilado de caña de azúcar + harina coco. [2] Error estándar de la media.

Fish meal, provided these amino acids in higher concentrations compared with the other protein sources used (9, 15). This difference in by-pass protein might be of importance for producers when adding protein to the diet of growing cattle.

Similar results have been reported by other authors. Van Nhiem *et al.* (2013) fed Laisind beef cattle with an urea-treated rice straw-based diet supplemented with two different protein sources, FM, and soybean cake and found that diets containing 100% soybean cake had higher DMD compared to FM.

da Silva *et al.* (2016) compared diets containing corn silage supplemented with SBM and urea and observed an increase in the ruminal digestibility of DM when SBM or urea were added, probably due to the addition of a highly digestible CP source.

The difference among diets of SBM, FM, and CCM in DMD may be explained by the small difference in RDP content. Also, these diets showed higher DM degradability parameters and faster degradation rates, probably due to the higher microbial degradation resulting from a good supply of protein that improved ruminal microbial growth.

The low DMD and ruminal turnover of the FM diets were due to a higher concentration of by-pass protein that may have resulted in limited microbial degradation of nutrients and decreased the efficiency of microbial synthesis in the rumen (14, 21).

Table 3 shows pH values; no differences among treatments were found (P > 0.05).

Table 3. Ruminal pH over time of the experimental diets.

Tabla 3. pH ruminal a través del tiempo de las dietas experimentales.

H	Treatments[1]				SEM[2]
	SBM	FM	CM	CCM	
-1	7.64	7.65	7.75	7.64	0.055
0	7.61	7.66	7.84	7.76	0.055
2	7.53	7.64	7.54	7.53	0.055
4	7.38	7.56	7.55	7.50	0.055
6	7.39	7.55	7.51	7.38	0.055
8	7.22	7.49	7.40	7.33	0.055
10	7.07	7.25	7.14	6.91	0.055
12	7.06	7.16	7.29	6.91	0.055
Average	7.36	7.49	7.51	7.37	0.060

Values preceded by or had an increase or diminution (P< 0.05) compared with the previous measurement in the same treatments. [1]SMB: sugarcane silage + soybean meal; FM: sugarcane silage + fish meal; CM: sugarcane silage + canola meal; CCM: sugarcane silage + coconut meal. [2]Standard error of the mean.

Los valores precedidos por o tuvieron un aumento o disminución (P <0,05) en comparación con la medición anterior en los mismos tratamientos. [1]SBM: ensilado de caña de azúcar + harina de soya; FM: ensilado de caña de azúcar + harina de pescado; CM: ensilado de caña de azúcar + harina de canola; CMM: ensilado de caña de azúcar + harina coco. [2]Error estándar de la media.

However, differences across sampling time, resulted signifficant. A reduction was recorded within treatment for SBM and FM at 4 h. Canola meal treatment showed the greatest pH value across incubation time, whereas SBM showed the lowest value. However, all pH values were higher than 7.0, except for CCM at 10 h and 12 h. Russell and Wilson (1996) state that rumen pH may change ruminal cellulose digestion. Low ruminal pH decreased activity, or number of cellulolytic microorganisms, in all experimental diets of their study, while the range of ruminal pH was around the optimum value (6.7-7.0) avoiding reductions in ruminal fermentation. García *et al.* (2008) and da Silva *et al.* (2016) reported pH values between 6.62 and 7.2, similar to those found in this experiment.

The high ruminal pH recorded when the experimental diets were fed could be attributed to the natural buffering capacity observed in rations that contain legumes and have high protein concentration (10).

Conclusion

The use of sugarcane silage with inoculum and additive in integral diets with the protein sources used in this study did not modify ruminal pH.

However, it reduced the DMD parameters when the protein source was FM, possibly due to its lower content of rumen-degradable protein.

References

1. AOAC. 2007. Association of Official Analytical Chemist-International. Official Methods of Analysis. 18th ed. AOAC, Arlington. Virginia. USA.
2. Aranda, E. M.; Mendoza, G. D.; Ramos, J. A.; Da Silva, I. C.; Vitti A. C. 2010. Effect of fibrolitic enzymes on rumen microbial degradation of sugarcane fiber. Ciência Animal Brasileira 11: 448-495.
3. Arroquy, J. I.; Cochran, R. C.; Villarreal, M.; Wickersham, T. A.; Llewellyn, D. A.; Titgemeyer, E. C.; Nagaraja, T. G.; Johnson, D. E.; Gnad, D. 2004. Effect of level of rumen degradable protein and type of supplemental non-fiber carbohydrate on intake and digestion of low-quality grass hay by beef cattle. Animal Feed Science and Technology. 115: 83-99.
4. Bohnert, D. W.; Del Curto, T.; Clark, A. A.; Merrill, M. L.; Falck, S. J.; Harmon, D. L. 2011. Protein supplementation of ruminants consuming low-quality cool- or warm-season forage: Differences in intake and digestibility. Journal of Animal Science. 89: 3707-3717.
5. da Silva, L. D.; Pereira, O. G.; Da Silva, T. C.; Valadares Filho, S. C.; Ribeiro, K. G. 2016. Effects of silage crop and dietary crude protein levels on digestibility, ruminal fermentation, nitrogen use efficiency, and performance of finishing beef cattle. Animal Feed Science and Technology. 220: 22-33.
6. Ferreira, D. A.; Gonçalves, L.; Molina, L. R.; Castro-Neto, A.; Tomich, T. R. 2007. Fermentation of sugarcane silage treated with urea, zeolita, bacteria inoculant and bacteria/enzymatic inoculants. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 59: 423-433.
7. García, H.; Abreu, M.; Soto, J. M. 2008. Digestion of residuals of the crop cane treatment with OHNa. 1. Determination of digestibility *in situ*. Revista Electronica de Veterinaria. 11: 1-8.
8. McDonald, I. 1981. A revised model for estimation of protein degradability in the rumen. Journal of Agricultural Science. 96: 251-252.
9. Mendoza, G. D.; Oviedo, M. F.; Pinos, J. M.; Lee-Rangel, H. A.; Vázquez, A.; Flores, R.; Pérez, F.; Roque, A.; Cifuentes, O. 2020. Milk production in dairy cows supplemented with herbal choline and methionine. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 52(1): 332-343.
10. Molina, A. S.; Sierra, J. F.; Febles, I. 1999. Sugar cane silage with protein synthesis: combined effect of additives and density. Cuban Journal of Agricultural Science. 33: 205-208.
11. Montañez-Valdez, O. D.; Solano-Gama, J. J.; Martínez-Tinajero, J. J.; Guerra-Medina, C. E.; Ley de Coss, A.; Orozco-Hernandez, R. 2013. Buffering capacity of common feedstuffs used in ruminant diets. Revista Colombiana de Ciencias Pecuarias. 26: 37-41.
12. Oliveira, A. C.; Garcia, R.; Pires, A. J. V.; Oliveira, H. C.; Almeida, V. V. S.; Silva, R. R.; Nascimento Filho, C. S.; Abreu Filho, G. 2015. Chemical composition and fermentation characteristics of sugar cane silage enriched with detoxified castor bean meal. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 67: 181-188.
13. Ørskov, E. R.; McDonald, L. 1979. Estimation of protein degradability in the rumen from incubation measurement weighted according to rate of passage. Journal of Agricultural Science. 96: 499-503.
14. Ortiz, M. A.; Ørskov, E. R.; Milne, J.; Galina, H. M. A. 2007. Effect of different sources of nitrogen on *in situ* degradability and feed intake of Zebu cattle fed sugarcane tops (*Saccharum officinarum*) Animal Feed Science and Technology. 139: 143-158.
15. Rezai, F.; Zamani, F.; Vatankhah, M. 2012. Effect of rumen undegradable protein (RUP) on colostrum quality and growth of lori Bakhtiari lambs. Global Veterinaria. 8: 93-100.
16. Rocha, K. D.; Pereira, O. G.; Valadares, F. S. C.; Oliveira, A. P.; Pacheco, L. B. B.; Chizzotti, F. H. M. 2006. Valor nutritivo de silagens de milho (*Zea mays* L.) producidas com inoculantes enzimobacterianos. Revista Brasileira de Zootecnia. 35: 389-395.
17. Rodríguez, D.; Martín, P. C.; Alfonso, F.; Enríquez, A. V.; Sarduy, L. 2009. Performance of crossbred steers (Holstein x Zebu) fed sugarcane forage alone or as part of the diet. Cuban Journal of Agricultural Science. 43: 231-234.

18. Russell, B. J.; Wilson, B. D. 1996. Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH? Journal of Dairy Science. 79: 1503-1509.
19. SAS. 1999. User, s Guide: Statistics. version 8.0. Ed. SAS Institute, Inc., Cary N. C. En CD-ROM.
20. Van Nhiem, D.; Berg, J.; Kjos, N. P.; Trach, N. X.; Tuan, B. Q. 2013. Effects of replacing fish meal with soy cake in a diet based on urea-treated rice straw on performance of growing Laisind beef cattle. Tropical Animal Health and Production. 45: 901-909.
21. Van Soest, P. J. 1994. Nutritional ecology of the ruminant. Cornell University Press.
22. Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. Journal of Dairy Science. 74: 3583-3597.
23. Vanzant, E. S.; Cochran, R. C.; Titgemeyer, E. C. 1998. Standardization of *in situ* techniques for ruminant feedstuff evaluation. Journal of Animal Science. 76: 2717-2729.

Development and characterization of nettle-leaves powder (*Urtica urens*) as a potential supplement for animal feed

Desarrollo y caracterización de un preparado en polvo de hojas de ortiga (*Urtica urens*) como un potencial suplemento para alimentación animal

Francisca Arros, Camila Garrido, Carolina Valenzuela*

Originales: *Recepción:* 04/03/2019 - *Aceptación:* 30/07/2019

Abstract

Nettle plants in Chile are an underutilized resource. Its use in animal nutrition is also limited. In addition, information about its composition (specifically fatty acids and aminoacids) is scarce. The objective of this work was to develop and characterize nettle-leaves powder (NP). This powder was characterized by means of proximate chemical analysis. Its concentration of minerals, and composition profiles for fatty acids and aminoacids, was determined. NP showed high dry basis content of proteins (24%) and nitrogen-free extract (32%), whereas crude fiber concentration was low (8.4%). Also, NP showed high concentrations of ash (29%), and mineral, specially for calcium (1.65%) and zinc (20 mg/100g of NP). Additionally, glutamic and aspartic acids, as well as leucine, were the major types of aminoacids found in NP. In conclusion, though nettle plants are an under-utilized resource, the elevated content of protein found in powders prepared from their leaves might become a compelling reason to include them as a protein supplement in animal diets.

Keywords
Urtica urens • chemical composition •minerals • aminoacids • fatty acids

Universidad de Chile. Facultad de Ciencias Veterinarias y Pecuarias. Casilla 2. La Granja. Santiago. Chile. * cvalenzuelav@u.uchile.cl

Resumen

Las plantas de ortiga en Chile son un recurso subutilizado y su uso en nutrición animal es limitado. Además, la información sobre su composición de ácidos grasos y aminoácidos es escasa. El objetivo de este trabajo fue desarrollar y caracterizar un preparado en polvo de hojas de ortiga (NP). El cual se caracterizó por análisis químico proximal, determinación de su concentración de minerales y perfiles de composición de ácidos grasos y aminoácidos. El NP mostró un alto contenido en base seca de proteínas (24%) y extracto libre de nitrógeno (32%), mientras que la concentración de fibra bruta fue baja (8,4%). Además, NP mostró altas concentraciones de ceniza (29%) y minerales, como calcio (1,65%) y zinc (20 mg/100 g de NP). Además, los ácidos glutámico y aspártico, así como la leucina, fueron los principales tipos de aminoácidos encontrados en este NP. En conclusión, aunque las plantas de ortiga son un recurso subutilizado, el contenido elevado de proteína que se encuentra en el preparado en polvo a partir de sus hojas, puede convertirse en una razón convincente para incluirlas como un suplemento proteico en las dietas de los animales.

Palabras clave

Urtica urens • composición química • minerales •aminoácidos • ácidos grasos

Introduction

The *Urticaceae* family comprises close to 54 genders and 2000 cosmopolitan, abundant plant species. These have been used from as far back as the Bronze Age in multiple medicinal applications (9, 10), especially *Urtica urens* and *Urtica dioica*, which have been more thoroughly studied. Both species are present in Chile, yet are currently an under-utilized resource.

Use of nettle plants in animal nutrition is limited as animals reject them due to their characteristic urticating hairs in leaves and stems, which on contact with the skin or mucosae trigger an erythematous macula, itchiness, and pain (6). However, their urticating trichomes are sensitive to heat, hence nettle is used for human medicine either as infusions or cooked (12). Therefore, a simple drying process could solve the aforementioned problem, resulting in the development of a dehydrated powder that could easily be homogenized in animal diets.

The fact that nettles are under-exploited for animal feeding is unfortunate, bearing in mind how interesting their nutritional composition is. For example, *Urtica urens* shows high concentrations of calcium, potassium, phosphorus, and zinc, as well as protein contents that range from 13 to 26 percent (1). In addition, some researchers have reported that its leaves show great antioxidant and antimicrobial properties (13, 15). However, few studies have reported on *Urtica urens* regarding other nutritional characteristics, such as fatty acids-whereas literature does provide this information for *Urtica dioica* (8, 18). Additionally, to the extent of our knowledge, we are not aware of studies reporting on their aminoacids profile (11).

Objective

To develop and characterize nettle-leaves powder (NP).

Materials and methods

Preparation of NP

Fresh nettle plants (*Urtica urens*) were collected when blossoming, in the Antumapu Campus of the University of Chile, Metropolitan Region, Santiago, Chile (latitude: 33°34'24.8", longitude: 70°37'47.9", height: 629 m) (photo 1A). After these nettles were identified by an agronomist, their leaves were clipped and refrigerated at 4°C for 48 hours. Then, leaves were weighed and placed in an aluminum tray to be dried in stove (Equilabmas, model ULM600, Germany), at 60°C for 48 hours. Once leaves were dried and cooled down to room temperature, they were ground in a mill (Thomas-Wiley Mill model 4, USA) to collect NP.

Characterization of NP

Appearance and yield

NP appearance was assessed by digital photography (Sony DSC-HX1, Sony Corporation, Japan) (photo 1B).

Yield of NP, was determined by calculating the weight difference between wet nettle leaves and NP.

Chemical composition

The NP was analyzed according to the Association of Official Analytical Chemists (4) for moisture content (method 945.15), crude protein (Kjeldahl method 945.18), ether extract (method 945.16) crude fiber (method 962.09) and ash (method 920.153). The Nitrogen-free extract was calculated by difference.

Mineral composition

Mineral contents (Ca, P, Mg, Cu, Fe, Zn) were determined according to the AOAC (1990). Mineral concentrations were measured at specific wavelengths for each element (Ca: 422.7, P: 630.0, Mg: 285.2, Cu: 324.7, Fe: 248.3 and Zn: 213.9 nm) using an atomic absorption spectrophotometer (GBC, 905AA, Victoria, Australia).

Photo 1. Nettle plants (A) and nettle-leaves powder (B).

Foto 1. Plantas de ortiga (A) y el preparado en polvo de hojas de ortiga (B).

Fatty acid composition

Lipids were saponified and derived according to the AOAC (1990) to obtain methyl esters (AOCS 1990; Method Ce 1b-89). These methyl esters were analyzed by gas chromatography–flame ionization detection (GC–FID), using a GC Agilent Technologies 6890N gas chromatograph, with a capillary column Omega wax 320 (30 mm x 0.25 mm x 0.25 µm) (Supelco, Bellefonte, PA, USA) and FID detector.

The temperature parameters were: injector 140-190-220°C-240°C and detector 270°C. The gas flows were as follows: N_2: 20 mL/min, H_2: 40 mL/min, syntheticgas (commercial mixture of N_2 and O_2) 250 mL/min.

Available pure standards of saturated, monounsaturated and polyunsaturated fatty acids (Sigma Co., St. Louis, Mo, USA) were used to identify each fatty acid methyl ester. Each result reported the average value of two analyses.

Aminoacids profile

Total amino acids were determined in samples following conventional hydrolysis (19). A High- Performance Liquid Chromatography (HPLC) method (HPLC Shimadzu, Kyoto, Japan), coupled with HPLC pump LC-20AD with diode-array detection (SPD-M20A detector; SIL-20A injector, Shimadzu Corporation, Kyoto, Japan) was used to identify and quantify amino acids. Derivatives were separated using a RP-18 (250 x 4.6 mm, 5-3 µm particle size; Inertsil® ODS-3, Shimadzu Corporation, Kyoto, Japan).

Statistical analysis

Descriptive statistics were calculated using mean ± standard deviation (Microsoft Excel 2010 software, Microsoft Corp., USA).

Results and discussion

Proximate and mineral composition

Table 1 presents the results from the proximate chemical analysis of NP. In particular, protein content was quite high, in line with ranges of 14-28% already reported by other authors in nettle species from India and South Africa (1, 12).

Tabla 1. Chemical and mineral composition of nettle-leaves powder in dry basis.

Tabla 1. Composición química y mineral de un preparado en polvo de hojas de ortiga en base seca.

Properties	Mean ± SD
Dry matter (%)	96 ± 1.2
Crude protein (N x 6.25) (%)	24 ± 2.8
Crude fiber (%)	8.4 ± 2.2
Ether extract (%)	2.9 ± 0.9
Ash (%)	29.1 ± 2.0
Nitrogen-free extract (%)	31.6 ± 2.1
Calcium (%)	1.65 ± 0.37
Magnesium (%)	0.44 ± 0.05
Phosphorus (%)	0.51 ± 0.02
Copper (mg/100g)	9.9 ± 0.2
Iron (mg/100g)	1.8 ± 0.5
Zinc (mg/100g)	20.1 ± 1.2

Contrarily, crude fiber percentage was lower than expected for an herbal product, according to reports from other researchers who found values ranging from 15-21% (1, 12, 14).

A possible explanation for low fiber contents might rely on several environment and climate factors, such as the intensity of sunlight, temperature, or even the mineral composition of soils (16). Bearing in mind that NP was prepared only from leaves-not roots neither stems-then such values for protein and fiber in NP are not surprising (17). As for the ether extract, it showed low values that were smaller than those reported in other works (1, 14). Meanwhile, ash contents almost accounted for one third of its total chemical composition, according to our expectations and in line with values of 26-28% that were previously reported by other authors (1, 14).

Table 1 (page 356), lists the mineral composition of NP. Interestingly, calcium and zinc were found at greater concentration than those reported in previous works by other authors (1, 12, 14), though calcium concentration was actually similar to previous reports on alfalfa meal (1.5%) (5).

Fatty acids profile

Table 2 details the fatty acids composition for NP. In particular, polyunsaturated fatty acids, such as α-linolenic and linoleic acids, were the two most abundant. The saturated palmitic acid was the third most important fatty acid found, while the oleic acid was the most significant monounsaturated one. Similarly, some studies have reported that α-linolenic acid was predominant in *U. dioica*, reaching up to 50% of its ether extract (18). Additionally, Guil-Guerrero *et al*. (2003) reported that α-linolenic acid was the main fatty acid found in nettle leaves, followed by palmitic and linoleic acids, though remarking that the fatty acid composition in nettle varies when measured in other parts of the plant.

Total aminoacids profile

Among sixteen aminoacids that were identified in NP (table 3, page 358), the greater concentrations (in decreasing order) were found for glutamic acid, aspartic acid, and leucine. Interestingly, neither cysteine nor tryptophan were found in our NP samples, which agrees whit finding from other researchers who did not detect these aminoacids in *U. urens* or *U. dioica* (11). Additionally, Lapinskaya *et al*. (2008) reported finding that glutamic and aspartic acids, as well as alanine and leucine were the major aminoacids present in *U. urens* and *U. dioica*.

Tabla 2. Fatty acid profile of nettle-leaves powder.

Tabla 2. Perfil de ácidos grasos de un preparado en polvo de hojas de ortiga.

Fatty Acid (g/100g)	Mean ± SD
C 18:3	2.19 ± 1.16
C 18:2	1.58 ± 0.46
C 16:0	0.77 ± 0.23
C 18:1	0.45 ± 0.13
C 18:0	0.20 ± 0.06
Gamma-C 18:3	0.10 ± 0.03
C 14:0	0.07 ± 0.03
C 12:0	0.06 ± 0.02
C 15:0	0.06 ± 0.02
C 16:1	0.02 ± 0.01
C 17:0	0.01 ± 0.00
C 21:1	0.01 ± 0.00
C 10:0	0.01 ± 0.00

Table 3. Total amino acids of nettle-leaves powder (NP), compared to soybean meal described by Batal *et al.* (2012)* and FAO/ WHO (1973)** and to alfalfa meal reported by Batal *et al.* (2012).

Tabla 3. Amino ácidos totales de un preparado en polvo de hojas de ortiga (NP), comparado con harina de soya descrito por Batal *et al.* (2012)* y FAO/WHO (1973)** y harina de alfalfa por Batal *et al.* (2012).

Amino Acid (%)	NP	Soybean meal	Alfalfa meal
Glutamic acid	2.34 ± 0.54	19**	Not reported
Aspartic acid	1.23 ± 0.10	1.3**	Not reported
Leucine	1.10 ± 0.22	3.8 *	1.10
Proline	1.00 ± 0.20	5.3**	Not reported
Lysine	0.85 ± 0.35	2.7 *	0.60
Isoleucine	0.71 ± 0.12	2.8 *	0.68
Threonine	0.66 ± 0.09	1.7 *	0.60
Valine	0.62 ± 0.15	2.2 *	0.84
Alanine	0.60 ± 0.07	5.0 *	Not reported
Serine	0.58 ± 0.10	5.8**	Not reported
Phenylalanine	0.35 ± 0.02	2.1 *	1.04
Glycine	0.26 ± 0.01	4.5**	Not reported
Histidine	0.16 ± 0.01	1.1 *	0.30
Arginine	0.13 ± 0.02	3.2 *	0.98
Methionine	0.11 ± 0.04	0.70*	0.23
Tyrosine	0.11 ± 0.03	3.7**	Not reported
Tryptophan	-	5.8 *	0.38
Cysteine	-	0.71*	0.17

Not reported: amino acid not reported in Feedstuffs, (-) amino acid not found in the NP sample.

No reportado: amino ácidos no reportados en Feedstuffs, (-) amino ácidos no encontrado en las muestras de NP.

NP might possibly be used as a supplement for animal diets due to its high protein content. To assess its suitability, we compared the results of this work with the total aminoacid content from soy meal-the major protein concentrate used in poultry and pig diets (table 3). As expected, soy meal contains much greater amounts for every aminoacid, but the difference in contents between both meals for aspartic acid and leucine were narrower than for the remaining amino-acids. Hence, NP can be deemed as a good source of both aminoacids.

Another interesting comparison for NP was against alfalfa meal, which is a widely used as forage in ruminant diets. As seen in table 3, with the sole exception of phenylalanine and arginine, most aminoacids present in NP were found at a similar concentration in alfalfa meal.

Conclusions

In this work, our research group was able to prepare a nettle powder formulation that showed elevated contents of protein (24%), calcium (1.65%), and zinc (20.1 mg/100 g of powder), as well as a low fiber content of 8.4%. Considering its nutritional composition, nettle powder is an under-utilized resource that might become an interesting ingredient to include in animal diets formulation.

References

1. Afolayan, A.; Jimoh, F. 2009. Nutritional quality of some wild leafy vegetables in South Africa. Int. J. Food Sci. Nutr. 60: 424-431.
2. American Oil Chemists' Society (AOCS). 1990. 4° ed. Official methods and recommended practices. Champaign. AOCS.
3. Association of Official Analytical Chemists - International (AOAC). 1990. 15° ed. Official methods of analysis Arlington. AOAC.
4. Association of Official Analytical Chemists - International (AOAC). 1996. 16° ed. Official methods of analysis. Gaithersburg. AOAC.
5. Batal, A.; Dale, N.; Persia, M. 2012. Feedstuffs ingredient analysis table. Available in: http://fdsmagissues.feedstuffs.com/fds/Reference_issue_2012/03_Ingredient%20Analysis%20Table%202012%20Edition.pdf (Accessed September 2018).
6. Bisht, S.; Bhandari, N.; Bisht, N. 2012. *Urtica dioica* (L): an undervalued, economically important plant. Agric. Sci. Res. J. 2: 250-252.
7. Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO). 1973. Energy and protein requirement. Report of a Joint FAO/WHO ad hoc Expert Committee. Geneva. FAO/WHO.
8. Guil-Guerrero, J. L.; Rebolloso-Fuentes, M. M.; Isasa, M. T. 2003. Fatty acids and carotenoids from stinging nettle (*Urtica dioica* L.). J. Food Compos. Anal. 16: 111-119.
9. Kavalali G. 2004. Urtica: The genus urtica. Londres. CRC Press. 112 p.
10. Kopyt'Ko, Y.; Lapinskaya, E.; Sokol'Skaya, T. 2012. Application, chemical composition, and standardization of nettle raw material and related drugs. Pharm. Chem. J. 45: 622-631.
11. Lapinskaya, E. S.; Kopyt'ko, Y. F.; Timokhina, E. A.; Krapivkin, B. A.; Levandovskii, G. S.; Dargaeva, T. D.; Sokol'skaya, T. A. 2008. Amino acids and cyclic dipeptides in stinging nettle (*Urtica dioica* and *U. urens*) homeopathic matrix tinctures. Pharm. Chem. J. 42: 650-653.
12. Lewu, M.; Kambizi, L. 2015. Nutritional assessment of selected leafy vegetables. International conference on biotechnology and food technology. Harare. Zimbabwe. p: 80-84.
13. Maaroufi, L.; Sazzad-Hossain, M.; Tahri, W.; Landoulsi, A. 2016. New insights of Nettle (*Urtica urens*): Antioxidant and antimicrobial activities. J. Med. Plants Res. 11: 73-86.
14. Manu Kumar, H.; Prathima, V.; Sowmya, S.; Thribhuvan, K. 2013. Study of nutritional quality, phytochemical constituents and antioxidant activities by different solvents of nettle (*Urtica urens*) from Madikeri-karnataka state. Int. Res. J. Pharm. Applied. 3: 112-119.
15. Mzid, M.; Ben Khedir, S.; Ben Salem, M.; Regaieg, W.; Rebai, T. 2017. Antioxidant and antimicrobial activities of ethanol and aqueous extracts from *Urtica urens*. Pharm. Biol. 55: 775-781.
16. Pettigrew, W. 2001. Environmental effects on cotton fiber carbohydrate concentration and quality. Crop. Sci. 41: 1108-1113.
17. Rafajlovska, V.; Kavrakovski, Z.; Simonovska, J.; Srbinoska, M. 2013, Determination of protein and mineral contents in stinging nettle. Qual. Life. 7: 1-5.
18. Rutto, L. K.; Xu, Y.; Ramirez, E.; Brandt, M. 2013. Mineral properties and dietary value of raw and processed stinging nettle (*Urtica dioica* L.). Int. J. Food Sci. 2013: 1-9.
19. White, J.; Hart, R.; Fry, J. 1986. An evaluation of the waters pico-tag system for the amino-acid analysis of food materials. J. Automat. Chem. 8: 170-177.

Fruit peels as sources of bioactive compounds with antioxidant and antimicrobial properties

Cáscaras de frutas como fuentes de compuestos bioactivos con propiedades antioxidantes y antimicrobianas

Miguel A. Aguilar-Méndez [1], Martha P. Campos-Arias [1], Cinthya N. Quiroz-Reyes [1], Elba Ronquillo-de Jesús [2], Miguel A. Cruz-Hernández [3]

Originales: *Recepción:* 08/10/2018 - *Aceptación:* 31/08/2019

Abstract

Recently, a major interest in searching for phytochemicals with nutritional and pharmaceutical purposes has arisen. In this regard, it is known that polyphenols present antioxidant properties as well as an inhibitory effect against some kinds of microorganisms. The aim of this study was to obtain aqueous-ethanolic extracts from peels of avocado, cocoa bean, coconut and cactus pear by ultrasound-assisted extraction. The extracts were characterized in terms of phenolics (Folin-Ciocalteu reagent), antioxidant potential (ferric reducing/antioxidant power assay), radical-scavenging ability (2,2-diphenyl-2-picrylhydrazyl free radical assay), and antimicrobial activity against *Staphylococcus aureus*, *Shigella dysenteriae* and *Candida albicans* (disk diffusion test). The results revealed that the avocado peel extract had the highest phenol content (36.5 mg EAG g^{-1} dry weight), the highest antioxidant activity (141.2 mME Trolox g^{-1} dry weight) and the lowest IC_{50} value (59 ppm). Furthermore, avocado and coconut peels demonstrated an inhibitory effect against the tested microorganisms.

Keywords
fruit peel • phenolic compound • antioxidant activity • antimicrobial activity

1 Instituto Politécnico Nacional. Centro de Investigación en Ciencia Aplicada y Tecnología Avanzada. Legaria 694. C. P. 11500. Ciudad de México. México.
* maguilarme@ipn.mx

2 Universidad Politécnica de Francisco I. Madero Dirección de Ingeniería Agroindustrial. Domicilio conocido. C. P. 42660. Tepatepec. Hidalgo. México.

3 Colegio de Postgraduados Campus Montecillo. Instituto de Edafología. Carretera México-Texcoco km 36.5. C. P. 56230. Texcoco. Estado de México. México

Resumen

En los últimos años se ha producido un gran interés en la búsqueda de fitoquímicos con fines nutricionales y farmacéuticos. A este respecto, se sabe que los polifenoles presentan propiedades antioxidantes, así como un efecto inhibidor contra algunos tipos de microorganismos. En este estudio se obtuvieron extractos acuoetanólicos de cáscaras de aguacate, cacao, coco y tuna mediante extracción asistida por ultrasonido. Los extractos se caracterizaron en términos de fenoles (reactivo de Folin-Ciocalteu), potencial antioxidante (prueba del poder reductor férrico/antioxidante), capacidad secuestradora de radicales (prueba del radical libre 2,2-difenil-1-picrilhidracilo) y actividad antimicrobiana contra *Staphylococcus aureus*, *Shigella dysenteriae* y *Candida albicans* (método de difusión con discos). Los resultados revelaron que el extracto de cáscara de aguacate presentó el contenido más alto de fenoles (36,5 mg EAG/g materia seca), la mayor actividad antioxidante (141,2 mME Trolox/g de materia seca) y el valor más bajo de IC_{50} (59 ppm). Además, las cáscaras de aguacate y coco demostraron un efecto inhibitorio contra los microorganismos testados.

Palabras clave

cáscara de fruta • compuesto fenólico • actividad antioxidante • actividad antimicrobiana

Introduction

Traditionally, plants and fruits have been used for obtaining compounds with biological activity (21, 22, 25). Specifically, fruit hulls or peels have been listed as potential sources of compounds with antioxidant and antimicrobial properties. This part of the fruit is non-edible material discarded during the manufacturing processes (15). Although they are typically considered waste material, it has been reported that several of these materials are promising sources of valuable components, such as phenolic compounds (polyphenols, flavonoids and tannins), and other bioactive components (8). Peel of many fruits has already been used for the extraction of phenolic compounds, for example kinnow (27), mango (1), melon (20), orange (11, 17), pear (15), pomegranate (29, 32), among others. It has even been reported that some peels obtained from fruits like apple, hawthorn, and pomegranate present a significantly greater amount of phenolic compounds in comparison with the pulp.

Polyphenols are secondary metabolites that play essential roles in plant physiology and have beneficial properties for human health, mainly as antioxidants and antimicrobials (3, 4, 6). Polyphenolic compounds display antioxidant activity through different mechanisms, in particular by free radical scavenging and by chelation of metal ions (12). On the other hand, the antimicrobial properties of polyphenols are related to their structural configuration, being the hydroxyl (-OH) group the responsible for inhibitory action (8). Recently, considerable interest in the use of natural compounds with antioxidant and antimicrobial activity has arisen, not only for food preservation and shelf life improvement, but also for increasing stability of fats and oils, and for controlling microbial diseases in both humans and plants (7, 13, 19).

Fruits like avocado, cocoa, coconut and cactus pear are native and/or major crops in Mexico. However, little has been studied about the use of by-products like the peel of these fruits. Therefore, the aim of this study was to evaluate the antioxidant and antimicrobial activity of aqueous ethanolic extracts obtained from peels of avocado, cocoa, coconut and cactus pear.

Materials and methods

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, Folin-Ciocalteu reagent, Tris-HCl Buffer, 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) and ferric chloride hexahydrate were purchased from Sigma-Aldrich (USA). Acetic acid, ascorbic acid, sodium acetate, sodium chloride and sulphuric acid were obtained from JTBaker (Mexico). Mueller Hinton agar (Bioxon) and chloramphenicol (Sophia Laboratories, Mexico) were used for microbiological analysis. All the solvents used were analytical reagent grade.

Preparation of plant material

Avocado (Hass), cocoa (Forastero), coconut (Acapulco) and cactus pear (San Martin) fruits were purchased at a local market in Mexico City. The fruits were washed with water and sanitized with a solution of sodium hypochlorite 1%. Subsequently, their peels were removed and dried in an oven at 40°C for 48 h. In the cases of cocoa and coconut, the inner shell (endocarp) was selected. Finally, the peels were pulverized in a disc mill (Model 148-2, The Bauer Bros Co., USA) and stored.

Ultrasound-assisted extraction

In all cases, extracts were obtained using a sample-solvent ratio of 1:20. Aqueous ethanolic extractions (70:30) were performed under sonication (25 kHz) for 30 min in an ultrasonic bath (TI-H-5, Elma, Germany). Subsequently, the extracts were centrifuged at 1750 rpm, filtered (Whatman no. 1), and concentrated (35°C) in a rotary evaporator (RE-500, Yamato, Japan). Finally, all samples were dried in a vacuum oven (Precision, Thelco, USA) at 35°C.

Quantification of total phenols

Total phenolic content was calculated from the reduction capacity of Folin-Ciocalteu using gallic acid as a standard (5). A 20-μL sample volume was added to 1.4 mL of distilled water, followed by 100 μL of Folin-Ciocalteu reagent. The final solution was allowed to stand for 5 min at room temperature. Subsequently, 300 μL of a sodium carbonate solution was added (20% w/v). After resting for 90 min in a dark room, absorbance was determined at a wavelength of 760 nm on a Cary 50 (Varian, USA) spectrophotometer. Results were expressed as mg gallic acid equivalents·g^{-1} dry weight.

Ferric-Reducing Antioxidant Power (FRAP) Assay

Antioxidant capacity was determined using the FRAP (Ferric Reduction Antioxidant Power) test, modified (16). This assay determines the antioxidant capacity of the polyphenols to reduce TPTZ-Fe^{3+} complex. The FRAP reagent is prepared by mixing 25 mL of a 0.3 M acetate buffer (pH 3.6), 2.5 mL TPTZ solution (0.01 M) and 2.5 mL of a solution of $FeCl_3 \cdot 6H_2O$ (0.02M) at 37°C. A sample of 150-μL extract was mixed with 2850 μL of FRAP solution and allowed to stand for 30 minutes in the dark.

Absorbance was recorded at a wavelength of 593 nm. The results were reported in mM Trolox eq·g^{-1} dry weight.

DPPH Radical Scavenging Ability

The antiradical capacity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (22). A 2-mL aliquot of extract was mixed with 500 µL of 0.1M Tris-HCl buffer by vortex mixing for 5 seconds. To this solution, 2 mL of a 200-µM DPPH solution were added. After 30 minutes, absorbance was determined at 517 nm. The control sample consisted of a solution of ascorbic acid. The percentage of DPPH reduction was calculated using (eq. 1).

$$DPPH\ inhibition\ \% = \left(1 - \frac{absorbance\ of\ sample}{absorbance\ of\ control}\right) x\, 100 \tag{1}$$

The EC$_{50}$ value was determined from the data contained in the DPPH reduction effect against the extract concentration graph.

Antimicrobial activity

The antimicrobial activity was evaluated *in vitro* by disk diffusion assay, modified (28). Extracts were dissolved in distilled water at a concentration of 200 mg mL^{-1} to evaluate their activity against *Staphylococcus aureus*, *Candida albicans* and *Shigella dysenteriae*. A standardized suspension of the microorganisms was spread on Mueller Hinton agar culture medium using swabs. Paper disks (6mm diameter) were impregnated with 20 µL of extract and placed on the inoculated agar. The petri dishes were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the zone of inhibition test against microorganisms. Chloramphenicol and distilled water were used as positive and negative controls, respectively.

HPLC analysis

The two extracts with the best antioxidant and antimicrobial properties were analyzed by HPLC for phenolic identification. HPLC-analyses were carried out in an Agilent 1200 chromatograph (Agilent Technologies, Germany) equipped with a multiple wavelength detector. Separations were conducted on a Zorbax Eclipse XDB-C18 (3.5 µm, 100x4.6 mm). The mobile phase consisted of water/formic acid (99.9/0.1) as eluent A and methanol/acetonitrile (50/50) as eluent B. The system was run with a gradient program: 13- 17% B for 21 min, 17-23% B for 14 min and 23-33% for 5 min. The Column temperature was set at 30°C, flow rate was 300 µL per min and the injection volume was 5 µL. Samples were previously dissolved in demineralized water/ethanol and filtered through a 0.45 µm membrane filter. Peaks of ascorbic acid, oxalic acid, ferulic acid, gallic acid, (+)- catechin, (-)-epicatechin, procyanidin B1 and procyanidin B2 were identified by comparing the retention times of samples with those of standards. Chromatograms were recorded at 280 nm (26).

Statistical analysis

All the analyses were performed in triplicate, and the results were analyzed using ANOVA (Design Expert 8). Differences between means were detected by the Duncan multiple range test. Differences were considered significant at a significance level (α) of 0.05.

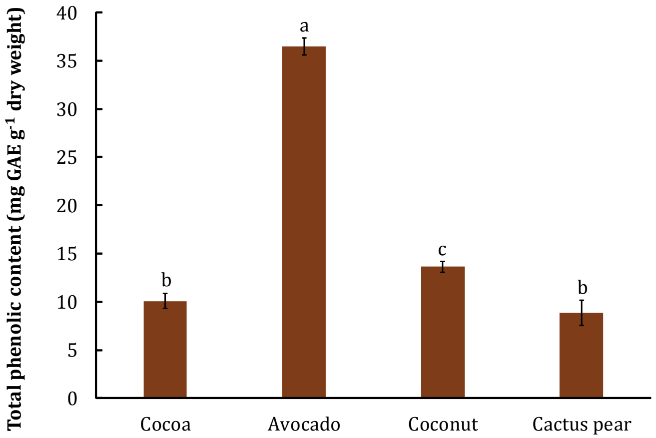
Results and discussion

Total phenol content

Many phenolic compounds found in fruits and vegetables have generated much interest due to their antioxidant potential. The total phenolic contents studied in this work are presented in figure 1, which shows that avocado peel has the highest content of total phenols (36.5±0.5 mg GAE·g^{-1} dry weight), followed by coconut (13.6±0.5 mg·GAE g^{-1} dry weight). However, no differences were detected between mean values obtained for cocoa and cactus pear extracts (P > 0.05). Phenolic contentsfound in this study resulted lower than those reported for mango peel (54-109 mg GAE·g^{-1} dry weight) (1) and pomegranate (55-89 mg GAE·g^{-1} dry weight) (28), but higher than those reported for peels from different pear varieties (2.6-11.2 mg GAE·g-1 dry weight) (15) and gac fruit (2.31-2.80 mg GAE·g^{-1} dry weight) (14).

Antioxidant capacity

Antioxidant activity mainly rests on redox properties of various compounds, that act as reducing agents or hydrogen atom donors (23). Phenolic compounds and pigments are the main groups of compounds that contribute to antioxidant activity in vegetables, fruits, cereals and other plant materials.



Values are expressed as mean±sd (n = 3). Means with different letters were significantly different (P < 0.05).

Valores expresados como media±de (n = 3). Medias con diferentes letras fueron significativamente diferentes (P < 0,05).

Figure 1. Total phenolic content of fruit peels extracts.

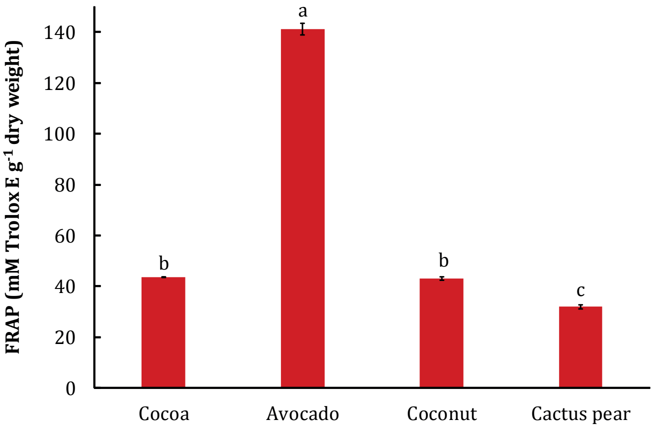
Figura 1. Contenido fenólico total de extractos de cáscaras de fruta.

According to figure 2, the avocado peel extract showed the highest antioxidant activity (141.23 mM Trolox equivalents·g^{-1} dry weight), as measured by the reduction of Fe^{3+} to Fe^{2+}, statistically different from the values obtained for the cocoa, coconut and cactus pear extracts ($P < 0.05$). These results could be positively correlated with total phenol content ($R^2 = 0.98$), indicating that the higher the phenolic content, the higher antioxidant activity. Nedamani *et al*. (2014) also reported a substantial relationship between total phenols and antioxidant activity in extracts of rosemary leaves and oak fruit.

Antiradical activity

The DPPH radical is a stable radical widely used to determine the ability of plant extracts acting as free radical scavengers or hydrogen donors. Figure 3 (page 366), shows radical inhibition *vs* concentration of the tested extracts. The avocado and coconut extracts showed inhibitory activity that increased rapidly in the range of 0-100 ppm, reaching inhibition values of up to 75% and remaining almost constant at higher concentrations. Meanwhile, cocoa and cactus pear extracts showed lower inhibitory response against the DPPH radical.

The EC_{50} is defined as the amount of antioxidant required to reduce the initial DPPH radical concentration by 50%. The lower the EC_{50} value, the greater the DPPH radical scavenging activity of the extracts (31).

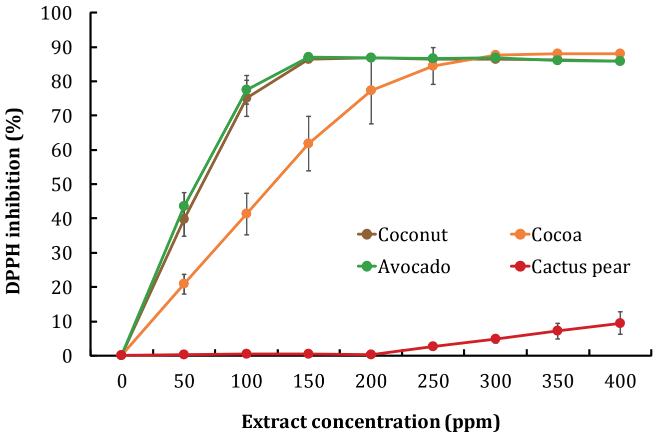


Means with different letters were significantly different (P< 0.05).

Medias con diferentes letras fueron significativamente diferentes (P<0,05).

Figure 2. Antioxidant capacity of fruit peels extracts measured by FRAP. Values are expressed as mean±sd (n = 3).

Figura 2. Capacidad antioxidante de extractos de cáscaras de frutas medida mediante FRAP. Valores expresados como media±de (n = 3).



Values are expressed as mean±sd (n = 3). / Valores expresados como media±de (n = 3).

Figure 3. DPPH radical scavenging activity of fruit peels extracts.
Figura 3. Actividad secuestradora del radical DPPH de extractos de cáscaras de frutas.

Table 1 shows the EC_{50} values for the different extracts. The avocado peel extract had the lowest value, whereas it was not possible to determine an EC_{50} value for the cactus pear extract because of its poor activity as DPPH radical scavenger.

Antimicrobial activity

Table 2 (page 367), shows the inhibition zone diameters caused by the extracts tested against microorganisms. It can be observed that the avocado and coconut peel extracts showed the highest inhibition values against *S. aureus, S. dysenteriae* and *C. albicans*.

In the case of cactus pear peel extract, no inhibition was observed against *S. dysenteriae*, while the cocoa extract only showed antimicrobial activity against *C. albicans*. It is possible that the differences in antimicrobial activity among extracts are due to variations in phenolic content, as well as microorganism sensitivity.

Table 1. EC_{50} values from fruit peels extracts.

Tabla 1. Valores de EC_{50} de extractos de cáscaras de frutas.

Extract	EC_{50} (ppm)
Cocoa	122.38±18.04[b]
Avocado	59.03± 5.86[a]
Coconut	64.60± 7.12[a]
Cactus pear	-

- Not detected. / - No detectado.

Means with different letters were significantly different (P < 0.05).

Medias con diferentes letras fueron significativamente diferentes (P < 0,05).

Table 2. Antimicrobial activity from fruit peels extracts.
Tabla 2. Actividad antimicrobiana de extractos de cáscaras de frutas.

Extract	Microorganism/inhibition zone (mm)		
	S. aureus	***S. disenteriae***	***C. albicans***
Cocoa	-	-	12.3±0.3
Avocado	11.3 ±0.2	10.6 ±0.2	15.3±0.1
Coconut	11.3 ±0.4	14.16±0.1	12.3±0.1
Cactus pear	8.33±0.2	-	11.0±0.1

- Not detected. / - No detectado.

According to several authors, the antimicrobial activity of phenolic compounds involves the reaction of phenols with cell membrane proteins and/or sulfhydryl protein groups, leading to bacterial death by precipitation of membrane proteins and inhibition of some enzymes (8, 9).

It should be noted that the extracts with higher antioxidant activity were also more active against the tested microorganisms, being this fact more evident for avocado extract. Jimenez *et al*. (2011) also reported a correlation between antioxidant capacity and antimicrobial activity in black cherry extracts (*Prunus serotina* subsp *capuli*).

HPLC

Typical chromatograms of avocado and coconut peel extracts obtained by sonication are shown in figure 4 (page 368).

The presence of oxalic acid, ascorbic acid, ferulic acid, gallic acid, procyanidin B1, (+)-catechin and caffeic acid were identified in the coconut chromatogram (figure 4-A, page 368). Meanwhile, ascorbic acid, procyanidin B1, (+)-catechin, procyanidin B2 and (-)-epicatechin were confirmed in the avocado extract (figure 4-B, page 368). Flavonols, which were present in both extracts (catechin and epicatechin), could have an important role in the observed antimicrobial properties. Alonso-Esteban *et al*. (2019) reported important antimicrobial properties of (+)-catechin and (-)-epicatechin against *B. cereus*, *L. monocytogenes*, *E. faecalis*, *E. coli*, and *S. typhymurium*. Catechins can increase the content of reactive oxygen species in cells and cause endogenous oxidative stress in bacteria such as *E. coli* (18).

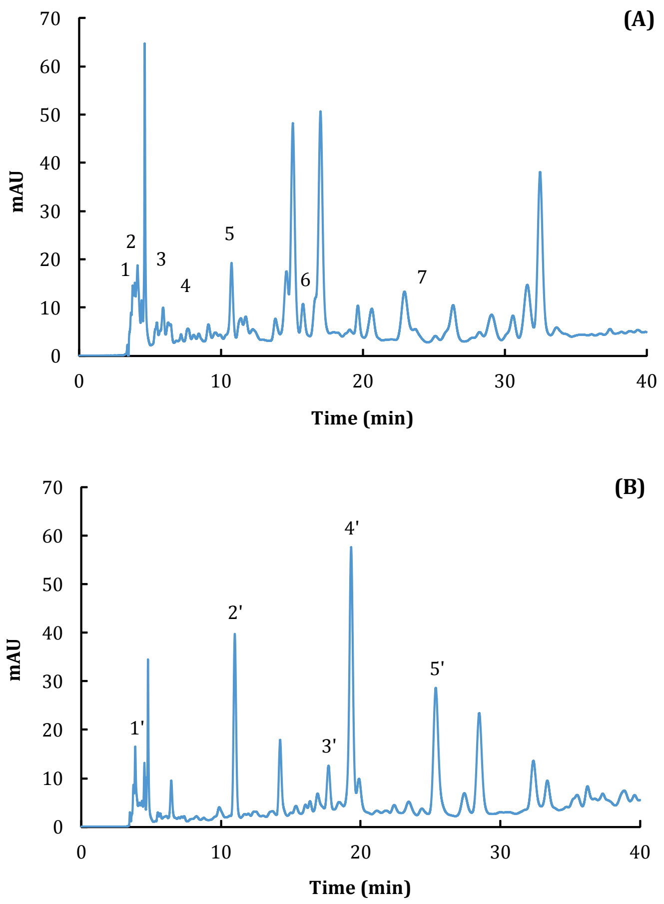


Figure 4. Chromatograms of coconut peel extract (A): (1) oxalic acid, (2) ascorbic acid, (3) ferulic acid, (4) gallic acid, (5) procyanidin B1, (6) (+)-catechin, (7) caffeic acid; and avocado peel extract (B): (1') ascorbic acid, (2') procyanidin B1, (3') (+)-catechin, (4') procyanidin B2, (5') (-)- epicatechin.

Figura 4. Cromatogramas de extracto de cáscara de coco (A): (1) ácido oxálico, (2) ácido ascórbico, (3) ácido ferúlico, (4) ácido gálico, (5) procianidina B1, (6) (+)-catequina, (7) ácido cafeico; y de extracto de cáscara de aguacate (B): (1') ácido ascórbico, (2') procianidina B1, (3') (+)-catequina, (4') procianidina B2, (5') (-)-epicatequina.

Conclusions

Bioactive compounds from avocado, cocoa, coconut, and cactus pear peels were obtained by ultrasound-assisted extraction. The aqueous ethanolic extracts from avocado peel presented the highest phenolic content and the best antioxidant and antiradical activities.

The results showed positive relationships between total phenolic content and antioxidant activity in all extracts. Furthermore, avocado and coconut peels extracts presented important inhibiting action against *S. aureus*, *S. dysenteriae* and *C. albicans*. We conclude that fruit by-products such as peels, could represent an important source of bioactive compounds with antioxidant and antimicrobial properties, and potential use in the pharmaceutical and food industries.

References

1. Ajila, C. M.; Naidu, K. A.; Bhat, S. G.; Prasada Rao, U. J. S. 2007. Bioactive compounds and antioxidant potential of mango peel extract. Food Chemistry. 105(3): 982-988.
2. Alonso-Esteban, J. I.; Pinela, J.; Barros, L.; Ćirić, A.; Soković, M.; Calhelha, R. C.; Torija-Isasa, E.; Sánchez-Mata, M. C.; Ferreira, I. C. F. R. 2019. Phenolic composition and antioxidant, antimicrobial and cytotoxic properties of hop (*Humulus lupulus* L.) seeds. Industrial Crops & Products. 134: 154-159.
3. Al-Zoreky, N. S. 2009. Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels. International Journal of Food Microbiology. 134(3): 244-248.
4. Bubonja-Sonje, M.; Giacometti, J.; Abram, M. 2011. Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. Food Chemistry. 127(4): 1821-1827.
5. Cardenas-Sandoval, B. A.; López-Laredo, A. R.; Martínez-Bonfil, B. P.; Bermúdez-Torres, K.; Trejo-Tapia, G. 2012. Advances in the phytochemistry of *Cuphea aequipetala*, *C. aequipetala* var. hispida and *C. lanceolata*: extraction and quantification of phenolic compounds and antioxidant Activity. Revista Mexicana de Ingeniería Química. 11(3): 401-413.
6. Daglia, M. 2012. Polyphenols as antimicrobial agents. Current opinion in Biotechnology. 23(2): 174-181.
7. González-Gómez, D.; Cardoso, V.; Bohoyo, D.; Ayuso, M. C.; Delgado-Adamez, J. 2014. Application of experimental design and response surface methodology to optimize the procedure to obtain a bactericide and highly antioxidant aqueous extract from orange peels. Food Control. 35(1): 252-259.
8. Gyawali, R.; Ibrahim, S. A. 2014. Natural products as antimicrobial agents. Food Control. 46: 412-429.
9. Ismail, T.; Sestili, P.; Akhtar, S. 2012. Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects. Journal of Ethnopharmacology. 143(2): 397-405.
10. Jiménez, M.; Castillo, I.; Azuara, E.; Beristain, C. I. 2011. Antioxidant and antimicrobial activity of capulin (*Prunus serotina* subsp capuli) extracts. Revista Mexicana de Ingeniería Química. 10(1): 29-37.
11. Khan, M. K.; Abert-Vian, M.; Fabiano-Tixier, A. S.; Dangles, O.; Chemat, F. 2010. Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel. Food Chemistry. 119(2): 851-858.
12. Khonkarn, R.; Okonogi, S.; Ampasavate, C.; Anuchapreeda, S. 2010. Investigation of fruit peel extracts as sources for compounds with antioxidant and antiproliferative activities against human cell lines. Food and Chemical Toxicology. 48(8-9): 2122-2129.

13. Kossah, R.; Zhang, H.; Chen, W. 2011. Antimicrobial and antioxidant activities of chinese sumac (*Rhus typhina* L.) fruit extract. Food Control. 22(1): 128-132.
14. Kubola, J.; Siriamornpun, S. 2011. Phytochemicals and antioxidant activity of different fruit fractions (peel, pulp, aril and seed) of thai gac (*Momordica cochinchinensis* Spreng). Food Chemistry. 127(3): 1138-1145.
15. Li, X.; Wang, T.; Zhou, B.; Gao, W.; Cao, J.; Huang, L. 2014. Chemical composition and antioxidant and anti-inflammatory potential of peels and flesh from 10 different pear varieties (*Pyrus* spp.). Food Chemistry. 152(1): 531-538.
16. Lim, Y. S.; Hui, L. S. S.; Chin, T. B. 2013. Antioxidant capacity and antibacterial activity of different parts of mangosteen (*Garcinia mangostana* Linn.) extracts. Fruits. 68(6): 483-489.
17. Luengo, E.; Alvarez, I.; Raso, J. 2013. Improving the pressing extraction of polyphenols of orange peel by pulsed electric fields. Innovative Food Science & Emerging Technologies. 17: 79-84.
18. Ma, Y.; Ding, S.; Fei, Y.; Liu, G.; Jang, H.; Fang, J. 2019. Antimicrobial activity of anthocyanins and catechins against foodborne pathogens *Escherichia coli* and *Salmonella*. Food Control. 106: 106712.
19. Majhenic, L.; Kerget, M. S.; Knez, Z. 2007. Antioxidant and antimicrobial activity of guarana seed extracts. Food Chemistry. 104(3): 1258-1268.
20. Mallek-Ayadi, S.; Bahloul, N.; Kechaou, N. 2017. Characterization, phenolic compounds and functional properties of *Cucumis melo* L. peels. Food Chemistry. 221(15): 1691-1697.
21. Mansilla, J. T.; Tarcaya, V. P.; Cufre, I. M.; Fabrizio, M. C.; Wright, E. R.; Broussalis, A. M.; Rivera, M. C. 2018. Control of *Rhizoctonia solani* with extracts from *Ovidia andina*. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 355-368.
22. Márquez, D. M.; Galeano, E.; Martínez, A. 2003. Productos naturales con actividad antimicrobiana. Parte I. Vitae. 10(2): 61-71.
23. Medini, F.; Fellah, H.; Ksouri, R.; Abdelly, C. 2014. Total phenolic, flavonoid and tannin contents and antioxidant and antimicrobial activities of organic extracts of shoots of the plant *Limonium delicatulum*. Journal of Taibah University for Science. 8(3): 216-224.
24. Nedamani, E. R.; Mahoonak, A. S.; Ghorbani, M.; Kashaninejad, M. 2014. Antioxidant properties of individual *vs.* combined extracts of rosemary leaves and oak fruit. Journal of Agricultural Science and Technology. 16: 1575-1586.
25. Preciado-Rangel, P.; Gaucín-Delgado, J. M.; Salas-Pérez, L.; Sánchez Chavez, E.; Mendoza-Vllarreal, R.; Rodríguez Ortiz, J. C. 2018. The effect of citric acid on the phenolic compounds, flavonoids and antioxidant capacity of wheat sprouts. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 119-127.
26. Quiroz-Reyes, C. N.; Aguilar-Méndez, M. A.; Ramírez-Ortiz, M. E.; Ronquillo-de Jesús, E. 2013. Comparative study of ultrasound and maceration techniques for the extraction of polyphenols from cocoa beans (*Theobroma cacao* L.). Revista Mexicana de Ingeniería Química. 12(1): 11-18.
27. Safdar, M. N.; Kausar, T.; Jabbar, S.; Mumtaz, A.; Ahad, K.; Saddozai, A. A. 2017. Extraction and quantification of polyphenols from kinnow (*Citrus reticulate* L.) peel using ultrasound and maceration techniques. Journal of Food and Drug Analysis. 25(3): 488-500.
28. Saravanan, S.; Parimelazhagan, T. 2014. *In vitro* antioxidant, antimicrobial and anti-diabetic properties of polyphenols of *Passiflora ligularis* Juss. fruit pulp. Food Science and Human Wellness. 3(2): 56-64.
29. Sood, A.; Gupta, M. 2015. Extraction process optimization for bioactive compounds in pomegranate peel. Food Bioscience. 12: 100-106.
30. Tabaraki, R.; Heidarizadi, E.; Benvidi, A. 2012. Optimization of ultrasonic-assisted extraction of pomegranate (*Punica granatum* L.) peel antioxidants by response surface methodology. Separation and Purification Technology. 98: 16-23.

31. Wootton-Beard, P. C.; Ryan, L. 2011. Improving public health?: The role of antioxidant-rich fruit and vegetable beverages. Food Research International. 44(10): 3135-3148.

32. Yasoubi, P.; Barzegar, M.; Sahari, M. A.; Azizi, M. H. 2007. Total phenolic contents and antioxidant activity of pomegranate (*Punica granatum* L.) peel extracts. Journal of Agricultural Science and Technology. 9: 35-42.

Acknowledgements

This work was financially supported by SIP-IPN (project: 20140034). M. P. Campos-Arias thanks COFAA-IPN and CONACYT for the scholarships received.

Authors are also grateful for the technical assistance and facilities provided by Dra. Adriana Cuadros and Dr. Jorge Yañez, respectively.

Opuntia ellisiana Griffiths as livestock feed in areas similar to USDA cold hardiness zones 6-7

Opuntia ellisiana Griffiths como alimento para el ganado en áreas similares a las zonas de resistencia al frío USDA 6-7

Josefina María Grünwaldt [1], Peter Felker [2], Juan Carlos Guevara [1],
Eduardo Guillermo Grünwaldt [1]

Originales: *Recepción:* 19/05/2018 - *Aceptación:* 09/09/2018

Index

1 Argentine Institute for Arid Land Research (IADIZA-CCT-CONICET-MENDOZA) Av. Adrián Ruiz Leal s/n. Parque Gral. San Martín. 5500. Mendoza. Argentina. jgrunwaldt@mendoza-conicet.gob.ar

2 D' Arrigo Bros. Co. of California. 21777 Harris Road Salinas. California. 93908 USA.

REVIEW

Abstract

The present review compiles the studies carried out so far on *Opuntia ellisiana* Griffiths. This species, of unknown origin, was first described at the beginning of the 20th century in southern Texas, USA, and introduced to Argentina in 1998. This species, like other *Opuntia* sps., can be cultivated in a wide range of environments and its lower transpiration per unit of carbon gained in relation to C_3 and C_4, has lead to in an important increase in water-use efficiency. While *O. ellisiana* has a lower growth and productivity than *O. ficus-indica* (L.) Mill. it stands out for its resistance to sub-zero temperatures. Fortunately, the intraspecies variation within *O. ellisiana,* shortens the time for its use after establishment. There is a wide variation in the nutrient content between the different forage species and clones of *Opuntia*. Due to the inherently low N availability in arid ecosystems, *O. ellisiana*, like the other species, has low protein content in natural unfertilized conditions. Some efforts, as the use of N-fertilizer, have been carried out to improve its protein level. About 15% protein levels have been obtained with other Opuntias. Other research has been directed to provide a favorable abiotic environment for a cactus to achieve higher biomass productivity and improved protein levels by interacting with nurse plants, such as *Prosopis* sps. The last alternative resulted in a significant increase in protein content and cladode quantity per plant of *O. ellisiana*.

Keywords
Opuntia ellisiana • phylogeny • ecophysiology • cold hardiness • productivity • nutrient content

Resumen

La presente revisión compila los estudios realizados hasta el presente sobre *Opuntia ellisiana* Griffiths. Esta especie, de origen desconocido, fue descripta primeramente a comienzos del siglo 20 en el sur de Texas, EE.UU. y fue introducida a la Argentina en 1998. Al igual que otras *Opuntia* sps. puede ser cultivada en un amplio rango de ambientes y su transpiración más baja por unidad de carbón ganada en relación con plantas C_3 y C_4 conduce a un importante incremento en la eficiencia del uso del agua. Mientras que *O. ellisiana* tiene un crecimiento y una productividad más bajos que *O. ficus-indica* (L.) Mill. se destaca por su resistencia a temperaturas sub-cero. Afortunadamente hay variación intra especie dentro de *O. ellisiana* que se puede utilizar para acortar el tiempo de uso después de su establecimiento. Existe una amplia variación en el contenido de nutrientes en las diferentes especies forrajeras y entre los clones de *Opuntia*. Debido a la baja disponibilidad de N en los ecosistemas áridos, *O. ellisiana*, al igual que las otras especies, tiene bajo contenido de proteínas en condiciones naturales sin fertilización. Algunos esfuerzos, como el uso de N como fertilizante, se han llevado a cabo para mejorar su nivel de proteína, alcanzando niveles proteicos cercanos al 15% en otras Opuntias. Algunas investigaciones han sido dirigidas a proporcionar un ambiente abiótico favorable para el cactus para lograr una mayor productividad de biomasa y niveles mejorados de proteína mediante la interacción con plantas nodrizas, tales como *Prosopis* sps. La última alternativa permitió incrementar significativamente el contenido de proteína y la cantidad de cladodios por planta de *O. ellisiana*.

Palabras clave
Opuntia ellisiana • filogenia • ecofisiología • resistencia al frío • productividad • contenido de nutrientes

Introduction

Cactaceae, have evolved to develop adaptive mechanisms that allow them to ensure their survival in highly hostile conditions and are now part of the natural environment and agricultural systems worldwide.

Plantations of drought-tolerant and water-efficient fodder shrubs, especially *Opuntia* species, have been established as buffer feed reserve, as a strategy to mitigate the effects of drought in animal production systems in various arid and semiarid areas of the world.

In this strategy the buffer reserve was aimed not only as "drought insurance" for inter-annual drought but also to bridge over a recurrent annual period of feed scarcity (40). *Opuntia* species have the ability to withstand prolonged drought, high temperatures, as well as wind and water erosion (26).

Cactus and other drought-tolerant and water-efficient fodder shrubs can survive under rainfall as low as 50 mm on a particular year, but with neither growth nor production. Mean annual rainfall of 100-150 mm corresponds to the minimum required to successfully establish rainfed plantations (41), provided soils are sandy and deep (42).

Within the Cactaseas family, the genus *Opuntia* is considered the one of greatest agronomic importance and there are many reasons behind its worldwide diffusion such as simple cultivation practices required to grow the crop; rapid establishment soon after introduction in a new area; ability to grow in very harsh conditions characterized by high or low temperature, lack of water and poor soil; possibility of massive propagation by *in vitro* culture of areoles when there is low availability of material for propagation; appreciated fruits; use of stems in the human diet and as forage for livestock; production of a wide range of industrial derivatives. These and other factors have contributed to such a wide distribution, from the regions of origin in Latin America to remote areas in different continents, cultures and traditions (38, 45).

Cacti have greater water-use efficiency due to Crassulacean Acid Metabolism (CAM) photosynthetic pathway (35, 52, 53) making them especially suited for forage productions in arid lands.

At the end of the 20th century, the area under cultivated O*puntia* for forage was reported to be 900,000 ha, greatly surpassing the reported area for fruit (100,000 ha). The succulence and nutritive value of *Opuntia* make it a valuable emergency crop, permitting livestock farmers in Brazil, Mexico, South Africa and USA to survive prolonged and severe droughts (61).

In Argentina, the cultivated area of cactus is estimated at 10,000 ha for forage and fruit production (11).

The major limitation to cultivation of cactus in many areas of the world is severe cold winter temperature as occurs in the region of Mendoza, Argentina (26), northern Mexico (4), the Mediterranean Basin (42), the arid highland steppes of western Asia (43) and the south-western United States (59).

Opuntia ellisiana is a slower growing species compared to other *Opuntia* species such as *O. ficus-indica.* Nevertheless *O. ellisiana* is the only spineless *Opuntia* fodder species that is completely cold resistant in Texas (34) and in Mendoza, Argentina (30, 31).

This review reports the findings of the studies on *Opuntia ellisiana* Griffiths with respect to phylogeny, ecophysiology, cold hardiness, productivity and nutrient content, and its interaction with *Prosopis* sps. as a nurse plant.

Phylogeny

The first systematic collections, descriptions and field testing of Opuntias from Mexico and southwestern USA for fruit, forage and cold hardiness was conducted in the first few years of the 20th century principally by David Griffiths (21, 23, 24, 25) including a 1906' with guidelines on the use of cactus in animal feed (19).

Among Griffith's extensive work there is one about *O. ellisiana* (20). In 1915 this species was described in a similar manner as in 1910 (22). This description indicated "Plant spreading, ascending, laxly to compactly branched, 1-1.5 m high, and 1.25-2 m in spread of branch, depending upon moisture and fertility conditions; joints light, pale, glaucous, green, when young, but yellowish shortly after maturity, broadly obovate, about 20 x 24 cm, slightly elevated at areoles when young; areoles at first almost cottony white, turning gray, and finally black, small, 2-3 mm in diameter, after leaves have fallen and maturity has approached, made up of a central papillum in which the spicules are produced surrounded by a depressed groove separating it from the outer zone of gray or white wool; leaves long, prominent, circular in sections or slightly flattened, subulate, cuspidate, broadly arched backward, 12-15 mm in length; spicules light yellow, never prominent, scarcely visible, few and only 1 mm or less in length, scarcely distinguishable except by feeling from the central papillum of wool in which they are situated; spines entirely absent; flowers deep yellow, changing to orange, reddish when closed, some of the outer perianth segments dull, greenish red in bud, about 6 cm in diameter when open, filaments and style white, stigma very light greenish yellow, 7-parted; fruit pyriform to hemispherical, deep reddish purple throughout, young ovary thickly beset above with small white subcircular areoles 3 mm apart, and 1.5 mm in diameter, the wool being prominently raised to 1 mm or more in a compact columnar tuft, from center of which are produced 1-2 delicate yellowish fugaceous spines, 2-3 mm long and 1-3 or 4 minute spicules 1 mm long or less, the lower part of ovary having only 1-3 spicules, and the areoles being much farther apart".

"The origin is not known, but it has evidently been in cultivation for a long time. It is now quite widely distributed in collections due to the efforts of the Department of Agriculture (Washington, D. C.) and Professor J. C. Ellis, who first found it cultivated by Mexicans in the outskirts of Corpus Christi. There are indications that it has been derived by selection from native forms of southern Texas; but the evidence is not conclusive. It is perfectly hardy at Austin, and as *Op. cacanapa*, and possibly as hardy as *Op. subarmata* (22). In growth it is not as fast as the other two; but it is much more smooth, approaching if not quite equaling in this respect, the smoother forms of the Indian-fig group. Another feature is lack of spicules on the fruits.

On this accounts, the species is quite promising for breeding purposes. While these three forms appear to be the most promising, and are the ones upon which the greatest effort is being expended at present, it is not at all impossible that other selections may be made of as great, if not even greater merit. One nearly spineless form recorded under my collection N° 9087, from Webb County, Texas, is a rapid, very succulent, wavy jointed, compact form, as good as any of the above, were it not for its few spines. It is probably very close to, if not the same as, forms of *Opuntia subarmata*. Another selection made last year is a remarkably smooth form of *Op. bentonii*. It is thus far devoid of spines, but has quite prominent spicules. This grows rapidly, but its joints are as thin as those of *Op. cacanapa*. The difference in cold resistance of these forms is not great. They will withstand from -6.7 to -11.1°C lower temperatures than the conventional spineless ones of today; and will probably all be hardy throughout the entire pear region of Texas" (22).

In 1919, *O. ellisiana* is mentioned as an unarmed species and known only from cultivated plants. It was stated by Griffiths that it is quite different from the *Ficus-indicae* series, which in much resembles, and is quite hardy in southern Texas. It may be a spineless race of the common *O. lindheimeri* of this region (6).

The taxonomic instability of the Opuntioideae is not restricted to the generic classification. *Opuntia* sps. in particular, the largest genus in the subfamily, is well known for being extremely difficult taxonomically at the species and lower levels as a consequence of a high incidence of inter specific hybridization and polyploidy, which have resulted in complex patterns of morphological variation.

O. ellisiana is mentioned as a species distributed and exclusively cultivated in the USA (35).

Wild *Opuntia* (62, 71) can be diploid, triploid, tetraploid and octaploid (n = 11). Evidently due to insect pollination of *Opuntia*'s perfect, self-fertile flowers, today's commercial *O. ficus-indica* land races for fruit use are octaploid (60). While a diploid spineless *O. ficus-indica* in Alpine, Texas (71), had been reported the species designation for this accession was later determined to be *O. ellisiana*. A later work confirms the diploidy of *O. ellisiana*: "Of the 164 species in the Opuntieae for which chromosome counts have been carried out, including our new counts, 26.2% are diploid, 13.4% are both diploid and polyploid, and 60.4% are polyploid reiterating that the frequency of genome duplication in the group is far more common than diploidy" (46).

Among other characteristics, phenotypically, *O. ellissiana* can be differentiated from *O. ficus-indica* by its lower height at maturity (1.0-1.5 m *vs*. up to 6 m); by the length of its cladodes (20 cm *vs* 20-50 cm) and by the shape (pyriform to hemispherical *vs* tuberculate, ovoid to oblong) and color of its fruits (dark red to almost purple *vs* yellow, orange, pink-green or reddish), respectively.

Texas A&M University, Kingsville, (TAMUK) has been involved since 1982 in collection and introduction of *Opuntia* to the USA, as well as agronomic research and extension (49). The programme focuses on the development of freeze-tolerant cultivars, as freeze damage is a common problem in the region (70). In 1996, the first round of crosses marked the beginning of a long-term breeding programme. In 1998, the genetic material, including *O. ellisiana* clone 1364, was transferred to National University of Santiago del Estero, Argentina by Peter Felker (pers. comm. 2018). At present, *O. ellisiana* existing in the Mendoza province descends from the clone 1364, taking part of research works for its use as fodder.

Ecophysiology

Crassulacean acid metabolism (CAM) photo-synthesis is known in 33 families with an estimated 15 to 20,000 species, including *O. ellisiana*. These CAM plants express the most plastic and tenacious photosynthesis known. They can switch photosynthesis pathways and live and conduct photosynthesis for years even in the virtual absence of external H_2O and CO_2, *i.e.*, CAM tenaciously protects its photosynthesis from both H_2O and CO_2 stresses (3).

CAM metabolism is one of the three metabolic pathways found in the photosynthetic tissue of vascular plants for assimilation for atmospheric CO_2. Elucidation of the complete pathway of carbon assimilation in CAM plants took nearly 150 years and encompassed many fundamental discoveries in plant biochemistry (73).

To understand CAM photosynthesis, several landmark discoveries were made at the following times, *i.e.*, daily reciprocal acid and carbohydrate cycles were found during 1870 to 1887; their precise identification, as malic acid and starch, and accurate quantification occurred from 1940 to 1954; and photosynthesis in two different types of cells was discovered from 1965 to approximately 1974. Therefore, by approximately 1980, CAM photosynthesis was finally rigorously outlined (3).

The physiological basis of the ecological success and agricultural usefulness of opuntias as a forage, in large measure reflects their daily pattern of stomatal opening. Most plants have daytime stomatal opening so that CO_2 uptake occurs concomitantly with photosynthesis, which uses the energy of light to incorporate CO_2 from the atmosphere into carbohydrates. Plants like opuntias, however, have nocturnal stomatal opening, so net CO_2 uptake and water loss occur during the cooler part of the 24-hour cycle (55) and like other CAM plants, accumulate and store malate in the vacuoles of the chlorenchyma cells. This gas exchange pattern is referred to as CAM because it was studied extensively in the Crassulaceae, although apparently first recognized in the Cactaceae (51, 68). In *O. ficus-indica*, net nocturnal CO_2 uptake had a relatively low optimal temperature, ranging from 11°C for plants grown at day/night air temperature of 10°C/0°C to 23°C at 45°C/35°C. Nocturnal CO_2 uptake and acid accumulation summed over the whole night were maximal for growth temperatures near 25°C/15°C, with CO_2 uptake decreasing more rapidly than acid accumulation as the growth temperature was raised (57).

CAM plants are native to arid and semi-arid regions, as well as to periodically dry microhabitats such as those occupied by epiphytes. Most of the 20,000 species of CAM plants are epiphytes growing on trees in tropical forests (52, 55, 72).

Since night-time temperatures are lower than diurnal ones, and relative humidity is generally higher, the transpiration of CAM plants is three to five times lower than that of C_3 and C_4 plants. The result is a tremendous increase in water-use efficiency and in the plant's ability to thrive in semi-arid environments characterized by a restricted water supply (200-300 mm of annual rainfall) or where long periods of drought and relatively high temperatures occur.

The mechanisms of adaptation to aridity are not necessarily valid in relation to high temperatures. Although they occur at night, CO_2 uptake and acid accumulation are strongly influenced by environmental variables such as air temperature, light, plant water status, nutrients and soil salinity (51). There are plantations in Aziza (Lybia) where the maximum temperature exceed 50°C (44). Cladodes of different opuntias species, included *O. ficus-indica*, cannot survive between 64 and 70°C (56). In Mexico the regions of greatest diversity for commercial fruit varieties are in the altiplano of central Mexico averaging 1,800 m elevation where the maximum daily temperatures are much lower than the lower elevations of north eastern Mexico.

The daily pattern and the magnitude of total net CO_2 uptake by *O. ficus-indica* mainly reflect nocturnal temperatures. Most cacti examined have a low temperature optimum (near 15°C) for net CO_2 uptake. Moreover, substantial net CO_2 uptake occurs at 0°C for *O. ficus-indica*, and *O. humifusa* can even have substantial net CO_2 uptake at air temperatures of -5°C. Thus, low nighttime temperatures are not disadvantageous for net CO_2 uptake by these cacti, whereas high nighttime temperatures, such as those above 30°C, can lead to appreciable stomatal closure and hence limited net CO_2 uptake, leading to poor plant growth and production and eventually to low crop value (58).

Cladode succulence acts as a buffer to maintain turgor in the photosynthetic tissue (chlorenchyma), making it possible for the cladode to continue photosynthesizing during dry periods (37). Daily fluctuations in cladode thickness may also represent an early indicator of dehydration stress. Young cladodes show more pronounced diel thickness fluctuations compared with older cladodes, and therefore serve as a suitable model for assessing plant responses to environmental factors. Under well-watered conditions, diel fluctuations of cladode thickness are directly related to temperature variations, but not under severe drought stress (65).

Water-use efficiency (WUE) has been defined as the amount of water used per unit of plant material produced (5). This definition may be too broad in that it embraces water-use efficiencies obtained in diverse time and process scales. The plant material can be expressed as carbon dioxide assimilation, total biomass, or yield; the water use can be expressed as transpiration, evapo-transpiration or total water input to the ecosystem; and the timescale can be instantaneous, daily or seasonal (66). These authors stressed that water-use efficiency obtained at different time and process scales should not be used interchangeably.

It has been widely accepted that, in general, plants possessing CAM have higher water-use efficiency than C_3 and C_4 plants. The transpiration ratio for CAM plants ranged from 50 to 125 kg H_2O/kg CO_2, while for C_3 plants: from 450 to 950 kg H_2O/kg CO_2 and for C_4 plants: from 250 to 350 kg H_2O/kg CO_2 (2). As nearly all of the 130 accessions of germplasm collection (34) have greater height growth than *Opuntia ellisiana* under the same rainfall conditions, it would appear that they might have even greater water-use efficiencies. These accessions are mainly represented by *O. ficus-indica*; *O. lindheimerii* and *O. megacantha*, from Mexico, Africa, USA, Chile and Brazil (P. Felker, pers. comm. 2018). The average WUE of *O. ellisiana* was 162 kg H_2O DM^{-1}. This is among the highest WUE of any plant species, including C_3 and C_4, measured under long-term field conditions. Considering that WUE measured for *O. ficus-indica* was 250-300 kg H_2O DM^{-1} (41), WUE of *O. ficus-indica* is about 55 to 85% lower than *O. ellisiana*. Thus, it can be assumed that the lower productivity of *O. ellisiana* could be explained by its higher transpiration rates.

Table 1 (page 380), presents the climatic and soil characteristics of three sites in which experiments with *O. ellisiana* have been carried out: CCT CONICET (CCT), El Divisadero Cattle and Range Experimental Station (DIV) both in Mendoza, Argentine, and Deming (DEM) New Mexico, USA. In the Mendoza plain, the dry season coincides with a cold winter, while the hot season, during which fruit and vegetative growth take place, correspond to the rainy season; whereas in Deming, NM; USA the dry season coincides with the hottest days, when the fruit develops and vegetative growth occurs.

The climatic conditions of the CCT, DIV and DEM are typical of arid zones with torrid summers, cold winters and great daily thermal amplitude, although in Deming it may be noted that the minimum absolute temperature is significantly lower, whereas no major differences in annual rainfall are among the three mentioned sites.

Soil composition presents its differences mainly in relation to nitrogen content and electrical conductivity; indicating that *O. ellisiana* has the capacity to develop under different soil characteristics.

Cold hardiness

During 1989, members of the Texas Prickly Pear Council discovered in Kingsville Texas, a thornless *Opuntia* that had survived a -12°C freeze without damage, when all *O. ficus-indica*, *O. robusta* types and others spineless types froze to ground level. This freeze hardy *Opuntia* appears to be *O. ellisiana* Griffiths (18). This species could be a useful forage variety in locations that are too cold for *O. robusta* Wendl. or *O. ficus-indica* (34). Other authors found that *O. ellisiana* was also completely tolerant to 20 hours below -7°C, with a minimum of -16°C (12). Although spineless varieties generally have less tolerance to freezing weather than spiny varieties (13), *O. ellisiana* suffered no damage when temperatures of -20°C were reached in a site located about 500 km north of Kingsville (70).

The experiments with *Opuntia* sps. as a fodder crop for drought periods began in the Mendoza plain, mid-west Argentina, at the end of 1995. The major limitation for cultivating this sort of cactus in this area, is the cold winter temperatures. Tolerance to sub-zero temperatures depends on the turgidity of cladode's chlorenchyma tissue, dehydration augmenting frost tolerance (42), while the relationship between water content of the cladodes and frost damage in general, showed no definite pattern (28).

Table 1. Climatic and soil characteristics in the CCT CONICET (Mendoza, Argentine), El Divisadero Cattle and Range Experimental Station (Mendoza Province, Argentine) and Deming (New Mexico, USA).

Tabla 1. Características climáticas y de suelo en CCT CONICET (Mendoza, Argentina), Estación Experimental de Ganado y Pasturas Naturales El Divisadero (provincia de Mendoza, Argentina) y Deming (New Mexico, USA).

Characteristics	CCT#	DIV##	DEM&&
Absolute Maximum temperature °C	38.2	41.1	43.3&
Absolute Minimum temperature °C	-4.9	-9.0	-20.0&
Mean Maximum Temperature °C	23.0	25.5	24.8&
Mean Minimum Temperature °C	16.9	10.5	15.8&
Medium temperature °C	14.3	18.2	6.7&
Annual rain fall mm	303.0*	326.4*	237.5** &

Soil order	Entisol‡	Entisol‡	Aridisols&&&
Texture	Sandy Loam‡	Sandy‡	Loam Clay&&&&
Nitrogen ppm	490‡	392‡	<10&&
MO %	1.56‡	0.81‡	0.25-1.5&&
P ppm	5.56‡	3.6‡	<10.0&&
K ppm	625‡	391‡	800-1,345&&
Ca meq/L	8.3‡	4.0‡	2.33&&
Mg meq/L	6.5‡	0.8‡	1.18&&
Na meq/L	2.0‡	0.6‡	0.48&&
Ph	7.7‡	8.5‡	≥ 8.5&&&
EC µS/cm 25°C	1,518‡	224‡	6,000&&&&

* Nearly 80% occurring during the growing season (October to March). / * Alrededor del 80% ocurren durante la estación de crecimiento (octubre a marzo).

** The most rain occurring in July. / ** La mayor parte de las lluvias ocurren en julio.

CCT= Campus Centro Científico Tecnológico CONICET Mendoza (32°53' S 68°52' W, 840 m a .s. l.)

DIV= Campo Experimental El Divisadero Mendoza (33°47' S 67°46' W, 520 m a. s. l.)

DEM= Deming, NM, USA= 32°15' N 107°45' W, 1,321 m a. s. l.)

\# Estación CRICYT. Means 2013-2016. http://www.prmarg.org

\## Estación J. Antunez. Means 2013-2016. http://www.mendoza-conicet.gob.ar/ladyot/red_iadiza/index.htm

‡ IADIZA phytochemical laboratory. / ‡ Laboratorio de fitoquímica del IADIZA.

& https://wrcc.dri.edu/cgi-bin/cliMAIN.pl?nmdemi

&& R. Flynn, pers. comm. 2018.

&&& https://www.nrcs.usda.gov/Internet/FSE_MANUSCRIPTS/new_mexico/lunaNM1980/Luna.pdf.

&&&& https://soilseries.sc.egov.usda.gov/OSD_Docs/H/HONDALE.html.

When night temperatures in El Divisadero Cattle and Range Experiment Station field trial dropped to -12.3°C in the fall 1996, almost all the 7-month-old plants of some species of *Opuntia* were affected by these low temperatures. Thereafter, at the same study site, an *Opuntia* sps. collection was established containing the specimens that had survived the 1996 freeze and others new accessions with potential for increased cold hardiness. When night temperatures dropped to -16 and -17°C on 2 consecutive days in August 1999, great variability in frost damage was observed (28). A similar situation was found in Algeria where *O. ficus-indica* clones were severely damaged, after many genotypes were submitted to night temperatures of -8 to -10°C for a week, with midday temperatures rising to around 5°C (39). Cold hardiness of *Opuntia* sps. clones used for fruit, forage or vegetable production has been reported (18, 39, 59, 64, 70). *Opuntia ellisiana* in Texas endured a -9°C without apparent damage (64).

In El Divisadero Cattle and Range Experimental Station, one-year-growth-period plants of *O. ellisiana* obtained by micropropagation (38) under 23.5 hours of temperatures ≤ -10°C in 2000 suffered no frost damage. The next year, the 2-year-growth-period plants suffered frost damage of about 0.9% under 19 hours at ≤ -10°C, 11 hours at ≤ -12°C and 5 hours at ≤ -13°C, indicating that it would not be necessary to protect the plants during winter for 1 or 2 years after planting. At the same time, *O. ficus-indica* suffered frost damage of 58.6%, statistically significant with respect to *O. ellisiana*, whose pads showed only slight necrosis around the margins. Frost damage for the 2-year-growth-period of *O. ellisiana* was significantly lower than for the 3-year-growth-period plants of *O. ficus-indica* and *O. spinulifera* from adjacent plantations.

The frost damage differences among *O. ellisiana* and the other two species would have been greater at a comparable plant age (30). In fact, frost damage proved to be inversely related to the age of the plant (28, 70). In the summer of 2016, Cushman of the University of Reno Nevada established a planting of ten spineless cold hardy progenies of *O. ficus-indica* x *O. lindheimerii* and *O. ellisiania* on the grounds of the University Campus. After the winter of 2016-2017 none of the cold hardy progenies of *O. ficus-indica* x *O. lindheimerii* survived while *O. ellisiana* survived with no damage. Temperature data for December, January and February found no temperatures as low as -12°C but recorded many days with temperatures of -5°C all day round. It seems that many days of below freezing weather, not necessarily reaching to an absolute minimum of -12°C, are lethal for the cold hardy forage clones (J. C. Cushman, pers. comm. 2018).

During the May-September 2009 period, the total hours with temperatures below 0°C and the absolute minimum temperatures in Ñacuñán (34°03' S, 67°58' W, similar to the study site: El Divisadero Cattle and Range Experimental Station), were 6 h and -1.8°C; 77 h and -7.1°C; 146 h and -6.1°C; 37 h and -4.7°C, and 54 h and -4.7°C in May, June, July, August and September, respectively. *O. ellisiana* suffered zero frost damage during this period (31).

Plants of *O. ellisiana* and *O. ficus-indica*, both obtained by micropropagation, were also established in Malargüe, Mendoza (35°28'8'' S, 69°35'07' W) in 1999. At this site, the mean daily minimum temperature of the coldest month is -4°C (29). Plants of *O. ficus-indica* froze to ground level while plants of *O. ellisiana* suffered no damage when temperatures dropped to -15°C in the winter of 2000 (30).

In an experiment carried out at the CCT Campus during 2014-2015, no frost damage was observed in the *O. ellisiana* cladodes, but it should be considered that winters of that period were not very rigorous, with a minimum temperature of -0.7°C (27).

Cladodes established in two places 5 km apart, with the same soil characteristics, a plain and a sand dune (10-15% slope) with north exposure, suffered frost damage that tended to be higher in the plain than in the dune. This difference can be explained by the cold protection provided by the dune (28). The latter could be taken as a recommendation for the implantation of *O. ellisiana* and other species in cold areas.

To arrive at a quantitative measure for freeze hardiness in perennials such as cacti, is difficult. Absolute minimum temperatures do not seem to be reliable indicators of freeze hardiness. Probably, a survey based on the average annual extreme minimum temperature during a 30-year period in the past, is more important than the lowest temperature ever occurred. Many plants that can survive a short period of exposure to cold, may not tolerate longer periods of cold weather. Therefore, using the USDA cold hardiness zones as a ranking criteria is advisable, given that most of the international botanical publications refer to them. Many other environmental factors, in addition to hardiness zones, contribute to the success or failure of plants such as light, soil moisture, temperature, humidity and duration of exposure to cold. The cold hardiness zones classification of some sites in which experiments with *O. ellisiana* have been carried out are 7 in Nevada, USA (69) and between 6 and 8 for Mendoza Province, Argentina (16).

It has been reported that in Deming, New Mexico, USA, USDA cold hardiness zone 7 (68), *O. ellisiana* never freezes. Clone 1364 of *O. ellisiana* was also not damaged by the worst freeze over 15 years in San Angelo, Texas, USA. Therefore it can be recommended for cold hardiness zone 7.

The adaptability of *O. ellisiana* to USDA cold hardiness zone 7 has important international ramifications. In North America, USDA cold hardiness zone 7 extends from the high elevation Chihuahua Desert of Mexico in the south, westward to southern Nevada and eastward through southern New Mexico, northern Texas, the states along the Gulf of Mexico and as far north as North Carolina. Tunisian researchers have obtained clones for testing in the foothills of the Atlas Mountains. Climates with USDA zone 7 are also located in the arid regions of southern Asia such as the foothills of the Himalayas in India and Pakistan (13). In Argentina, USDA cold hardiness zone 7 includes locations such as La Quiaca, Santa Rosa, Neuquén, Río Colorado, Villa Reynolds and Ushuaia (16).

Productivity and nutritive value

The production of *O. ellisiana* to *O. ficus-indica* ratio ranged from about 0.30 - 0.35 (1, 34) to 0.5 (H. Le Houérou, pers. comm. 1995). While *O. ellisiana* is not economically promising as it does not produce edible fruit and presents insufficient immature growth on a year-round basis to be a vegetable (nopalito) variety (34), it is an encouraging species to be used as forage in cold areas.

In Mendoza, Argentina, after a 2-year growth period, *O. ellisiana* plants obtained by micropropagation, established at 1 m x 5 m spacing, had reached 20.9, 22.2, and 24.8 cm in height under no irrigation, with 30 mm every 30 days and 15 mm every 15 days respectively, totaling

150 mm during the whole growing season. Plant height for the 30 mm every 30 days treatment was significantly higher than that for the no-irrigation treatment. However, irrigation did not significantly affect above-ground biomass production (g DM $plant^{-1}$) and stem-area index (SAI) (cm^2 $plant^{-1}$). Nevertheless, dry-matter production and SAI tended to be higher in the irrigated plots than in the non-irrigated ones. Total biomass production (kg DM ha^{-1}) was 146, 157, and 208 for no irrigation, 15-15 and 30-30 treatments, respectively (30). These dry-matter productions were obtained with a total water input during the growing season of 383 mm in 1999-2000 (150 mm irrigation plus 233 mm rainfall) and 369 mm in 2000-2001 (150 mm irrigation plus 219 mm rainfall).

The 2-year-growth-period average production (170 kg DM ha^{-1}) represented only 2.8% of the production for 2-year-growth-period plants of *O. ellisiana* established at 1.5 x 1 m spacing in Texas (34). Several hypotheses could be advanced to explain this great difference in biomass between the two field trials. First, the Texas plantation was fertilized annually to avoid fertility limitation on water-use efficiency, while also receiving 1,691 mm rainfall in the 2-year growth period. In contrast, the plantation of the study carried out in El Divisadero Cattle and Range Experiment Station, was not fertilized and it received 700 mm rainfall plus 300 mm irrigation from the establishment date to the date when the biomass production was estimated. Second, the planting material probably had lower size (in terms of cladode dry weight) than the single cladodes used (34) and hence, the number and size of the shoots produced during the first year of growth in the field were lower in the plantation (48). Third, the low biomass from plants after the 2-year growth period seems to be due to a lower SAI that reached only 0.028, 0.029, and 0.038 for no irrigation, 15-15 and 30-30 treatments, respectively, while the Texas plantation had and SAI of 0.39 for the 2-year growth period (34). These authors found that biomass productivity was very low until a SAI of 2 was reached. At a SAI of 4 to 5 productivity of *O. ficus-indica* considered maximal (54).

Opuntia has low height growth compared to grasses, *e.g.*, *O. ellisiana* only grows about 40 cm per year and *O. ficus-indica* only about 100 cm per year (34). However, due to annual extension of as many as 100 cladodes of 1.5 kg fresh weight in average (ca. 150 g dry weight) over the surface of several year old plant, productivity can be higher. For example, when *O. ellisiana* achieved a leaf area index of 2, it had a dry matter productivity of 17 Mg ha^{-1} yr^{-1} with only 662 mm rainfall (34). However at 4 yr with 38 dry t per ha, the height of this stand was only about 1 m. With a typical composition of 90% water (fresh weight basis), 6% protein (dry weight basis), 4% calcium (dry weight basis), 75% *in vitro* dry matter digestibility, and 72% digestible protein, cactus offers a highly digestible source of energy, a rich source of calcium for lactating animals, and high water content to offset the animal drinking requirements during drought periods. Indeed, the yearly fresh weight of 194,187 kg ha^{-1} in the fourth year would be sufficient for 4,315 cows day^{-1} at a feeding rate of 45 kg day^{-1} (12).

For *O. ellisiana* with slow height growth, grazing could not begin before the third year. Subsequent grazing is possible at 1 to 2 yr intervals depending on rainfall and weed control (13).

There is considerable variation in nutritional quality of *Opuntia* forage for various species or clones, growing conditions, and cladode ages (15, 17, 28, 50).

In arid conditions, the low quality of the forage and water shortage could be attenuated by the introduction of Opuntia species. In this regard, there is evidence of the beneficial effect of the inclusion of cactus in the ruminant´s diets. Cladodes contain high soluble carbohydrate, calcium and carotene contents, while they are low in protein and fiber (27, 32).

Some parameters related to nutritional quality of the 1-year-growth-period cladodes of *O. ellisiana* obtained by micropropagation expressed in percentage of dry matter (DM), were: crude protein (CP), 5.8; *in vitro* dry matter digestibility (IVDMD), 78.3; ash, 17.3 and organic matter (OM), 82.7 (30). Crude protein content of *O. ellisiana* cladodes was similar to that of 1-year-growth-period cladodes of *O. paraguayensis* (currently *O. elata*), *O. ficus-indica* and *O. robusta* clones growing in the same conditions in the study site, whose average was 5.9% (28). These protein values are somewhat higher than 4.1%, as the mean found for *O. robusta*, *O. lindheimerii* and *O. ficus-indica* in Mexico (15).

The IVDMD was high and similar to the overall value reported for opuntias. Given its low protein content, to supplement animal rations based on unfertilized cactus with protein, mineral, and vitamin supplements, such as soybean or cotton seed meal (12).

Opuntia interaction with nurse plants, such as *Prosopis* sps., have been directed to improve the cacti protein level The adaptation of *Prosopis* to herbivory is the main reason for its dominance in silvopastoral systems in arid and semiarid areas of America. Several species grow well usually under *Prosopis* canopy, responding to a higher soil nutrient content. *O. ellisiana* was implanted under and outside the canopy of isolated *Prosopis* sps. in Mendoza Province (32°53'45" S, 68°52'28" W, 840 m a. s. l.). After one year, the totality of cladodes was harvested. Frost damage was not observed under, nor outside the canopy (27).

Under the *Prosopis* canopy, the nutritional values of *O. ellisiana* were increased; nitrogen in particular, doubled its value. Productivity of cladodes per plant and concentrations of moisture, OM, acid detergent fiber, neutral detergent fiber, K and P in the cladodes were also significantly higher under *Prosopis*, while Ca and Na were higher outside the canopy. Magnesium values were not affected by the position (table 2, page 385). Under the crown of the tree, effective precipitation is greater than outside of it, due to the signifficant runoff of the branches and trunk (36), which favors the leaching of Na in the soil; while an increase in N and P reduces the uptake of Ca and Na (9).

The improvement in the forage value of this cactus under the *Prosopis* crown evidences the better condition of the site as a result of the higher nutrient content of the soil and the contribution of OM as mulch, resulting in the formation of fertility islands (63). In arid and semiarid ecosystems, dominant woody plants are likely to cause changes in microclimate and soil properties by mitigating harsh environmental conditions (*e.g.*, high temperature and radiation) and by modifying soil characteristics, resource availability (*e.g.*, water and nutrients) and spatial distribution of nutrients (7, 33).

Table 2. Means and standard deviations of bromatological values and yield of cladodes per plant of *O. ellisiana* planted outside and under *Prosopis* canopy in Mendoza Province, Argentine.

Tabla 2. Medias y desviaciones estándar de valores bromatológicos y producción de cladodios por planta de *O. ellisiana* bajo y fuera de la canopia de *Prosopis* en la provincia de Mendoza, Argentina.

Parameters	Outside the canopy (n=23)	Under the canopy		
		North (n=9)	South (n=9)	North and South (n=18)
Moisture (%)	89.1±1.3a	91.9±0.9b	91.9±1.2b	91.9±1.0b
Organic matter (%)	76.7±2.5a	80.2±2.7b	79.4±1.0b	79.8±2.1b
Na (%)	0.04±0.01a	0.02±0.01b	0.02±0.005b	0.02±0.01b
K (%)	3.1±0.4a	3.5±0.4b	3.7±0.4b	3.6±0.4b
P (%)	0.07±0.02a	0.11±0.02b	0.12±0.02b	0.11±0.02b
Ca (%)	5.5±1.4a	4.3±1.2b	4.7±0.4b	4.4±0.9b
Mg (%)	1.9±0.2a	1.9±0.2a	1.8±0.2a	1.9±0.2a
Crude protein (%)	4.4±1.0a	8.1±1.3b	8.0±0.5b	8.1±1.0b
Acid detergent fiber (%)	13.7±2.4a	17.7±1.9b	18.8±1.0b	18.2±1.6b
Neutral detergent fiber (%)	29.0±4.2a	31.8±2.4b	31.2±1.3b	31.5±1.9b
Cladode quantity per plant	3.1±1.2a	5.7±2.7b	5.6±2.1b	5.7±2.3b

Hotelling test Alfa=0.05. Means with a letter in common are not significantly different (p>0.05).

Prueba de Hotelling Alfa=0,05. Medias con letra en común no son significativamente diferentes (p>0,05).
Source: (27). / Fuente: (27).

The nurse effect of *Prosopis* improves nitrogen content of cladodes in the same way as cattle manure applied to soil (10). High doses of applied chemical fertilizers almost doubled the CP mean content of the 1-year-old cladodes when compared with the treatment in which no fertilizer was added: 7.8 and 4.3% DM, respectively (31).

Even though all the aforementioned, Cactus growing under *Prosopis* would die due to shade. In Texas, *Opuntia* sps. never grows under the canopy of mature forest but forms extensive growth just outside of it, if that area is cleared giving access to high levels of sunlight (P. Felker, pers. comm. 2018). However, other authors cite that *Prosopis* coverage facilitates the establishment of different species that do not settle in exposed areas (47). It has been suggested that the relationships of cacti and nurse species are primarily the result of intricate biotic relationships rather than differences in simple physical conditions (67). Strong interactions between soil and shaded effect were found when soil beneath *Prosopis articulata* shade, increased the biomass of *Pachycereus pringlei* (8).

Possibilities for genetic improvement

Crosses among many accessions of *O. ficus-indica* and between *O. ficus-indica* and *O. lindheimerii* have been examined (70). However, unsuccessful attempts in crossing *O. ellisiana* with *O. lindheimerii* in the Kingsville collection were carried out. It was later found that *O. lindheimerii* was hexaploid. Since *O. ellisiana* is a diploid, this should be expected. Much in the same way as (14), crossed *O. lindheimerii* with *O. ficus-indica* and obtained spineless progeny with much more cold hardiness than any *O. ficus-indica*, it would be very interesting to cross *O. ellisiana* with a faster growing diploid *Opuntia*. A spiny diploid *O. lindheimerii* that has been found (R. Puente, pers. comm. 2018) would be very interesting to cross with *O. ellisiania*. To completely eliminate spines on the progeny, it might be necessary to make an additional back cross to the *O. ellisiana* parent. As the estimated time expended from making a cross till evaluation of a 80 cm tall seedling, and subsequent additional backcrossing is on the order of 3-4 years, this is highly feasible.

Conclusions

Although *Opuntia ellisiana* with respect to other opuntias is not economically promising as a producer of forage, fruit and vegetables as other opuntias, it could be a useful forage variety in locations that are too cold, as evidenced by the results found in Argentina and the USA. Besides, this species has demonstrated its hardiness for its cultivation under different conditions. The results obtained from the association of *O. ellisiana* with *Prosopis* sps. are also encouraging in relation to the increase of cladode productivity per plant and important nutrient content improvement, like protein.

Plants of *O. ellisiana* obtained by micropropagation appear to be tolerant to freezing temperatures attained in areas with extremely cold winters, and in contrast with other *Opuntia* sps., to protect the plants in winter for 1 or 2 years after planting, seems to be unnnecessary.

The little available information on nutrient content of *O. ellisiana* should inspire further research, considering that it has a promising future as a fodder in cold areas.

References

1. Barrientos-Pérez, F.; Borrego Escalante, F.; Felker, P. 1992. Collaborative Mexico/United States initiative to breed freeze tolerant fruit and forage *Opuntia* varieties, in: Felker, P. (Ed.). Third Annual Prickly Pear Council. Texas A&M University. Kingsville. Texas. p. 49-55.
2. Black, C. C. Jr. 1973. Photosynthetic carbon fixation in relation to net CO_2 uptake. Annual Review of Plant Physiology. 24: 253-286.
3. Black, C. C.; Osmond, C. B. 2003. Crassulacean acid metabolism photosynthesis: 'working the night shift'. Photosynthesis Research. 76: 329-341.
4. Borrego-Escalante, F.; Murillo-Soto, M. M.; Parga-Torres, V. M. 1990. Potencial de producción en el norte de México de variedades de nopal (*Opuntia* spp.) tolerantes al frío, in: Felker, P. (Ed.). Proceedings of the First Annual Texas Prickly Pear Council. Caesar Kleberg Wildlife Research Institute. Kingsville. Texas. p. 49-73.
5. Briggs, L. J.; Shantz, H. L. 1914. Relative water requirements of plants. Journal of Agricultural Research. 3: 1-63.
6. Britton, N. L.; Rose J. N. 1919. The Cactaceae. Descriptions and Illustrations of plants of the cactus family. Volumes I. New York. Dover Publications. Inc. p. 166. Available online at: http://publicationsonline.carnegiescience.edu/publications_online/cactaceae/Britton_Rose_Cactaceae_1.pdf (accessed: October 2017).
7. Callaway, R. M. 1995. Positive interactions among plants. The Botanical Review. 61: 306-349.

8. Carrillo-Garcia, A.; Bashan, Y.; Bethlenfalvay, G. J. 2000. Resource-island soils and the survival of the giant cactus, cardon, of Baja California Sur. Plant and Soil. 218: 207-214.
9. Da Silva, J. A.; Bonomo, P.; Donato, S. L. R.; Pires, A. J. V.; Rosa, R. C. C.; Donato, P. E. R. 2012. Composição mineral em cladódios de palma forrageira sob diferentes espaçamentos e adubações química. Revista Brasileira de Ciências Agrárias. 7: 866-875.
10. Donato, P. E. R.; Vieira Pires, A. J.; Rodrigues Donato, S. L.; Da Silva, J. A.; De Aquino, A. A. 2014. Valor nutritivo da palma forrageira 'gigante' cultivada sob diferentes espaçamentos e doses de esterco bovino. Revista Caatinga. 27: 163-172.
11. Dubeux Jr, J. C. B.; Muir, J. P.; Santos, M. V. F.; Cavalcante, M.; dos Santos, D. C. 2013. Potential da palma forrageira na América do sul, in: Nazareno, M. B.; Ochoa, M. J.; Dubeux Jr., J. C. (Eds.). Proceedings of the Second Meeting for the Integral use of cactus pear an other cacti and First South American Meeting of FAO-ICARDA CACTUSNET. Santiago del Estero. Argentina. 29-40.
12. Felker, P. 1995. Forage and fodder production and utilization, in: Barbera, G.; Inglese, P.; Pimienta- Barrios, E. (Eds.), Agro-ecology, cultivation and uses of cactus pear. FAO. Rome. Italy. 144-154.
13. Felker, P.; Paterson, A.; Jenderek, M. M. 2006. Forage potential of *Opuntia* clones maintained by the USDA. National Plant Germoplasm System (NPGS) collection. Crop Science. 46: 2161-2168.
14. Felker, P.; Zapata, R.; Wang, X; Medina, D.; Bunch, R; Paterson, A. 2010. Fruits characters among apomicts and sexual progeny of a cross of the Texas native *Opuntia lindheimerii* (1250) with a commercial fruit type *Opuntia ficus–indica* (1281). Journal of the Professional Association for Cactus Development. 12: 48-66.
15. Fuentes-Rodríguez, J. 1997. A comparison of the nutritional value of *Opuntia* and *Agave* plants for ruminants. Journal of the Professional Association for Cactus Development. 2: 20-24.
16. Grassia, J. A. 2009. Zonas climáticas en Argentina. Available online at: http://palmasenresistencia.blogspot.com.ar/2009/09/zonas-climaticas-en-argentina.html (accessed: September 2017).
17. Gregory, R. A.; Felker, P. 1992. Crude protein and phosphorus contents of eight contrasting *Opuntia* forage clones. Journal of Arid Environments. 22: 323-331.
18. Gregory, R. A.; Kuti, J. O.; Felker, P. 1993. A comparison of *Opuntia* fruit quality and winter hardiness for use in Texas. Journal of Arid Environments. 24: 37-46.
19. Griffiths, D. 1906. Feeding prickly pear to stock in Texas. U.S. Department of Agriculture, Bureau of Animal Industry. Bulletin N° 91. 44 p.
20. Griffiths, D. 1910. Illustrated Studies in the Genus Opuntia-III. Missouri Botanical Garden Annual Report Vol. 1910. 165-174. Published by: Missouri Botanical Garden Press Stable Available online at: http://www.jstor.org/stable/2400129 Accessed: June 2017 12: 57 UTC).
21. Griffiths, D. 1913. Behavior, under cultural conditions, of species of cacti Known as Opuntia. U. S. Department of Agriculture. 24 p.
22. Griffiths, D. 1915. Hardier spineless cactus**:** present commercial varieties of prickly pear suited to very limited range-selection of favorable variations in native species gives promise of providing forms that will stand zero temperature. The Journal of Heredity. 6: 182-191.
23. Griffiths, D. 1916. New Species of Opuntia. Bulletin of the Torrey Botanical Club. 43(2): 83-92. Available online at: http://www.jstor.org/stable/pdf/2479824.pdf (accessed: November 2017).
24. Griffiths, D.; Hare, R. F. 1907. Summary of recent investigations of the value of cacti as stock food. U.S. Government Printing Office. 16 p.
25. Griffiths, D.; Hare, R. F. 1907. The tuna as food for man. U.S. Government Printing Office. 73 p.
26. Grünwaldt, J. M.; Guevara. J. C.; Grünwaldt, E. G.; Martínez Carretero. E. 2015. Cacti (*Opuntia* sps.) as forage in Argentina dry lands. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 47(1): 263-282.
27. Grünwaldt, J. M.; Guevara. J. C.; Martínez Carretero. E.; Grünwaldt, E. G. 2018. Effect of *Prosopis* spp. as a nurse plant on nutrient content and productivity of *Opuntia ellisiana* Griffiths. Accepted for publication in Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 129-137.

28. Guevara, J. C.; Gonnet, J. M.; Estevez, O. R. 2000. Frost hardiness and production of *Opuntia* forage clones in the Mendoza plain, Argentina. Journal of Arid Environments. 46: 199-207.
29. Guevara, J. C.; Estevez, O. R. 2002. *Opuntia* spp. for fodder and forage production in Argentina: experiences and prospects, in: Mondragón-Jacobo, C.; Pérez-González, S. (Eds.), Cactus (*Opuntia* spp.) as forage. FAO Plant Production and Protection Paper. 169: 63-71.
30. Guevara, J. C.; Silva Colomer, J. H.; Juárez, M. C.; Estevez, O. R. 2003. *Opuntia ellisiana*: cold hardiness, above-ground biomass production and nutritional quality in the Mendoza plain, Argentina. Journal of the Professional Association for Cactus Development. 5: 55-64.
31. Guevara, J. C.; Felker, P.; Balzarini, M. G.; Paez, S. A.; Paez, M. N.; Antúnez, J. C. 2011. Productivity, cold hardiness and forage quality of spineless progeny of the *Opuntia ficus-indica* 1281 x *O. lindheimerii* 1250 cross in Mendoza plain, Argentina. Journal of the Professional Association for Cactus Development. 13: 48-62.
32. Guevara, J. C.; Estevez, O. R. 2018. Sustainable use of rangelands of the Mendoza plain (Argentina). Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 295-307.
33. Gutiérrez, J. R.; Meserve, P. L.; Contreras, L. C.; Vásquez, H.; Jaksic, F. M. 1993. Spatial distribution of soil nutrients and ephemeral plants underneath and outside the canopy of *Porlieria chilensis* shrubs (Zygophyllaceae) in arid coastal Chile. Oecologia. 95: 347-352.
34. Han, H.; Felker, P. 1997. Field validation of water-use efficiency of the CAM plant *Opuntia ellisiana* in south Texas. Journal of Arid Environments. 36:133-148.
35. Hernández, H. M.; Gómez-Hinostrosa, C.; Bárcenas, R. T.; Puente, R.; Reyes-Agüero, J. A. 2014. A checklist of the subfamily Opuntioideae (Cactaceae) in North and Central America, in: Hunt, D. (Ed.), Further Studies in the Opuntioideae (Cactaceae). The Manse, Chapel Lane, Milborne Port DT9 5DL. England. 185-200.
36. Horno, M. E. 1993. Interceptación de la precipitación por algarrobo, in: IADIZA (Ed.), Contribuciones Mendocinas a la Quinta Reunión Regional para América Latina y el Caribe de la Red de Forestación del CIID. Conservación y mejoramiento de especies del género *Prosopis.* Mendoza. Argentina. IADIZA. 93-97.
37. Inglese, P.; Liguori, G.; de la Barrera, E. 2017. Ecophysiology and reproductive biology of cultivated cacti, in: Inglese, P.; Mondragon, C.; Nefzaoui, A.; Sáenz, C. (Eds.), Crop ecology, cultivation and uses of cactus pear. FAO. Rome. Italy. 29-41.
38. Juárez, M. C.; Passera, C. B. 2002. *In vitro* propagation of *Opuntia ellisiana* Griff. and acclimatization to field conditions. Biocell. 26:319-324.
39. Le Houérou, H. N. 1971. Les Bases Ecologiques de l'amélioration de la Production Fourragère en Algérie. FAO, Rome. Italy. 58 p.
40. Le Houérou, H. N. 1991. Feeding shrubs to sheep in the mediterranean arid zone: intake, performance and feed value, in: Gaston, A.; Kernick, M.; Le Houérou, H. N. (Eds.), Proceedings of the Fourth International Rangeland Congress CIRAD (SCITS) Montepellier. France. 639-644.
41. Le Houérou, H. N. 1994. Drought-tolerant and water-efficient fodder shrubs (DTFS), their role as a "drought insurance" in the agricultural development of arid and semi-arid zones in southern Africa. WRC. Pretoria. South Africa. Report N° KV 65. 139 p.
42. Le Houérou, H. N. 1996a. The role of cacti (*Opuntia* spp.) in erosion control, land reclamation, rehabilitation and agricultural development in the Mediterranean Basin. Journal of Arid Environments. 33: 135-159.
43. Le Houérou, H. N. 1996b. Utilization of fodder trees and shrubs (TRUBS) in the arid and semi-arid zones of western Asia and northern Africa (WANA): history and perspectives. A review. ICARDA/CIHEAM. Hammamet. Tunisia. 51 p.
44. Le Houérou, H. N. 2002. Cacti (*Opuntia* spp.) as a fodder crop for marginal lands in the Mediterranean basin. Acta Horticulturae. 581: 21-46.
45. Louhaichi, M.; Nefzaoui, A.; Guevara, J. C. 2017. Cactus ecosystem goods and services, in: Inglese, P.; Mondragón, C.; Nefzaoui, A.; Sáenz, C. (Eds.), Crop ecology, cultivation and uses of cactus pear. FAO. ICARDA. Rome. Italy. 159-169.

46. Majure, L. C.; Puente, R.; Pinkava, D. J. 2012. Miscellaneous chromosome numbers in Opuntieae Dc. (Cactaceae) with a compilation of counts for the group. Haseltonia. 18: 67-78.
47. Mares, M. A.; Enders, F. A; Kingsolver, J. M.; Neff, J. L.; Simpson, B. B. 1977. Prosopis as a niche component, in: Simpson, B. B. (Ed.). Mesquite. Its biology in two Desert Scrub Ecosystems. 123-149. U.S./ibp synthesis series 4. Dowden, Hutchinson & Ross, Inc.
48. Mondragón-Jacobo, C.; Pimienta-Barrios, E. 1995. Propagation. in: Barbera, G.; Inglese, P.; Pimienta-Barrios, E. (Eds.), Agro-ecology, cultivation and uses of cactus pear. Rome. Italy. FAO. 64-70.
49. Mondragón Jacobo, C.; Pérez González, S. 2001. Germplasm resources and breeding opuntia for fodder production, in: Mondragón Jacobo, C.; Pérez González, S. (Eds.), Cactus (*Opuntia* spp.) as forage. FAO. Rome. Italy. p. 21-28.
50. Monjauze, A.; Le Houérou, H. N. 1965. Le rôle des *Opuntia* dans l'Economie agricole Nord Africaine. Extrait du Bulletin de l'Ecole Nationale Supérieure d'Agriculture de Tunis. 8-9: 85-164.
51.Nobel, P. S. 1988. Environmental biology of agaves and cacti. New York. USA: Cambridge University Press. 270 p.
52. Nobel, P. S. 1991. Achievable productivities of CAM plants: basis for high values compared with C_3 and C_4 plants. Tansley Review 32. New Phytologist. 119: 183-205.
53. Nobel, P. S. 1994. Remarkable agaves and cacti. Oxford University Press, New York. 180 p.
54. Nobel, P. S. 1995. Environmental biology, in: Barbera, G.; Inglese, P.; Pimienta-Barrios, E. (Eds.), Agroecology, cultivation and uses of cactus pear. FAO Plant Production and Protection Paper N° 132. 36-48.
55. Nobel, P. S. 2001. Ecophysiology of *Opuntia-ficus-indica,* in: Mondragón-Jacobo, C.; Pérez-González, S. (Eds.), Cactus (*Opuntia* spp.) as forage. FAO Plant Production and Protection Paper. 169: 13-20.
56. Nobel, P. S. 2002. Cactus physiological ecology, emphasizing gas exchange of *Platyopuntia* fruits. Acta Horticulturae. 581: 143-150.
57. Nobel, P. S.; Bobich, E. G. 2002. Environmental Biology, in: Nobel, P. S. (Ed.), Cacti: biology and uses. University of California Press. Berkeley. CA. USA. 57-74.
58. Nobel, P. S.; Hartsock, T. L. 1984. Physiological response of *Opuntia ficus-indica* to growth temperature. Physiologia Plantarum. 60: 90-105.
59. Parish, J.; Felker, P. 1997. Fruit quality and production of cactus pear (*Opuntia* spp.) fruit clones selected for increased frost hardiness. Journal of Arid Environments. 37: 123-143.
60. Pimienta B., E. 1990. El Nopal Tunero. Departamento de Investigación Científica y Superación Académica de la Universidad de Guadalajara. UG. Guadalajara Jalisco. México. 246 p.
61. Pimienta, E. 2002. Preface, in: Mondragón-Jacobo, C.; Pérez-González, S. (Eds.), Cactus (*Opuntia* spp.) as forage. FAO Plant Production and Protection. Paper 169.
62. Powell, A. M.; Weedin, J. F. 2001. Chromosome numbers in Chihuahuan Desert Cactaceae. III. Trans Pecos Texas. American Journal of Botany. 88: 481-485.
63. Pugnaire, F. I.; Haase, P.; Puigdefabregas, J. 1996. Facilitation between higher plant species in a semiarid environment. Ecology. 77: 1420-1426.
64. Russell, C. E.; Felker, P. 1987. The prickly pears (*Opuntia* spp., Cactaceae): a source of human and animal food in semiarid regions. Economic Botany. 41: 433-445.
65. Scalisi, A.; Morandi, B.; Inglese, P.; Lo Bianco, R. 2015. Cladode growth dynamics in *Opuntia ficus-indica* under drought. Environmental Experimental Botany. 122: 158-167.
66. Sinclair, T. R.; Tanner, C. B.; Bennett, J. M. 1984. Water-use efficiency in crop production. Bioscience. 34: 36-40.
67. Suzán-Azpiri, H.; Sosa, V. J. 2006. Comparative performance of the giant cardon cactus (*Pachycereus pringlei*) seedlings under two leguminous nurse plant species. Journal of Arid Environments. 65: 351-362.
68. Ting, I. P. 1985. Crassulacean acid metabolism. Annual Review of Plant Physiology. 36: 595-622.
69. USDA, NRCS. 2012. Available online at http://planthardiness.ars.usda.gov/PHZMWeb/ (accessed: September 2017).

70. Wang, X.; Felker, P.; Paterson, A. 1997. Environmental influences on cactus pear fruit yield, quality and cold hardiness and development of hybrids with improved cold hardiness. Journal of the Professional Association for Cactus Development. 2: 48-59.
71. Weedin, J. F.; Powell, A. M. 1978. Chromosome numbers in Chihuahuan desert Cactaceae. Trans-Pecos Texas. American Journal of Botany. 65: 531-537.
72. Winter, K. 1985. Crassulacean acid metabolism, in: Barber, J.; Baker, N. R. (Eds.), Photosynthetic mechanisms and environment. Elsevier. Amsterdam: 329-387.
73. Winter, K.; Smith, J. A. C. 1996. An introduction to Crassulacean Acid Metabolism. Biochemical Principles and Ecology Diversity, in: Winter, K.; Smith, J.; Andrew, C. (Eds.), Crassulacean Acid Metabolism. Biochemistry, Ecophysiology and Evolution. Springer-Verlag Berlin Heidelberg: 1-13.

Rev. FCA UNCUYO. 2020. 52(1): 391-405. ISSN (en línea) 1853-8665.

Cadmium phytotoxicity: issues, progress, environmental concerns and future perspectives

Fitotoxicidad del cadmio: problemas, avances, preocupaciones ambientales, y perspectivas futuras

Essa Ali [1, 2], Abid Hussain [3], Izhar Ullah [3], Fahad Said Khan [4], Shamaila Kausar [5], Shaikh Abdur Rashid [6], Imran Rabbani [7], Mohammad Imran [8], Kaleem Ullah Kakar [9], Jawad Munawar Shah [10], Ming Cai [1], Lixi Jiang [2], Nazim Hussain [2], Peilong Sun [1*]

Originales: *Recepción:* 02/11/2017 - *Aceptación:* 19/09/2018

Index

1 Zhejiang University of Technology. Department of Food Science and Technology. China. essaali@upr.edu.pk / * sun_pl@zjut.edu.cn
2 Zhejiang University. College of Agriculture and Biotechnology. Zhejiang Key Laboratory of Crop Gene Resources.
3 The University of Poonch. Department of Pharmacy. Rawalakot. AJ&K.
4 The University of Poonch. Department of Eastern Medicine. Rawalakot. AJ&K.
5 University of Central Punjab. Department of Microbiology. Lahore.
6 Gomal University. Faculty of Pharmacy. DI Khan.
7 Kohat University of Science and Technology. Department of Pharmacy. Kohat.
8 Mohi-ud-din Islamic Institute of Pharmaceutical Sciences. Mohi-ud-din Islamic University. Mir Pur.
9 Department of Biotechnology, Faculty of Life Sciences & Informatics, Balochistan University of Information Technology, Engineering and Management Sciences Quetta.
10 Bahauddin Zakarya University Bahadur sub campus Layyah. College of agriculture.

Abstract

Cadmium, a high toxicity element, is a potential threat to plant and human health, and a dangerous pollutant in the environment. Uptake and accumulation by crops represent the main entry pathway for potentially health-threatening toxic metals into human and animal food. Crops and other plants take up Cd from the soil or water and may distribute it in their roots and shoots. Soil and/or water are usually contaminated with Cd through natural sources, industrial effluent, and anthropogenic activities. In this review, the sources of Cd contamination, evaluation of the phytotoxic effects on plants, and mode of action of Cd toxicity, were summarized. Plant defensive strategies upon excess Cd are also considered in this review. Cd-induced effects include oxidative stress, disintegration of the photosynthetic apparatus, reduction in gas exchange parameters, nutrient imbalance, and subcellular organelle degradation. In addition, Cd severely impairs biomolecules such as DNA, protein, and lipids. Although plants are sessile in nature, they are equipped with certain mechanisms to cope with unfavorable conditions. These mechanisms include synthesis of metal-chelating proteins, expression of enzymatic and non-enzymatic antioxidants, organic acids, and plant root–mycorrhiza association. The built-in system of plant tolerance to Cd can be further enhanced by the application of exogenous organic and inorganic metal sources. This review will broaden the knowledge about the Cd accumulation in plants and the responses to metal exposure, as well as our understanding of metal tolerance and overcoming this serious issue for sustainable agriculture and human health worldwide.

Keywords
Cadmium • antioxidant enzymes • phytochelaton • metal transporters

Resumen

El Cadmio, con su alta tasa de toxicidad, constituye una amenaza potencial para la salud humana y las plantas, es un contaminante peligroso en el medio ambiente. La absorción y acumulación en los cultivos representan la principal vía de entrada de metales tóxicos en alimentos para humanos y animales, potencialmente peligrosos para la salud. Los cultivos y otras plantas absorben Cd del suelo o del agua y pueden distribuirlo en sus raíces y brotes. El suelo y/o el agua se contaminan con Cd generalmente a través de fuentes naturales, efluentes industriales y actividades antropogénicas. En esta revisión, se resumieron las fuentes de contaminación de Cd, la evaluación de los efectos fitotóxicos en las plantas y el modo de acción de la toxicidad. Además, las estrategias de las plantas para protegerse del exceso de Cd. Los efectos inducidos por Cd incluyen el estrés oxidativo, la desintegración del aparato fotosintético, la reducción de los parámetros de intercambio de gases, el desequilibrio de nutrientes y la degradación de los orgánulos subcelulares. Además, el Cd deteriora gravemente las biomoléculas como el ADN, las proteínas y los lípidos. Aunque las plantas son de naturaleza sésil, están equipadas con ciertos mecanismos para hacer frente a condiciones desfavorables. Estos mecanismos incluyen la síntesis de proteínas quelantes de metales, la expresión de antioxidantes enzimáticos y no enzimáticos, ácidos orgánicos y la asociación de la raíz de la planta y la micorriza. El sistema incorporado de tolerancia de plantas al Cd puede mejorarse aún más mediante la aplicación de fuentes orgánicas e inorgánicas de metales exógenos. Esta revisión ampliará el conocimiento sobre la acumulación de Cd en plantas y las respuestas a la exposición a metales, así como la comprensión de la tolerancia al metal y la superación de este grave problema para la agricultura sostenible y la salud humana en todo el mundo.

Palabras clave

Cadmio • enzimas antioxidantes • fitoquelaton • transportadores de metales

Abbreviations

Pn: Photosynthetic rate • Tr: Transpiration rate • Gs: Stomatal conductance • SOD: Superoxide dismutase • CAT: Catalase • APX: Ascorbate peroxidase • POD: Peroxidase • MDA: Malondialdehyde • PCs: phytochelatons

Introduction

Heavy metals are major environmental pollutants given their harmful effects on ecological, evolutionary, nutritional, and environmental ins and outs. A metallic element with a relatively high density (greater than 4 g/cm^3 or at least 5 times greater than water) and is toxic even at a low concentrations, can be categorized as a heavy metal (2, 3, 35).

The industrial revolution triggered the regular and uncontrolled release of hazardous materials into the environment as industrial effluents. Therefore, heavy metals, especially Cadmium (Cd), are constantly added into the soil-plant-environment system (36). Over the past decades, Cd has been listed 7th out of 275 compounds in the priority list of hazardous materials (9). Over 2×10^7 acres of farmland in PR China have been contaminated by heavy metals, which is almost one fifth of the total arable farmland area. China suffers a 10,000,000 t loss of crop output per year because of heavy metal pollution (60).

Cd accumulation is an irreversible process; remaining in soil for 15-1100 years (29). In addition, Cd has high plant-soil mobility and easily accumulates in plant tissues. High accumulation of Cd in different tissues of crops, especially edible tissues, reduces growth and quality of crops and poses a danger to the organisms feeding on such crops. Humans are the first victims of Cd toxicity because they are at the top of the food chain (61). A health risk study conducted by Wang (2005), found that the health risk in adults living in Ding Li, Tianjin, China was mainly associated with the intake of Cd through vegetable and fish consumption. Cd causes hepatotoxicity, nephrotoxicity, pulmonotoxicity, neuro-toxicity, bone toxicity, and carcinogenesis in humans. Moreover, Cd is deposited and stored in the human liver ($t_{1/2}$ = 4-19 year) and kidney ($t_{1/2}$ = 6-38 year) (10).

Plants are the main vector of Cd transfer to humans; therefore, extensive studies have been carried out on the effects of Cd on plants including its accumulation and translocation. Though plant responses to Cd stress vary among species and cultivars, the mechanisms of response are almost the same (21).

Effects of Cd on crops include decreased gas exchange (28), photosynthetic pigment degradation (38), deficiency of nutrient elements (19), subcellular changes (58), and modulation of antioxidant enzyme activity (1, 6). Additionally, exposure to Cd results in the inhibition of cell elongation (17), and alterations in root morphological characteristics (28). In brief, Cd contamination in soil and plants has posed a serious issue to sustainable agriculture and human health worldwide (62).

Source of contamination

Cd can be introduced to the environment from different sources ranging from natural to anthropogenic. In China, most areas are contaminated with Cd by mining and smelting operations (25). Water usually contains small amounts of Cd. Seawater has an average Cd concentration of approximately 0.1 mg/L or less, and river water contains dissolved Cd concentrations up to approximately 0.5 mg/L; although higher values have been reported under certain conditions. Atmospheric concentrations of Cd are usually 5 ng/m^3 in rural areas, 5-15 ng/m^3 in urban areas, and up to 60 ng/m^3 in industrial areas (26).

Geologic materials and rock outcropping are the major natural sources of Cd contamination in the environment. According to Climino (1983), 10×10^6 kg of Cd are emitted from Mount Etna every year. Agriculture soil is often contaminated with Cd by the application of different kinds of fertilizers, pesticides, and fungicides. In addition, Cd is introduced to agricultural soil by sewage sludge, animal manure, and limes (63). Nitrate and Phosphate fertilizers are also one of the sources of Cd contamination in the agricultural soil (48). However, , increased Cd accumulation in soil depends on the sources, types, and quantity of the contaminant and the types of agricultural soil.

Heavy metals, particularly Cd, are constantly added to the environment because of industrial revolution and uncontroled release of effluent. These industrial activities include mining, transport of ores, smelting and metal finishing, and recycling of metals. Smelting and castings emit Cd in vapor form that combines with water and gets spread in the environment. In addition, coal-burning power plants, petroleum combustion, nuclear power stations, and high tension lines contribute Cd to the environment (57). Cd is also released as a by-product of Zn, and occasionally Pb, refining (35).

Effects of cadmium toxicity in higher plants

Cd is soluble in water and is highly mobile. Cd-induced toxicity to plants results in physiological to molecular and biochemical changes. Generally, Cd in plants causes leaf rolling and chlorosis and reduces growth of both roots and stems. Some of the effects are described in this study.

Effect of Cd toxicity on Gas exchange in plants

Cd severely decreases gas exchange parameters including photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (g_s). Under severe Cd stress, plants adapt by closing stomata and reducing reduce the uptake of Cd to the upper parts of the plants. Stomatal movements are not directly affected by Cd, but rather due to the strong interference of Cd with K^+, Ca^{+2} and abscisic acid in the guard cells (12). Stomatal closure is followed by a subsequent decrease in Tr and Pn. This phenomenon explains the reduced growth of plants under stress. However, tolerant plants use other strategies to cope with high Cd instead of stomatal closure and reduced Tr.

Effect of Cd toxicity on photosynthetic apparatus and pigments

In the photosynthetic system, photosynthetic pigments are considered indicators of damage induced by environmental stressors (39). In *Brassica napus*, Cd reduced total chlorophyll content and carotenoid content, while increasing non-photochemical quenching (5). The decrease in chlorophyll content is primarily caused by the destruction of chloroplast structures induced by Cd, as well as further inhibition of chlorophyll synthesis and increased degradation (37). Cd also damages the light harvesting complex II (32), and photosystems II and I (51).

Deficiency of nutrient elements under Cd stress

Plants exposed to Cd stress showed disturbances in their macro- and micronutrients homeostasis (46), which indirectly affects the processes where these compounds are involved. Cd stress decreases the absorption of essential nutrient elements such as Ca, Mg, Zn, and Fe (40). As Cd is a non-essential element for plants; it can be transported via other metal transporters such as Ca, Mg, Zn, and Fe. Therefore, as Cd competes with these elements, in excess Cd, the absorption of these nutrients is reduced; causing deficiency of essential elements. In addition, the inhibition of root Fe (III) reductase induced by Cd results in Fe (II) deficiency seriously affecting photosynthesis (4). Finally, reduced Tr under Cd stress could also be a cause of nutrient deficiency in plants given that transpiration is involved in the movement of essential elements to the upper parts of the plant.

Modulation of antioxidant enzymes under Cd stress

Plant cells are equipped with enzymatic machinery (SOD, POD, APX, CAT, GPX, and GR) that actively participate in stress conditions. SOD produces hydrogen peroxide (H_2O_2) from reactive oxygen species (ROS) generated during oxidative stress. H_2O_2 is reduced to water and oxygen by CAT and GPX (in the cytoplasm and other cellular compartments) or APX (in the ascorbate-glutathione cycle) (8).

Cd has inhibitory, as well stimulatory, effects on the activity of these antioxidant enzymes. In *B. napus* leaves, SOD, POD, APX, GR, and GPX showed increased activity, whereas CAT activity decreased (5). In *Helianthus annus* leaves, Cd decreased the activity of superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and dehydroascorbate reductase, whereas lipoxygenase activity and MDA content were increased (20). In *Phaseolus vulgaris* roots and leaves, Cd enhanced GPX and APX activity and lipid peroxidation (15).

Varying responses to Cd-induced oxidative stress are probably related to levels of Cd supplied, duration of treatment, and crop species (44). Modulation of enzyme activities is one of the strategies used by the plants to cope with unfavorable conditions.

Harmful effects of Cd on subcellular organelles

Once Cd enters the cell, it deleteriously affects biomolecules and subcellular organelles. For instance, Cd damaged nucleoli of cells in the root tip of *Allium cepa* (37). Cd also altered the synthesis of RNA and inhibited ribonuclease activity in rice (50). In addition, Cd degrades chloroplasts and mitochondria, compromises the integrity of the plasma membrane (5), and increases the number and size of plastoglobuli (23). The increase in number and size of plastoglobuli under Cd stress is responsible for the synthesis and recycling of lipophilic compounds produced during oxidative metabolism (41). High concentrations of Cd also cause structural changes in the chloroplast through the decrease in photosynthetic activity (14), as previously mentioned.

Mechanism of Cd phytotoxicity

Oxidative stress is a disturbance of the cellular redox balance and can lead to disruption of cellular components including proteins, DNA, chloroplast, mitochondria, and cell membrane (figure 1) (56).

Cd is a bivalent heavy metal unable to directly generate free radicals through Fenton and/or Haber Weiss reactions in biological systems under physiological conditions. However, the production of ROS after Cd exposure has been reported in multiple studies (42, 64). Cd indirectly produces cellular ROS by increasing the free Fe-concentration, possibly via replacement in various proteins (18). Free redox-active metals directly enhance the production of $^{\bullet}OH$ (hydroxyl) radicals through the Fenton reaction. Reduction of the oxidized metal ion can be achieved by the Haber-Weiss reaction with superoxide radicals ($O_2^{\bullet-}$) as a substrate.

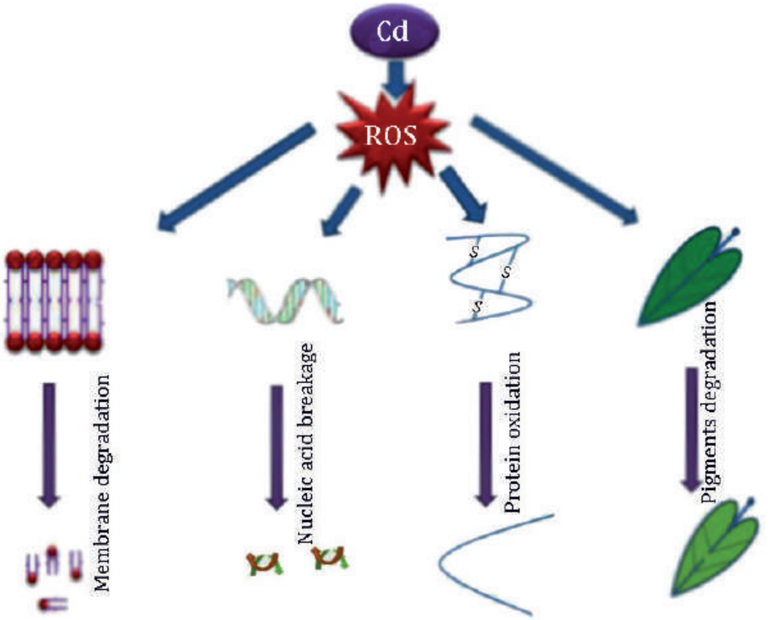


Figure 1. Schematic representation of damage caused by cadmium in plants.

Figura 1. Representación esquemática del daño causado por el cadmio en las plantas.

Other reducing agents, such as ascorbate, can catalyze this reaction. These ROS are responsible for most of the oxidative damage in biological systems. Hydroxyl radicals produced in response to Cd stress can mutate or degrade nucleic acid by adding or removing H^+ from DNA bases or the sugar-phosphate backbone (45). These ROS are responsible for 10^4-10^5 DNA base modifications per cell per day (figure 1, page 396) (7).

Apart from nucleic acid, Cd is believed to oxidize protein as well, , given that most enzymes require a metal as a cofactor for their activities. These cofactors are replaced by Cd ions under Cd stress inhibiting enzymatic activity. These modifications correspond to site-specific processes; with amino acid residues at metal binding sites being specific targets. Therefore, histidine, arginine, lysine, proline, methionine, and cysteine residues are the most common sites of oxidation in proteins. A major consequence of oxygen free radical damage to proteins is making them targets for degradation by proteases (figure 1, page 396) (47).

In addition, ROS produced during Cd stress can compromise the integrity of the plasma membrane by peroxidation of membrane lipids, which can be demonstrated by the increase in MDA content, , being MDA the byproduct of peroxidation of membrane lipids (figure 1, page 396) (49).

In line with the aforementioned, the chloroplast is once more, the candidate target for ROS accumulation in the cell. The chloroplast membrane is rich in polyunsaturated fatty acids such as linoleic and linolenic acids. These fatty acids are very susceptible to oxidation by ROS. After chloroplast degradation, a subsequent obvious decrease in photosynthetic pigments can be observed (figure 1, page 396).

Detoxification mechanism

Reducing Cd absorption from soil

The uptake of hazardous materials can be restricted in plants by the following methods.

The symbiotic relationship between the roots of higher plants and some fungi reduce metal uptake by roots (figure 2, page 398). These fungi secrete metal chelators that bind Cd; thus, making Cd unavailable for the plant (27). Sousa (2012), inoculated *Pinus pinaster* seedlings with *Suillus bovinus*, resulting in seedlings with higher growth parameters, increased Cd tolerance, and low Cd accumulation in the upper parts.

The soil provides a good habitat for fungi and bacteria. Different types of bacterial colonies that chelate metals (especially Cd) are present in the soil (figure 2, page 398). Application of *Pseudomonas aeruginosa* to black gram (*Vigna mungo*) seeds, pumpkin, and mustard seedlings reduced Cd accumulation in the upper parts and enhanced growth. In another study, tomato seedlings were inoculated with *Methylobacterium oryzae* or *Burkholderia sp*., and they restricted the bioavailability of Cd to plants by secreting metal chelators that bind Cd (52). Plant roots constantly secret high and low molecular weight compounds known as root exudates. These exudates, including organic acids, sugars, and polysaccharides, are believed to protect the plants from the harmful effects of Cd and other heavy metals by binding Cd and reducing its bioavailability (figure 2, page 398) (11). These exudates also change the pH of the rhizosphere, inhibiting the uptake of Cd to the root system (13).

The cell wall is composed of suberin and pectin and acts as the first check post for Cd entry; thus, reducing its transport across the cell. Pectin usually binds bivalent ions such as Cd; thus, inhibiting its entry into the cytosol of the cell (figure 2) (33).

The root epidermis provides a reservoir for metal precipitates. Cd is usually restricted as Cd phosphate (Cd-P) precipitate in the root epidermal wall in hyper-accumulator plants (figure 2) (34). Having all these examples in mind, one can conclude that plants have the potential to reduce the bioavailability of Cd by structural modifications and secretions of certain metabolites and defense chemicals.

In brief, plant roots reduce the uptake of Cd by five means:

a) Mycorrhizal association between fungi and roots of higher plants are believed to restrict Cd uptake by the roots. These fungi release metal chelators that form a complex with Cd that cannot be absorbed by plant roots.

b) Soil bacteria, such as *Pseudomonas aeruginosa,* also release some chemicals that bind Cd and inhibit its uptake by roots.

c) Roots release organic acid exudates that bind Cd and inhibit its entry into the root cells.

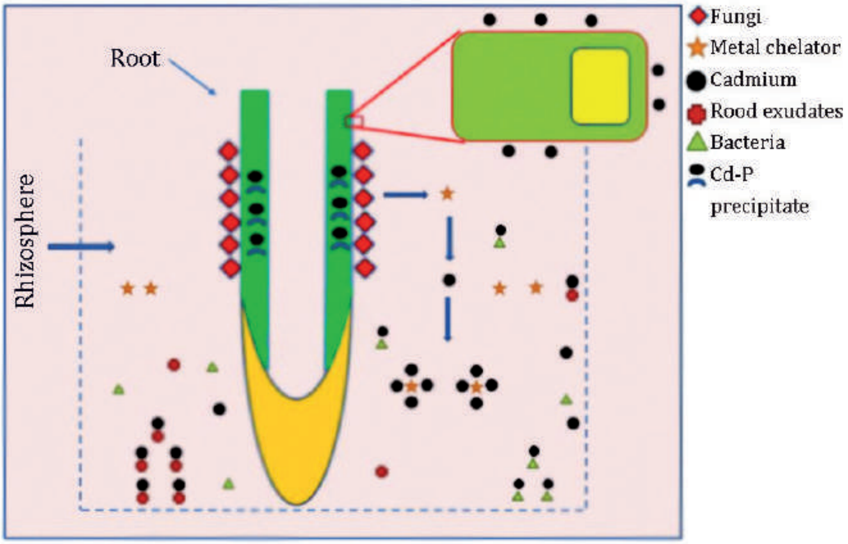


Figure 2. Schematic representation of processes involved in reducing Cd uptake in plant roots.

Figura 2. Representación esquemática de los procesos implicados en la reducción de la absorción de Cd en las raíces de las plantas.

d) d) Suberin and pectin from root cell walls, bind divalent elements such as Cd. This representation is given in the top right portion of the figure.

e) Root epidermis cell wall precipitates Cd in the form of Cd-P and reduces its entry into the cytosol.

Regulation of metal influx to the cytosol

Once Cd breaches the cell wall, it faces the plant cell membrane. Cell membranes are provided with different kinds of metal transporter proteins whose expression are tightly regulated and depend on the quantity and type of metals. These transporters are mostly metal-specific.

To date, a specific Cd transporter protein has not been found in plants. Cd chemically resembles Zn; thus, Cd is believed to cross the cell membrane via the ZIP transporter family (ZRT-IRT like protein; zinc-regulated transporter, iron-regulated transporter Protein) (22, 31, 43).

The production of ZIP transporters is inhibited at the transcriptional or posttranscriptional level to inhibit the influx of Cd into the cytosol (figure 3).

Metal chelation

The entry of Cd to the cytosol triggers undesired interactions with biomolecules including DNA and chloroplast among others. Therefore, Cd needs to be chelated in order to inhibit harmful effects to the biomolecules. As previously mentioned, to sequester Cd and/or other heavy metals, plant produce several kinds of metal chelators. These metal chelators inhibit the interaction of Cd with the biomolecules and restrict it to a site, such as the vacuole. These metal chelators are often oligopeptides, amino acids (cystin), organic acids (malic acid), or proteins.

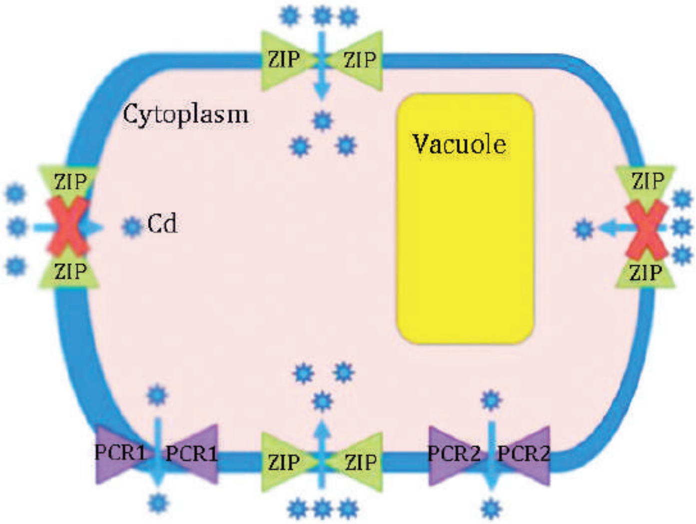


Figure 3. The molecular mechanism of metal homeostasis.

Figura 3. El mecanismo molecular de la homeostasis del metal.

Nicotianamine (NA) and PCs are examples of compounds that form complexes with Cd. Binding strategies are almost the same for most of the chelators, but the transportation sites could be different. Likely, the NA-Cd complex can be transported through the cell membrane by YSL proteins or to the vacuole by ZIF1 proteins (24). The PC-Cd complex can be transported to the vacuole by the two ABCC-type transporters ABCC1 and ABCC2 in *A. thaliana* (figure 4) (54).

Enhancement of Cd efflux

In addition to metal chelation, another strategy used by metal hyper-accumulators and non-hyperaccumulators is metal efflux through the cell membrane. The direction of metal efflux in non-hyperaccumulator plants is towards the soil. By contrast, in hyper-accumulators Cd is loaded into the xylem and transported towards the shoot.

Another way to release these metals from the cells, is by carrying them out via other metal transporters.

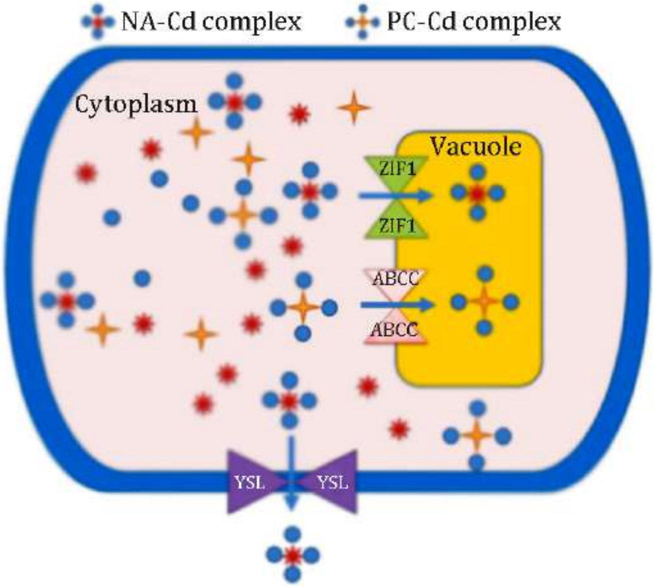


Figure 4. A schematic representation of processes involved in chelation and sequestration of Cd.

Figura 4. Una representación esquemática de los procesos implicados en la quelación y secuestro de Cd.

In *A. thaliana,* two transporters (PCR-1 and PCR-2) are used for the efflux of Cd from the cell (figure 3, page 399) (53). PCR-1 and PCR-2 are, actually, Zn transporters.

Cd sequestration and distribution

The epidermal cell wall and vacuoles are sites of Cd sequestration in case plants are unable to restrict influx or increase efflux of the metal. These organelles are the alternative sites for Cd storage , preventing excess cytoplasmic Cd concentration. In *N. caerulescens*, the Cd-hyper-accumulating ecotype Ganges can significantly store more Cd in the cell walls of epidermal cells than the low Cd-accumulating ecotype Prayon (figure 2, page 398) (30). Sequestration is important to prevent the transport of Cd to the photosynthetic organelles where it can cause serious damage.

Summary and future perspectives

In summary, Cd is an immense threat not only for crop growth and yield, but for humans as well. Cd induces morphological, physiological, and biochemical responses in plants. Reduced growth, organelle dysfunction, inhibition of photosynthesis, deregulation of membrane metal transporters, modulation of metabolic pathways, and distorted gene expression are some of the Cd-induced impairments.

However, plants launch a range of defensive mechanisms to cope with the adverse effects of Cd including reduced uptake from the soil, binding of the absorbed Cd to the epidermis of cell walls, sequestration by the vacuole, and detoxification by metal chelators (organic acids, phytochelatins, and metallothioneins).

In the past few decades, tremendous progress has been reached regarding the molecular mechanisms of plant tolerance to toxic non-essential metals such as Cd. Some literature on the entrance pathway of Cd is also available.

However, a detailed and quantitative understanding of Cd accumulation in plants is lacking. Moreover, finding the associated genes is also important because low Cd content of edible plant parts might be one important target for future crop breeding programs.

Towards this end, breeding and selection of plants showing reduced ability to accumulate Cd in the cells and tissues, and/or its efficient binding, complexation, and compartmentation, along with strategies like seed and foliar application of osmo-protectants, mineral nutrients, and plant-growth regulators, are among the important strategies for mitigating Cd toxicity in plants in the future.

References

1. Ahmad, P.; Nabi, G.; Ashraf, M. 2011. Cadmium-induced oxidative damage in mustard (*Brassica juncea* L.) Czern & Coss.] plants can be alleviated by salicylic acid. South African Journal of Botany. 77: 36-44.

2. Alcalá Jáuregui, J.; Rodríguez Ortiz, J. C.; Hernández Montoya, A.; Filippini, M. F.; Martinez Carretero, E.; Díaz Flores, P. E.; Rojas Velázquez, A. N.; Rodríguez-Fuentes, H.; Beltrán Morales, F. A. 2018. Heavy metals in atmospheric dust deposited in leaves of *Acacia farnesiana (Fabaceae)* and *Prosopis laevigata (Fabaceae)*. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(2): 173-185.

3. Alcalá Jáuregui , J.; Rodríguez Ortíz, J. C.; Hernández Montoya, A.; Filippini, M. F.; Martínez Carretero, E.; Diaz Flores, P. E. 2018. Capacity of two vegetative species of heavy metal accumulation. Revista de la Facultad de Ciencias Agrarias. Universidad Nacional de Cuyo. Mendoza. Argentina. 50(1): 123-139.
4. Alcantara, E.; Romera, F. J.; Canete, M.; De La Guardia, M. D. 1994. Effects of heavy metals on both induction and function of root Fe (III) reductase in Fe-deficient cucumber (*Cucumis sativus L.)* plants. Journal of Experimental Botany. 45: 1893-1898.
5. Ali, E.; Maodzeka, A.; Hussain, N.; Shamsi, I. H.; Jiang, L. 2015. The alleviation of cadmium toxicity in oilseed rape (*Brassica napus*) by the application of salicylic acid. Plant Growth Regulation. 75: 641-655.
6. Ali, E.; Hussain, N.; Shamsi, I. H.; Jabeen, Z.; Siddiqui, M. H.; Jiang, L. 2018. Role of jasmonic acid in improving tolerance of rapeseed (*Brassica napus* L.) to Cd toxicity. Journal of Zhejiang University Science B. 19: 130-146.
7. Ames, B. A.; Shingenaga, M. K.; Park, E. M. 1991. Oxidative damage and repair: chemical, biological and medical aspects. In: Elmsford (ed) Pergamon Press. New York. 181-187.
8. Asada, K. 1994. Production and action of active oxygen species in photosynthetic tissue. In: Foyer CH, Mullineaux PM (eds) Causes of photo-oxidative stress and amelioration of defense system in plants. CRC Press. Boca Raton. 77-104.
9. ATSDR. 2008. Draft toxicological profile for cadmium. US Department of Health and Human Services. Atlanta.
10. ATSDR. 2012. Toxicological profile for cadmium. US Department of Health and Human Services, Atlanta.
11. Bais, H. P.; Weir, T. L.; Perry, L. G.; Gilroy, S.; Vivanco, J. M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology. 57: 233-266.
12. Barcelo, J.; Poschenrieder, C. 1990 Plant water relations as affected by heavy metal stress: a review. Journal of Plant Nutrition. 13: 1-37.
13. Bertrand, M.; Poirier, I. 2005. Photosynthetic organisms and excess of metals. Photosynthetica. 43: 345-353.
14. Carginale, V.; Sorbo, S.; Capasso, C.; Trinchella, F.; Cafiero, G.; Basile, A. 2004. Accumulation, localization, and toxic effects of cadmium in the liverwort. Protoplasma. 223: 53-61.
15. Chaoui, A.; Mazhoudi, S.; Ghorbal, M. H.; El Ferjani, E. 1997. Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). Plant Science. 127: 139-147.
16. Climino, G.; Ziino, M. 1983. Heavy metal pollution part VII emissions from Etna volcanic. Geophysical Research Letter. 10: 31-38.
17. Di Toppi, L. S.; Gabbrielli, R. 1999. Response to cadmium in higher plants. Environmental and Experimental Botany. 41: 105-130.
18. Dorta, D. J.; Leite, S.; DeMarco, K. C. 2003. A proposed sequence of events for cadmium-induced mitochondrial impairment. Journal of Inorganic Biochemestry. 97: 251-257.
19. Feng, R. W.; Wei, C. Y.; Tu, S. X.; Ding, Y. Z.; Song, Z. G. 2013b. A dual role of Se on Cd toxicity: evidences from the uptake of Cd and some essential elements and the growth responses in paddy rice. Biological Trace Element Research. 151: 113-121.
20. Gallego, S. M.; Benavides, M. P.; Tomaro, M. L. 1996. Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Science. 121: 151-159.
21. Grant, C. A.; Clarke, J. M.; Duguid, S.; Chaney, R. L. 2008. Selection and breeding of plant cultivars to minimize cadmium accumulation. Science of the Total Environment. 390: 301-310.
22. Guerinot, M. L. 2000. The ZIP family of metal transporters. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1465: 190-198.
23. Hakmaoui, A.; Ater, M.; Boka, K.; Baron, M. 2007. Copper and cadmium tolerance, uptake and effect on chloroplast ultrastructure. Studies on *Salix purpurea* and *Phragmites australis*. Zeitschrift für Naturforschung C. 62: 417-426.

24. Haydon, M. J.; Kawachi, M.; Wirtz, M.; Hillmer, S.; Hell, R.; Kramer, U. 2012. Vacuolar nicotian amine has critical and distinct roles under iron deficiency and for zinc sequestration in Arabidopsis. The Plant Cell. 24: 724-737.
25. Herawati, N.; Suzuki, S.; Hayashi, K.; Rivai, I. F.; Koyoma, H. 2000. Cadmium, copper and zinc levels in rice and soil of Japan, Indonesia and China by soil type. Bulletin of Environmental Contamination and Toxicology. 64: 33-39.
26. Jensen, A.; Bro-Rasmussen, F. 1992 Environmental cadmium in Europe. Reviews of Environmental Contamination and Toxicology. 125: 101-181.
27. Jentschke, G.; Godbold, D. 2000. Metal toxicity and ectomycorrhizas. Physiologia Plantaram. 109: 107-116.
28. Jia, Y.; Tang, S. R.; Ju, X. H.; Shu, L. N.; Tu, S. X.; Feng, R. W.; Giusti, L. 2011. Effects of elevated CO_2 levels on root morphological traitsand Cd uptakes of two Lolium species under Cd stress. Journal of Zhejiang University Science B. 12: 313-325.
29. KabataPendias, A.; Pendias, H. 1992. Trace Elements in Soils and Plants. 2nd ed. Baton Rouge: CRC Press. Fl.
30. Kim, D. Y.; Bovet, L.; Maeshima, M.; Martinoia, E.; Lee, Y. 2007. The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant Journal. 50: 207-218.
31. Korshunova, Y. O.; Eide. D.; Gregg, C. W.; Lou Guerinot, M.; Pakrasi, H. B. 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Molecular Biology. 40: 37-44.
32. Krupa, Z. 1988. Cadmium-induced changes in the composition and structure of the light-harvesting complex II in radish cotyledons. Physiologia Plantaram. 73: 518-524.
33. Krzesłowska, M. 2011. The cell wall in plant cell response to trace metals: polysaccharide remodeling and its role in defense strategy. Acta Physiologia Plantarum. 33: 35-51.
34. Kupper, H.; Lombi, E.; Zhao, F. J.; McGrath, S. P. 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta. 212: 75-84.
35. Lenntech Water Treatment and Air Purification. 2004. Water treatment. Lenntech, Rotterdam seweg, Netherlands.
36. Lima, A. I. G.; Pereira, S. I. A.; de Almeida, P.; Figueira, E. M.; Caldeira, G. C. N.; de Matos Caldeira, H. D. Q. 2006. Cadmium detoxification in roots of *Pisum sativum* seedlings: relationship between toxicity levels, thiol pool alterations and growth. Environmental and Experimental Botany. 55: 149-162.
37. Liu, D.; Jiang, W.; Wang, W.; Zhai, L. 1995. Evaluation of metal ion toxicity on root tip cells by the Allium test. Israelian Journal Plant Science. 43: 125-133.
38. López-Millán, A. F.; Sagardoy, R.; Solanas, M.; Abadıa, A.; Abadıa, J. 2009. Cadmium toxicity in tomato (*Lycopersicon esculentum*) plants grown in hydroponics. Environmental and Experimental Botany. 65: 376-385.
39. Maxwell, K.; Johnson, G. N. 2000. Chlorophyll fluorescence-a practical guide. Journal of Experimental Botany. 51: 659-668.
40. Metwally, A.; Finkermeier, I.; Georgi, M.; Dietz, K. J. 2003. Salicylic acid alleviates the cadmium toxicity in barley seedlings. Plant Physiology. 132: 272-281.
41. Olmos, E.; Kiddle, G.; Pellny, T. K.; Kumar, S.; Foyer, C. H. 2006. Modulation of plant morphology, root architecture, and cell structure by low vitamin C in *Arabidopsis thaliana*. Journal of Experimental Botany. 57: 1645-1655.
42. Pathak, N.; Khandelwal, S. 2006. Oxidative stress and apoptotic changes in murine splenocytes exposed to cadmium. Toxicology. 220: 26-36.
43. Pence, N. S.; Larsen, P. B.; Ebbs, S. D.; Letham, D. L. D.; Lasat, M. M.; Garvin, D. F.; Eide, D.; Kochian, L. V. 2000. The molecular physiology of heavy metal transport in the Zn/ Cd hyperaccumulator *Thlaspi caerulescens*. Proceedings of the National Academy of Sciences. 97: 4956.

44. Pichorner, H.; Korori, S. A. A.; Thur, A.; Ebermann, R. 1993. The two and the four electron transfer to molecular oxygen mediated by plant peroxidase in the presence of thiols. In: Welinder KG, Rasmussen SK, Penel C, Greppin H. (Eds.), Plant Peroxidases: Biochemistry and Physiology. University of Geneva. 131-136.
45. Pryor, W. A. 1988. Why is the hydroxyl radical the only radical that commonly adds to DNA? hypothesis: it is a rare combination of high electrophilicity, high thermo chemical reactivity, and a mode of production that occurs near DNA. Free Radical Biology and Medicine. 4: 219-223.
46. Ramos, I.; Esteban, E.; Lucena, J. J.; Garate, A. 2002. Cadmium uptake and subcellular distribution in plants of *Lactuca sativa* sp. Cd, Mn interaction. Plant Science. 162: 761-767.
47. Roseman, I. E.; Levine, R. L. 1987. Purification of a protease from *Ochelichia coli* with specificity for oxidized glutamine synthetase. Journal of biological chemistry. 262: 2101-2110.
48. Ross, S. M. 1994. Toxic metals in soil-plant systems. Wiley. Chichester. p. 469.
49. Scholz, R. W.; Graham, K. S.; Wynn, M. K. 1990. Interaction of glutathione and a-tocopherol in the inhibition of lipid peroxidation of rat liver microsomes. In: Eddy CC, Hamilton GA, Madyastha KM (eds) Biological oxidation systems. Academic Press. San Diego. 841-867.
50. Shah, K.; Dubey, R. S. 1998. Effect of cadmium on proline accumulation and ribonuclease activity in rice seedlings: role of proline as a possible enzyme protectant. Biologia Plantaram. 40: 121-130.
51. Siedlecka, A.; Krupa, Z. 1996. Interaction between cadmium and iron and its effects on photosynthetic capacity of primary leaves of *Phaseolus vulgaris*. Plant Physiology and Biochemistry. 34: 833-841.
52. Sinha, S.; Mukherjee, S. K. 2008. Cadmium-induced siderophore production by a high Cd-resistant bacterial strain relieved Cd toxicity in plants through root colonization. Current Microbiology. 56: 55-60.
53. Song, W. Y.; Martinoia, E.; Lee, J.; Kim, D.; Kim, D. Y.; Vogt, E.; Shim, D.; Choi, K. S.; Hwang, I.; Lee, Y. 2004. A novel family of cys-rich membrane proteins mediates cadmium resistance in Arabidopsis. Plant Physiology. 135: 1027.
54. Song, W. Y.; Park, J.; Mendoza-Cózatl, D. G.; Suter-Grotemeyer, M.; Shim, D.; Ho¨rtensteiner, S.; Geisler, M.; Weder, B.; Rea, P. A.; Rentsch, D. 2010. Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proceedings of the National Academy of Sciences. 107: 21187-21192.
55. Sousa, N. R.; Ramos, M. A.; Marques, A. P. G. C.; Castro, P. M. L. 2012. The effect of ectomycorrhizal fungi forming symbiosis with *Pinus pinaster* seedlings exposed to cadmium. Science of Total Environment. 414: 63-67.
56. Thévenod, F. 2009. Cadmium and cellular signaling cascades: to be or not to be? Toxicology and Applied Pharmacology. 238: 221-239.
57. Verkleji, J. A. S. 1993. The effects of heavy metals stress on higher plants and their use as bio monitors. In: Markert B (ed) Plant as bio-indicators: indicators of heavy metals in the terrestrial environment. VCH. New York. 415-424.
58. Wang, F.; Chen, F.; Cai, Y.; Zhang, G.; Wu, F. 2011. Modulation of exogenous glutathione in ultrastructure and photosynthetic performance against Cd stress in the two barley genotypes differing in Cd tolerance. Biological Trace Element Research. 144: 1275-1288.
59. Wang, X. L.; Sato, T.; Xing, B. S.; Tao, S. 2005. Health risks of heavy metals to the general public in Tianjin, China via consumption of vegetables and fish. Science of Total Environment. 350: 28-37.
60. Wang, X. L.; Ma, X. Q. 2008. Advance in the research of phytoremediation in heavy metal contaminated soils. Subtrop. Agriculture Research. 4: 44-49.
61. Wenzel, W. W.; Unterbrunner, R.; Sommer, P.; Sacco, P. 2003. Chelate-assisted phytoextraction using canola (*Brassica napus* L.) in outdoors pot and lysimeter experiments. Plant Soil. 249: 83-96.
62. Wu, F. B.; Dong, J.; Qian, Q. Q.; Zhang, G. P. 2005a. Subcellular distribution and chemical form of Cd and Cd-Zn interaction in different barley genotypes. Chemosphere. 60: 1437-1446.

63. Yanqun, Z.; Yuan, L.; Jianjun, C.; Haiyan, C.; Li, Q.; Schratz, C. 2005. Hyper accumulation of Pb, Zn and Cd in herbaceous grown on lead-zinc mining area in Yunnan, China. Environ International. 31: 755-762.
64. Zhou, Y. J.; Zhang, S. P.; Liu, C. W. 2009. The protection of selenium on ROS-mediated apoptosis by mitochondria dysfunction in cadmium-induced LLC-PK1 cells. Toxicology in Vitro. 23: 288-294.

Acknowledgements

Key Project of Research and Development Plan of Zhejiang (2018C02SA780973), Natural 323 Science Foundation of China (31401506).