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Phone: (571) 316 5355 / 316 5000 ext. 10265

Fax: 316 5176

E-mail: agrocol_fabog@unal.edu.co

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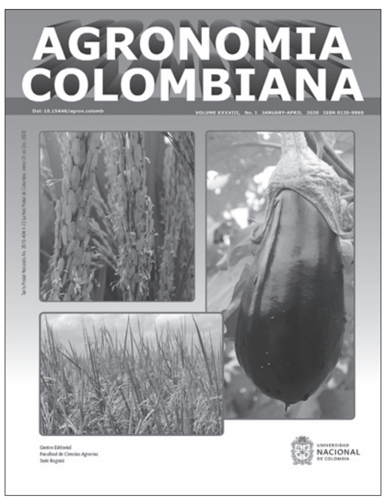

Our cover:

Selection of sowing date and biofertilization as alternatives to improve the yield and profitability of the F68 rice variety. Article on pages: 61-72

Characterization of eggplant producers in the Caribbean region of Colombia: socio-economic aspects and local production technology. Article on pages: 120-132

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AGRONOMIA COLOMBIANA

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TABLE OF CONTENTS

SCIENTIFIC NOTE / NOTA CIENTÍFICA

APPENDIX / ANEXOS

Path correlation and Bayesian analysis on popping expansion components in popcorn hybrids

Correlación de ruta y análisis Bayesiano sobre componentes de expansión en híbridos de maíz pipoca

Gustavo Hugo Ferreira De Oliveira[1]*, Gabriela Pelegrini[2], Tâmara Rebecca Albuquerque De Oliveira[3], Maisa Nascimento Carvalho[1], and Gustavo Vitti Môro[2]

ABSTRACT

Knowing the cause and effect among two or more traits can help to increase the selection accuracy of superior genotypes. The main objective of this study was to evaluate the cause and effect relationship between expansion volume and kernel size in popcorn hybrids using path analysis and Bayesian network. A total of 41 popcorn hybrids were evaluated through a randomized complete block design (RCBD) with two replicates in the city of Jaboticabal, Brazil. The assessed traits were grain length (GL), grain thickness (GT), grain width (GW), caryopsis roundness index (CRI), mass of 50 grains (MG), and expansion volume (EV). Measurements were performed on individual grains, using three 50-grain samples from each plot. Pearson's correlation coefficient, path analysis and Bayesian network were estimated. A negative correlation was detected among EV and the traits, except for GT. Path analysis indicated that MG has a direct and positive effect on EV and the negative correlation observed is mainly due to the indirect effects by GL and GT. Bayesian networks did not detect a direct association between kernel size and expansion volume while indicating that GT is the only trait that can affect popcorn flake size.

Key words: *Zea mays* L. var. everta, Bayesian networks, direct and indirect effect, correlation.

RESUMEN

El conocimiento sobre la causa y el efecto entre dos o más rasgos puede ayudar a aumentar la precisión de la selección de genotipos superiores. El objetivo principal de este estudio fue evaluar la relación de causa y efecto entre el volumen de expansión y el tamaño del grano en los híbridos de palomitas de maíz utilizando análisis de ruta y red bayesiana. Se evaluó un total de 41 híbridos de palomitas de maíz siguiendo un diseño de bloques completos al azar (DBCA) con dos repeticiones en la ciudad de Jaboticabal, Brasil. Los rasgos evaluados fueron la longitud del grano (LG), el grosor del grano (GG), el ancho del grano (AG), índice de redondez de la cariopsis (IRC), la masa de 50 granos (MG) y el volumen de expansión (VE). Las mediciones se realizaron en granos individuales, utilizando tres muestras de 50 granos de cada parcela. Se estimaron el coeficiente de correlación de Pearson, el análisis de ruta y la red bayesiana. Se detectó una correlación negativa entre VE y los rasgos, a excepción de GT. El análisis de ruta indicó que MG tiene un efecto directo y positivo sobre VE y la correlación negativa observada se debe principalmente a los efectos indirectos de LG y GG Las redes bayesianas no detectaron una asociación directa entre el tamaño del núcleo y el volumen de expansión, mientras que indicaban que GG es el único rasgo que puede afectar el tamaño de las hojuelas de palomitas de maíz.

Palabras clave: *Zea mays* L. var. everta, redes bayesianas, efecto directo e indirecto, correlación.

Introduction

The correlation between traits is of paramount importance for breeding programs, especially for the selection of superior genotypes. Therefore, for traits that are difficult to measure or have low heritability, indirect selection uses an easily measurable trait with moderate to high heritability, thus allowing the breeder to obtain faster progress compared to direct selection using a difficult trait. Cruz *et al*. (2012) state that associations can generate indirect gains when the studied traits show a genetic correlation, especially on truncated selection of low heritability traits, which may increase selection efficiency.

It is known that the popping expansion (PE) can be genetically correlated with other grain characteristics such as kernel size, length, width and mass (Cabral *et al*., 2016; Ribeiro *et al*., 2016). However, these results vary since other authors

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1 Universidade Federal de Sergipe, Nossa Senhora da Glória, Sergipe (Brazil).
2 Universidade Estadual Paulista Júlio de Mesquita Filho, Jaboticabal, São Paulo (Brazil).
3 Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro (Brazil).
* Corresponding author: gustavo.melhorista@gmail.com

report a positive correlation or a lack of linear correlation between the main kernel characteristics of popcorn, as described by Carpentieri-Pípolo *et al*. (2002) and Daros *et al*. (2004). Soylu and Tekkanat (2006) reported a significant and not significant positive correlation between PE and kernel size and PE and mass of 1000 grains, respectively.

In the literature, several studies on the association between traits show that the concomitant use of path analysis and Bayesian networks may generate accurate information for genetic improvement of popcorn (Felipe *et al*., 2015; Amaral *et al*., 2019). Path analysis is the unfolding of the correlation coefficient that informs not only the magnitude of the correlation values but also allows obtaining the cause and effect information of the correlation between two variables and how many other variables may influence this correlation (Cruz *et al*., 2012).

Bayesian networks (BN) are an alternative modeling approach, in which criteria are applied for selection models. In turn, it is important in the graphical representation of the causality relations between the system variables (Marques and Dutra, 2002), revealing information on the relationship of conditional dependence, which is represented by the joint distribution of a set of variables. In addition, it is an intuitive way to understand the direct and indirect relationships between variables (Felipe *et al*., 2015). The Directed Acyclic Graph (DAG) is the graphical representation produced by this probabilistic graphical model that represents a set of random variables and their conditional dependencies, connected directly by arrows (Margaritis, 2003).

The main advantage of the BN is that the modeling involves searching for a structure that is compatible with the joint distribution of the data, and the conditional dependence brought by the BN structure allows generating the most parsimonious representation of the dataset joint distribution (Amaral *et al*., 2019). This feature makes this model interesting for the purpose of predicting and selecting variables for genetic improvement.

It is known that the greatest expansion capacity is a desired trait in the commercial production of popcorn and that this capacity depends on factors, such as grain size, grain flake size and grain mass, among others, as well as chemical properties (Cabral *et al*., 2016). Furthermore, the evaluation and measurement methods are not clearly addressed in the literature. Thus, the objective of this work was to evaluate the cause and effect relationship between expansion capacity, weight, and grain size in popcorn hybrids using path analysis and Bayesian network.

Materials and methods

A total of 185 S_3 inbred lines of popcorn were obtained from nine populations from different origins. The lines were separated by similar agronomic traits and within each population. The seeds of the lines were mixed in the same proportion to build up the population of origin, thus, obtaining nine synthetic populations. In the 2013/2014 crop, the synthetic populations were sown in Jaboticabal, São Paulo, Brazil (2º15'17"S, 48º19'20"W, and 605 m a.s.l.) The climates in both places are classified as tropical Aw according to the Koppen classification, with 60 mm average rainfall in the driest month and average temperature of 18°C in the coldest month of the year. The average temperature in the rainy season is 29°C. The soils are classified as Eutrophic Red Latosol and Dystrophic Red Latosol, respectively (Embrapa, 2006) and crossed in a complete diallel scheme by manual pollination. The progenies (diallel hybrids) were harvested, hand-milled, and part of the seeds was packed in a dry chamber, while another part was sown on the same site in the 2014/2015 crop.

The randomized block experiment consisted of 41 diallel hybrids with two replicates, in plots of two 5 m lines, spaced 0.8 m between rows and 0.2 m between plants. Crop management followed the requirements of the culture (Cruz, 2010). All ears in the plot were manually harvested at physiological maturity. After harvesting, all ears were threshed manually and kept in tagged paper bags in a dry chamber.

From each replicate, three 50-grain samples were prepared and further used to evaluate the traits studied in this work.

The work was divided into two steps. The first step consisted in estimating the traits for each 50-grain samples as follows: grain length (GL), defined as the distance between the tip and the base of the grain; grain width (GW), the distance from end to end of the widest part of kernel; grain thickness (GT), the distance between the two faces of the kernel, as proposed by Pordesimo *et al*. (1990), and caryopsis roundness index (CRI), the relationship between GL, GW and GT using the following equation proposed by Mohsenin (1970).

$$CRI = (GL \times GW \times GT)^{1/3} \qquad (1)$$

The traits GL, GW, and GT were measured with a digital caliper and expressed as millimeters. The second step consisted of estimating the traits for the complete 50-grain sample as follows: mass of 50 grains (MG) (considering a grain moisture content of 13%), measured using a precision scale and expressed as grams and popping expansion (PE), evaluated using a microwave (Brastemp®, 30 L, 0.8 KW/h, 800 W, 127 V, 30 x 53.9 x 42 cm, 16 kg). The grains were placed in paper bags and two repetitions per each genotype were used to subject the samples to the same popping time (2 min and 30 sec). PE was given by the ratio between flake volume (ml) and its sample mass (g) and expressed in ml g^{-1}. The flake volume (VOL) was determined using a graduated 1000 ml cylinder, and homogenized by inverting the cylinder once, whereas flake size (FS) was given by the ratio between VOL and the number of popped kernels in the sample. VOL and FS were used only in the Bayesian network analysis.

The phenotypic (r_f), genotypic (r_g) and environmental (r_a) correlations were estimated using the following expressions:

$$r_f = \frac{Covf(x,y)}{\sqrt{\sigma^2_{fx}.\sigma^2_{fy}}};\ r_g = \frac{Covg(x,y)}{\sqrt{\sigma^2_{gx}.\sigma^2_{gy}}};\ r_a = \frac{Cova(x,y)}{\sqrt{\sigma^2_{ax}.\sigma^2_{ay}}} \quad (2)$$

where: *Covf(x,y)*, *Covg(x,y)*, and *Cova(x,y)* are, respectively, the phenotypic, genotypic and environmental correlations between the x and y variables; r_f, r_g and r_a are the phenotypic, genotypic, and environmental variances, respectively. The significance of coefficient b was evaluated by the F test and the correlation coefficient was analyzed by the t-test.

A multicollinearity test was performed between the variables and, according to this criterion, VOL and FS were removed from the database. Then, the r_f matrix was used for unfolding the correlation coefficient into cause and effect studies of kernel traits on grain expansion capacity determined by the phenotypic path analysis (FPA). The correlation and path analyses were performed using the Genes software (Cruz, 2013).

As a complement, a graphical analysis (Directed Acyclic Graph) was performed using Bayesian networks (BN). The BN is a graphical representation of a probability distribution over a set of variables (Felipe *et al*., 2015). The Directed Acyclic Graph (DAG) represents the BN using nodes connected by arrows, and is used as an output to the modeling approach. In this case, it is used to illustrate the association between traits. This graph characterizes a joint probability of the data, which brings scale benefits due to the factorization (Aliferis *et al*., 2010). In a set of variables {X1, X2, …, Xp} with joint distribution Pr(X1, X2, …, Xp) and a DAG D that is compatible with this joint distribution (Pearl, 2000), the following factorization can be performed:

$$\Pr(X_1, X_2, \ldots, X_p) = \prod_{i=l}^{p} \Pr(X_i|\mathrm{Pa}_i) \quad (3)$$

where: Pa_i are the parents of X_i in D.

The BN analysis involves searching for a structure that is compatible with the joint distribution of the data. The selected structure has already been used as a prediction tool, as described by Felipe *et al.* (2015). In this study, the BN was only used in the context of traits association.

For the present work, the Hill-Climbing algorithm ("search and score" approach) was used to construct the BN from the means of each plot. The model was adjusted using the package "bnlearn" of the R software (Scutari, 2009).

Results and discussion

The majority of phenotypic (r_f), genotypic (r_g) and environmental (r_a) correlation coefficients were significant (Tab. 1). Regarding the genetic correlation, six had a positive and significant r_g (0.59, 0.62, 0.44, 0.84, 0.57, 0.33 to GL x GW, GL x MG, GW x GT, GW x MG, GT x MG and CRI x PE, respectively). Also, five had negative and significant r_g (-0.72, -0.43, -0.49, -0.48, -0.39 to GW x CRI, GW x PE, GT x PE, CRI x MG and MG x PE, respectively). A positive correlation indicates that as one trait increases, the other trait also increases and that selection aiming at gain on one trait may reflect on the other trait (Vencovsky and Barriga, 1992). In addition, five genetic correlations had higher values than the environmental correlation, indicating that the observed linear association between these pairs of traits is little influenced by the environmental factors.

TABLE 1. Phenotypic (r_f), genotypic (r_g) and environmental (r_a) correlations between six grain traits of 41 diallel popcorn hybrids.

Traits		GW	GT	CRI	MG	PE
GL	r_f	0.59**	0.21	0.19	0.58**	-0.19
	r_g	0.59**	0.24	0.13	0.62**	-0.20
	r_a	0.68**	-0.14	0.46	0.10	0.01
GW	r_f		0.43**	-0.63**	0.82**	-0.41**
	r_g		0.44**	-0.72**	0.84**	-0.43**
	r_a		0.06	0.03	0.42**	-0.04
GT	r_f			-0.26	0.56**	-0.48**
	r_g			-0.26	0.57**	-0.49**
	r_a			-0.44**	0.36*	-0.22
CRI	r_f				-0.44**	0.29+
	r_g				-0.48**	0.33*
	r_a				-0.34*	0.07
MG	r_f					-0.38*
	r_g					-0.39**
	r_a					-0.15

*, **: significant at 5 and 1% by t-test; PE: popping expansion; GL: grain length; GW: grain width; GT: grain thickness; CRI: caryopsis roundness index; MG: mass of 50 grains.

On the other hand, of the significant 11 r_g values, six (GL x GW, GW x CRI, GW x PE, GT x PE, CRI x MG and MG x PE) had environmental correlations higher than the respective genetic correlations. According to Vencovsky and Barriga (1992), environmental correlations occur when the same variation of environmental conditions affects two distinct traits, in which negative and positive values indicate that the environment favored one trait to the detriment of another or harmed both due to the same causes of environmental variations.

Several authors point out the importance of the correlations between traits of popcorn hybrids (Soylu and Tekkanat, 2006; Cabral *et al*., 2016) especially regarding the degree of genetic and environmental association between the main commercial attributes of the crop. This is important because genetic correlations are inheritable and, therefore, can be used in breeding programs in the direct or indirect selection of superior genotypes. In addition, information on the correlations between grain traits may increase the accuracy of genotype selection in breeding programs, especially regarding popping expansion.

The highest significant r_f estimates were calculated for GW and MG (0.82) and GW and CRI (-0.63), although the second one is a negative correlation. Among the r_g estimates, the highest was also observed for GW and MG (0.84) and for GW and CRI (-0.72). The highest r_a estimate was obtained between GL and GW (0.68) and between GT and CRI (-0.44) (Tab. 1).

Regarding PE, r_f and r_g were positive and significant for CRI and negative for GL, GW, GT, and MG. A positive significant genotypic correlation (r_g) 0.33 was observed between PE and CRI, and negative but not significant between PE and GL. Non-significant correlations imply no linear correlation but not necessarily lack of association between two traits (Cruz and Regazzi, 1997). The significant genotypic correlations between PE x GT and PE x GW were calculated as -0.49 and -0.43, respectively. The three main grain dimensions suggest that smaller grains have higher popping expansion during popping. It is known that grain shape greatly influences its popping expansion (Lyerly, 1942). Cabral *et al.* (2016) calculated the genetic correlations between these three characteristics and PE and reported negative values for GL and GW, but positive for GT. Lyerly (1942) stated that round grains have higher PE than elongated grains.

The MG trait is related to the overall kernel size of the sample. In this study, the genotypic correlation (r_g) between PE and MG was significant and negative, -0.39. It is noteworthy that MG had positive and significant correlation coefficients with all-grain dimensions (GW, GT, and GL). Carpentieri-Pípolo *et al.* (2002) reported a negative correlation between popping expansion and ear total mass and grain mass per plant, important traits of crop grain yield. Daros *et al.* (2004) reported non-significant values for PE and grain yield. These results confirm the important effect of kernel size on PE. Cabral *et al.* (2016) suggested transforming these measurements into popcorn volume (PV) (Super-trait) ha^{-1} (PV, m^3 ha^{-1}) to reduce the effect of these correlations on the selection of productive parents with popping expansion above the national average.

To split the correlation coefficients in path analysis, a multicollinearity analysis was performed between variables (Cruz *et al*., 2012). The obtained condition number (NC) was 81.41, classified as weak; therefore, path analysis can be performed without statistical problems. Coimbra *et al.* (2005) stated that in the presence of multicollinearity, inconsistent estimates of the regression coefficient could be observed, while the direct effects of the explanatory variables on the response variable would be overestimated, and the irrational values would not explain the studied biological phenomenon.

It was observed that the highest values of positive direct effects on PE were for MG, GL and CRI and negative values for GT and GW (Tab. 2). These effects corroborate the results of r_g and r_f shown in Table 1.

TABLE 2. Estimates of the direct and indirect effects of grain traits on the popping expansion (PE) of popcorn.

Effect		GL	GW	GT	CRI	MG
Direct on PE		0.03	-0.34	-0.39	0.012	0.11
Indirect via GL			0.018	0.006	0.005	0.02
Indirect via GW		-0.20		-0.15	0.219	-0.29
Indirect via GT		-0.08	-0.16		0.105	-0.22
Indirect via CRI		0.002	-0.007	-0.003		-0.005
Indirect via MG		0.65	0.09	0.064	-0.05	
Total		-0.19	-0.41	-0.48	0.29	-0.38
R^2	0.28					
Residual effect	0.84					

PE: popping expansion; GL: grain length; GW: grain width; GT: grain thickness; CRI: caryopsis roundness index; MG: mass of 50 grains.

The highest total effect on PE (-0.47) was observed for GT. However, of that value, -0.15 is represented by GW indirectly. The second-largest total effect (-0.41) was observed for GW while GT contributed with -0.16, the largest share.

These results confirm that PE is strongly influenced by kernel size and that increasing kernel size may negatively affect PE. The total effect of MG on the PE was -0.38 and the direct positive effect was 0.11. In this case, the path analysis indicates a rather pronounced complex relationship between MG and PE, with the negative correlation between MG and PE due to the indirect effects of kernel size, especially GW and GT. However, when the three dimensions (GL, GW, and GT) are transformed into CRI, a positive total and direct effect on PE is observed. These results suggest that the CRI trait should be used for indirect selection aiming at genetic gains in EC. The residual effect was 0.84, indicating that a correlation in path analysis, to be statistically and genetically relevant, would need to be greater than the residual value of 0.84 (Souza, 2013).

The associations between the evaluated traits using BN can be visualized using Directed Acyclic Graph (DAG) (Fig. 1). In this study, BN was used only to visualize the relationship between the traits of popcorn, in order to assess the dependence relationship of the studied variables.

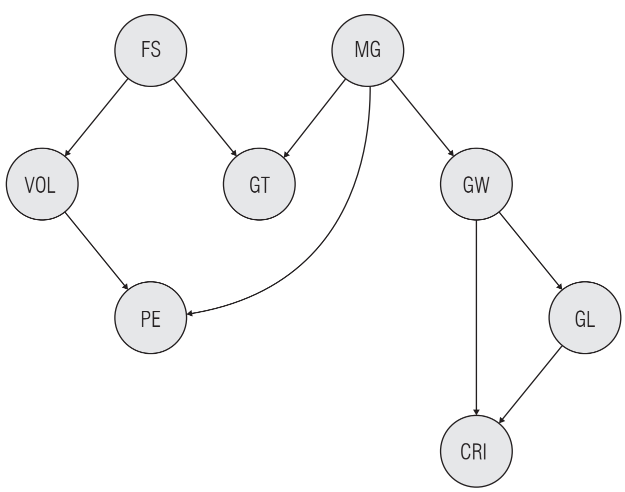

FIGURE 1. Bayesian network for the kernel traits of popcorn: grain width (GW), grain length (GL), caryopsis roundness index (CRI), flake volume (VOL), flake size (FS), grain thickness (GT), mass of 50 grains (MG), and popping expansion (PE).

In the Bayesian network, the associations are arranged hierarchically, so it becomes easy to visualize the importance in terms of prediction and conditional dependence of the direct association between the evaluated traits (Felipe *et al.*, 2015). Thus, DAG reveals that only MG and VOL are directly related to PE, indicating dependence on the mass of 50 grains and the popped volume of the popcorn. It is known that this relationship exists since PE is determined by the relationship between these two traits.

According to Yu *et al.* (2004), Bayesian networks can be used to demonstrate biological phenomena in experimental data. Thus, grain dimensions such as width, length, thickness, and diameter are not directly related to PE and do not need to be used in prediction models (Fig. 2). It is noteworthy that among the dimensions measured in the grain, only GT can influence flake size (FS). All the above considering that flake volume depends exclusively on FS and that, based on these results, increasing kernel thickness may lead to genotypes with low EC values.

DAG shows no direct or indirect relationship of GL and CRI with PE. It is also observed that GT and GW are directly associated with MG and grain size and can indirectly influence the PE. These results corroborate the path analysis (Tab. 2) and those presented by Cabral *et al.* (2016) using correlation and path analysis.

Although the analyses had few divergences, the concomitant use of these strategies can increase the selection efficiency on traits aiming at increasing PE in popcorn breeding programs.

Conclusions

There is a significant negative genetic correlation between the popping expansion and grain width and thickness. The correlations between the mass of 50 grains with the popping expansion are due to the indirect effects of grain width and thickness.

Grain thickness, width, length and diameter are not directly associated with the popping expansion according to BN.

We suggest using the Bayesian network, together with path analysis to aid in the selection and evaluation of traits in popcorn breeding programs.

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Fruit quality attributes of ten Colombian blackberry (*Rubus glaucus* Benth.) genotypes

Atributos de calidad de frutos en diez genotipos de mora colombiana (*Rubus glaucus* Benth.)

Erika Sánchez-Betancourt[1], María Cristina García-Muñoz[1]*, Jorge Argüelles-Cárdenas[1], Viviana Franco-Flórez[1], and Víctor Núñez-Zarantes[1]

ABSTRACT

Colombia requires blackberry genotypes with remarkable traits that improve the competitiveness of this product in the country. The objective of this work was to evaluate the characteristics of the quality attributes in fruits of ten Colombian blackberry genotypes in order to assess their potential in the berry markets but also as a genetic source to be included in breeding programs. The weight of fruits, the equatorial and longitudinal diameters, volume, firmness, color, juice content, flesh content, seed/fruit weight percentage, soluble solids content, acidity, pH and maturity index were evaluated in fruits collected during three different harvest periods in the province of Cundinamarca. Thirty fruits for every genotype and period were harvested and analyzed. These 10 evaluated genotypes showed important differences that allowed separating them into five different groups; however, genotypes 8, 4 and 6 showed the best results regarding size, color, juice content, total soluble solids content, maturity index, firmness and low acidity. These features make them good candidates for incorporating them into breeding programs. Nonetheless, their soluble solids content was considerably lower than values reported for genotypes Prime Ark, Natchez, Ouachita, and Osage that are recognized for these remarkable traits.

Key words: soluble solids content, acidity, firmness, color, seed/fruit weight percentage, juice content.

RESUMEN

Colombia requiere identificar materiales de mora con características sobresalientes que permitan impulsar el crecimiento de esta cadena de producción en el país. Por esta razón el objetivo de ese trabajo fue evaluar los atributos de calidad de fruta de 10 genotipos de mora para determinar su potencial en los mercados de bayas, y como fuente genética para incluirlos en los programas de mejoramiento. El peso, diámetro ecuatorial y longitudinal volumen, firmeza, color, contenido de jugo, contenido de pulpa y porcentaje de peso de semilla/peso de fruta, contenido de sólidos solubles, acidez, pH y el índice de madurez fueron evaluados en frutas recolectadas en tres períodos de cosecha diferentes, en el departamento de Cundinamarca. Se evaluaron y analizaron 30 frutos por genotipo y periodo. Los 10 genotipos mostraron diferencias importantes que permitieron separarlos en cinco grupos diferentes; sin embargo, los genotipos 8, 4 y 6 mostraron los mejores resultados en cuanto a tamaño, color, contenido de jugo, contenido total de sólidos solubles, índice de madurez, firmeza y baja acidez. Estas características los hacen buenos candidatos para ser incorporados en los programas de propagación. Finalmente, el contenido de sólidos solubles fue considerablemente más bajo que los valores reportados para los genotipos Prime Ark, Natchez, Ouachita y Osage, los cuales son reconocidos por estas características sobresalientes.

Palabras clave: sólidos solubles, acidez, firmeza, color, porcentaje de peso de semilla/peso de fruta, contenido de jugo.

Introduction

The blackberry (*Rubus glaucus* Benth.) belongs to the Rosaceae family. It is a perennial shrub with semi-erect stems and is cultivated between 1,800 and 3,200 m a.s.l. (Afanador-Kafuri *et al*., 2015). Blackberry has become one of the most important berry fruits in markets of the United States and the European Union, along with strawberries, raspberries, and blueberries (Clark and Finn, 2011). Blackberry shipments to final markets in the U.S. increased more than 530% in the first decade of this century. In addition, production in the United Kingdom and Mexico for fresh markets and in China for processed product markets has contributed to the growth of blackberry marketing worldwide. Among the reasons that this market has increased are the development of genotypes with longer shelf life, better handling facilities, enhanced marketing and promotion, and growing interest of consumers in functional products (Lewers *et al*., 2010; Ali *et al*., 2011; García and Vaillant, 2014; García *et al*., 2014) as well as the fact that these berries are more profitable than other similar raspberries (Clark and Finn, 2011). Given the importance of this fruit in the

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[1] Corporación Colombiana de Investigación Agropecuaria - Agrosavia, Centro de investigación Tibaitatá, Mosquera, Cundinamarca (Colombia).
[*] Corresponding author: mcgarcia@agrosavia.co

world market, breeding programs were developed looking for an improvement of major traits. At the beginning of blackberry breeding programs, fruit size that was related to yield was the primary objective. However, plant productivity, stem architecture, diseases, and insect resistance as well as plant adaptations were also considered. However, fruit quality is currently the driving force in breeding programs (Brock, 2017). Different traits relating to fruit quality that satisfy the preferences of the consumer as well as different shipment characteristics that facilitate commercialization of long-distance fresh markets have been considered. Furthermore, for the processed product market, fruits are sold fresh and frozen to be used in jams, juice, ice cream and yogurt flavoring (Morales *et al*., 1996). Also, health properties associated with their consumption, such as their antioxidant capacity and other functional properties, have aroused growing interest among scientists (Seeram *et al*., 2006; Espin *et al*., 2007; García and Vaillant, 2014).

There are currently more than 400 genotypes worldwide due to important advances reached in breeding programs in the United States, New Zealand, Mexico, and Brazil. Genotypes like Brazo and Tupy became successful in Brazil and Mexico. The U.S. has developed the largest number of genotypes and hybrids with special characteristics, such as 'Navaho', 'Ouachita', 'Boysen' and 'Chester Thornless', and others (Clark and Finn, 2011).

In Colombia, despite the social and economic importance of the crop, clearly identifiable certified planting material is scarce; and in some cases, it has low genetic quality due to the lack of current breeding. Previous studies on blackberry fruit quality have been carried out, and the main variables have been described. Espinosa *et al*. (2009) focus their research on fruit length, weight, width, and maturity, as well as total soluble solids (TSS) content and acidity. Grijalba *et al*. (2010) evaluate weight, TSS, and number of basal stems in thorny and thornless genotypes, but they do not find significant differences between these two genotypes. Carvalho and Betancour (2015) mention fruit weight, TSS content, titratable acidity, maturity index, color index, and firmness as the most suitable parameters for characterizing the quality of Andean blackberry.

These studies are based on non-certified plant material, since in Colombia there are as yet no registered varieties. Therefore, future results must be carefully compared. In this research, the blackberry breeding program of Corporacion Colombiana de Investigación Agropecuaria (Agrosavia) established an observation plot with promising genotypes for quality or yield, based on the previous results. The plot was established in Silvania, in the province of Cundinamarca. This is the largest blackberry production zone at the national level. In 2017, blackberry production was 30,970 t with a yield of 9.4 t ha^{-1}. The climate of Silvania is tropical with significant rainfall during most of the months and a short dry season (Agronet, 2017).

Plant material characterized for several traits of interest in certain geographical regions will allow a better understanding of the relationships between variables and genotypes and may benefit a plant breeding program (Lewers *et al*., 2010). Considering that fruit size, soluble solids content, acidity, shape, and color are important characteristics for both fresh and processed markets, the aim of this research was to evaluate the fruit quality attributes of 10 Colombian blackberry genotypes in order to identify genotypes that have remarkable potential traits in the berry market and as a genetic source to be included in breeding programs.

Materials and methods

Field experiment

In 2016, a blackberry observation plot was established in Silvania, Cundinamarca, Colombia (04°24'10.6" N, 74°19'16.3" W and 2,215 m a.s.l.). The climatic conditions of Silvania are characterized as having a mean annual temperature of 11.5°C and 689 mm precipitation. Soil texture in the blackberry orchards was sandy with a medium percentage of organic matter (8.93%); soil pH measured with the potentiometric method was 5.18. The content of available P was 87.15 mg kg^{-1}; K, Ca and Mg measured with ammonium acetate 1 N pH 7.0 were 0.17 cmol(+) kg^{-1}, 1.48 cmol(+) kg^{-1}, and 0.56 cmol(+) kg^{-1}, respectively.

Plant material and experimental procedure

Blackberry (*Rubus glaucus* Benth.) plants of 10 genotypes were maintained in field plots in Silvania, Cundinamarca. These plants were established in performance plots for the project "Selected, identified and registered blackberry cultivars for commercial certification" included in the macro-project "Integrated development of sustainable production models for the cultivation of blackberry in Colombia". These genotypes were sown in rows spaced every 3 m in a completely randomized block design with four replicates per genotype with 10 fruits per block and genotype. Blackberry fruits of these ten genotypes were selected because of their interesting fruit quality traits, which were evaluated in three harvest dates throughout 2016 during the dry and rainy periods (Tab. 1).

TABLE 1. Origin and multiplication method used in the evaluated blackberry genotypes.

Genotype code	Common name	Town and/or department of origin	Propagation institution	Multiplication method
VERG 1	San Antonio	Antioquia	Universidad Católica de Oriente - UCO	*in vitro*
VERG 2	Sin espinas	Antioquia		
VERG 3	Francesa	La Ceja, Antioquia		
VERG 4	UTP1	Santa Rosa de Cabal, Risaralda*	Universidad Tecnológica de Pereira - UTP	
VERG 5	UTP5	Manizales, Caldas*		
VERG 6	ILS 2268 SNBGV 5014	Manizales, Caldas	AGROSAVIA	Sexual seed
VERG 7	Guapante	Guarne, Antioquia		
VERG 8	Pantanillo	La Ceja, Antioquia		
VERG 9	San Antonio	Envigado, Antioquia		
VERG 10	ILS 2281 SNBGV 5001	Envigado, Antioquia		

*Source: Marulanda *et al.* (2011).

The first fruit quality trial was carried out at the beginning of January 2016 after 10 months of production. This period was characterized by an absence of rainfall (0.0 mm), a mean day-time temperature of 19.4°C, and a mean night-time temperature of 17.2°C. The second trial was carried out in February, one month later, in which the precipitation increased to 3.2 mm. The mean temperatures for day and night during February were 18.8°C and 16.3°C, respectively. The third harvest was carried out in May, with 6.5 mm of precipitation and a mean temperature of 18°C.

Physicochemical fruit quality analyses

Ten mature blackberry fruits (stage 5 of the color scale of the Colombian Technical Regulation NTC 4106, published by Icontec, 1997) were evaluated for each block and cultivar with four repetitions per each genotype. The assessment comprised every fruit quality parameter in each of the three harvest dates. The berries were transported to the laboratory the same day they were harvested for sample preparation and analysis. The following quality parameters were assessed: seed/fruit weight percentage, fruit weight and diameter, firmness, color, volume, soluble solids content, total acidity, and pH. These parameters are described below.

Fruit weight and diameter

Fruit weight (FW), as well as equatorial (ED) and longitudinal or polar diameter (LD) of 10 fruits, were recorded with a calibrated electronic scale (Mettler PE 300 digital scale, Ohio, USA) and a digital caliper (Mituyoyo, São Paulo, Brazil).

Firmness

Fruit firmness (kgf) was measured for 10 fruits using a texturometer (Chatillon TCD200, Ametek MTC, Largo, Florida, USA) with a flat probe of 10.92 mm of diameter, 3.45 mm thickness and a speed of 0.60 mm/min. Since fruits were not completely spherical, the test was performed perpendicularly to the flatter face of the fruit and around the equatorial zone.

Color

The three standard CIELAB parameters (L*, a*, b*) were established on the two opposite sides of the fruit that offer the largest surface area. A Minolta chromameter (Minolta, Ramsey, NJ, USA) was used. The parameters (a) (redness) and (b) (yellowness) were used to calculate two indices that express color (HUE angle) as well as the chroma or color intensity (McGuire, 1992). The HUE angle is calculated from the arctangent of b*/a*, and represents red-purple at an angle of 0°, yellow at 90°, bluish-green at 180°, and blue at 270°. The chroma was calculated using the following equation:

$$(a^{*2} + b^{*2})^{½} \quad (1)$$

Volume

This characteristic was measured through water displacement in a graduated cylinder. The juice, pulp and seed/fruit weight percentages were established after weighing and squeezing 10 blackberry fruits and filtering the mass using muslin to separate the juice from the pulp and seeds. The extracted juice was measured and weighed, and the seeds were separated from the residue. Both the pulp and seeds were weighed.

Soluble solids content, total acidity, and pH

The extracted juice was analyzed for the following parameters: Soluble solids content (SSC) using an ATAGO PAL-1 refractometer (Atago Co. Ltd, Tokyo, Japan); pH using a digital potentiometer (Mettler Toledo AG, Schwerzenbach, Switzerland); and total acidity (TA) by titration of 2 ml of juice diluted with 24 ml of distilled water and titrated to pH 8.2 with 0.1 N NaOH. TA was calculated as malic acid. The maturity index (MI) was calculated as the relationship between SSC (°Brix) and acidity.

Statistical analysis

The data obtained were subjected to an analysis of variance complemented with a Tukey's multiple comparison test, using the GLM procedure ($\alpha \leq 0.05$) found in the SAS software (Statistical Analysis System, Inc., version 9.3). Furthermore, a correlation analysis among all attributes was performed according to the Pearson's test ($\alpha \leq 0.05$).

Clustering genotypes

In order to classify these 10 evaluated genotypes into homogeneous groups according to their quality attributes, a principal component analysis (PCA) was carried out using the PRINCOMP procedure of the SAS software (Statistical Analysis System, Inc., version 9.4). This analysis was also performed in order to eliminate the redundant information contained in all average variables (fruit weight, total sugar content, acidity, pH, juice content, etc.). Based on the principal components (PC) retained, a cluster analysis was performed using the CLUSTER procedure (Ward algorithm) of the SAS software. In order to describe the defined groups, basic statistics were generated (mean, minimum and maximum values and coefficient of variation).

Results and discussion

Physical parameters of fruits

Weight

From the global analysis, significant differences in fruit weight were found among genotypes. Genotype 3 showed the highest mean value (5.99 g), and genotypes 10, 2 and 1 reported the lowest values (4.52 g), as illustrated in Figure 1.

Fruit weight values found in all these genotypes were in the same range as those found by Hassimotto *et al.* (2008) and Farinango (2010) in studies conducted in Brazil and Ecuador, respectively. However, the values found in this study were lower than those found by Ayala *et al.* (2013), who report weights between 5.07-9.06 g for blackberries cultivated in Tolima, Colombia. According to the NTC 4106, all materials studied are classified as having medium or small fruits, corresponding to a commercial category C or D.

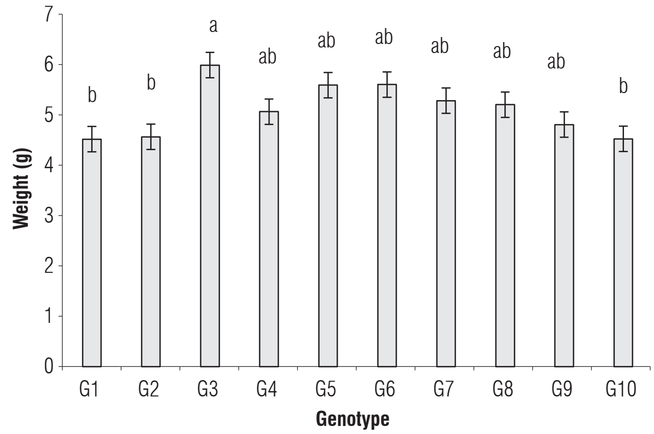

FIGURE 1. Mean fruit weight of ten blackberry genotypes assessed in Silvania, Cundinamarca. Each value represents the mean ± SE of ten berries per replicate with four replicates over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

Clark and Finn (2011) recommend a weight around 8 or 10 g; regarding these results, all ten of the assessed blackberry genotypes have a similar size compared to important genotypes developed by this industry such as 'Osage' and 'Ouachita', although they were a bit smaller than 'Prim-Ark® Traveler'. Regarding size, these genotypes could probably be accepted in both national and international markets and would be more appropriately prepared as frozen berry mixes similar to raspberries.

Longitude and equatorial diameter

The 10 materials evaluated in the current study showed longitude values between 21.4 mm and 24.1 mm without showing significant differences. However, the equatorial diameter showed significant differences between genotype 8 and genotypes 2 and 1. Genotype 8 showed the highest value (21.07 mm), whereas genotypes 2 and 1 reported values of 18.27 and 19.11 mm, respectively (Fig. 2). The size of most of the blackberry fruits studied corresponds to the commercial categories B or C according to NTC 4106. Taking into account that all genotypes were classified as C or D for fruit weight means that these fruits are less dense or less compact than those reported in the NTC. Results found for genotype 2, which correspond to a thornless genotype, match the results described by Grijalba *et al.* (2010), who report that this thornless genotype is characterized by having a low fruit weight and a small size. The size of these genotypes is in the range found for blackberry varieties cultivated and commercialized in Colombia and other countries, such as Ecuador and Brazil (Hassimotto *et al.*, 2008; Farinango, 2010). The correlation between diameters (LD and ED) was 0.53; therefore, both estimators should be taken into consideration for future studies. These two characteristics are important as they are related to the size of the fruit (Ayala *et al*., 2013) and both the industry and consumers have preferences for large fruits. Most people associate large size with quality, but this correlation is not completely true.

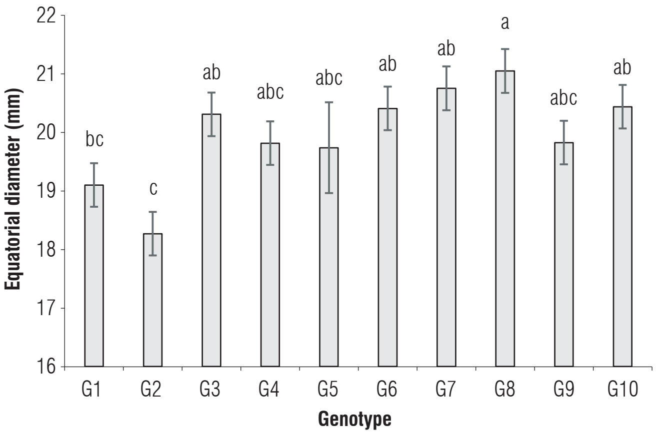

FIGURE 2. Mean equatorial values for the diameter of ten blackberry genotypes assessed in Silvania, Cundinamarca. Each value represents the mean ± SE of ten fruits per replicate with four replicates over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

Firmness

This is an important index for blackberry shelf life since it is directly related to postharvest handling potential, considering that it is necessary to remember that fruit becomes softer with ripening. Therefore, the challenge is to achieve ripe fruit with high soluble solids content, low acidity, and that are shiny and with a uniform color, a developed flavor, and enough firmness for commercial shipping and handling. Genotype 1 achieved the highest firmness value with 4.1 kgf and showed significant differences from genotypes 4 and 3, which obtained the lower values of 2.46 and 2.03 kgf. These results show that fruit firmness was lower for all the genotypes under study (Fig. 3), compared to the blackberry genotypes that García and García (2001) report. However, these were firmer than thornless genotypes such as Brazos and Navaho, reported by Perkins-Veazie *et al*. (2000), with values of 1.86 and 2.45 kgf; but they were also in the same range as those reported by Moreno and Deaquiz (2016) and Segantini *et al*. (2018).

According to these results, genotypes 1, 8 and 10 could be used to improve postharvest handling potential in a breeding program. However, genotypes 1 and 10 also showed the lowest fruit weight, which reduces their market potential. Thus, genotype 8 could be a good candidate for a breeding program.

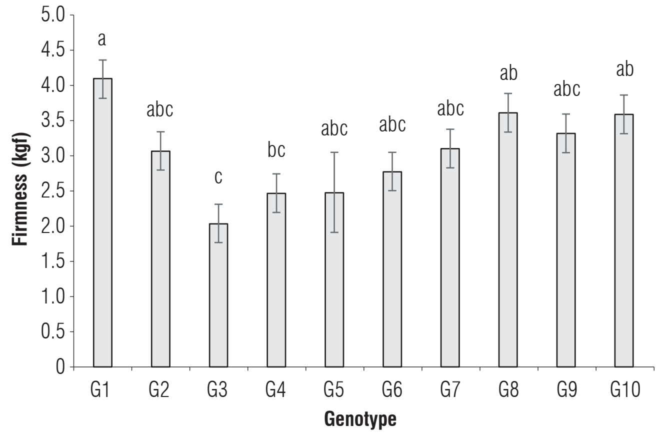

FIGURE 3. Mean firmness values of ten blackberry genotypes assessed in Silvania, Cundinamarca. Each value represents the mean ± SE of ten blackberry fruits per replicate with four replicates over three harvest dates per genotype. Means followed by the same letter within a single bar are not significantly different ($\alpha = 0.05$).

Fruit volume and juice, pulp content and seed/fruit weight percentage

The fruit volumes obtained for all genotypes showed acceptable values that were adequate for commercialization. However, despite the large differences in volume from 4 cm^3 to 5.9 cm^3, there was no significant difference among genotypes. Nonetheless, a significant correlation of 0.794 (α<0.0001) between fruit volume and weight was found. A small volume could be considered an advantage as fruit can be packed more easily compared to large blackberry fruits, especially because all genotypes showed fruits with uniform conical or barrel shape and uniform drupelets. However, the market prefers large fruits as consumers associate size with quality.

Juice content reported significant differences among the genotypes (Fig. 4). Genotypes 1, 2, 4, 5 and 6 achieved the highest juice content compared to genotype 9, 10, 7 and 8 that showed the lowest values.

Genotypes 8 and 7 reached the highest value (51%) for pulp content, while genotypes 6 and 5 reported the lowest value. However, before recommending any genotype, it is important to point out that genotypes 6 and 4 showed the lowest seed/fruit weight percentage (3.85%) and these genotypes showed markedly significant differences with genotypes 10 (5.64%) and genotypes 7, 9 and 8 (5.3%) (Fig. 5). This characteristic relating to low seed perception is critical for both fresh and processed berries (Clark and Finn, 2011).

Parameters such as juice and seeds are very important for fresh and processed fruit markets as they determine their potential yield and the production cost related to downstream operations.

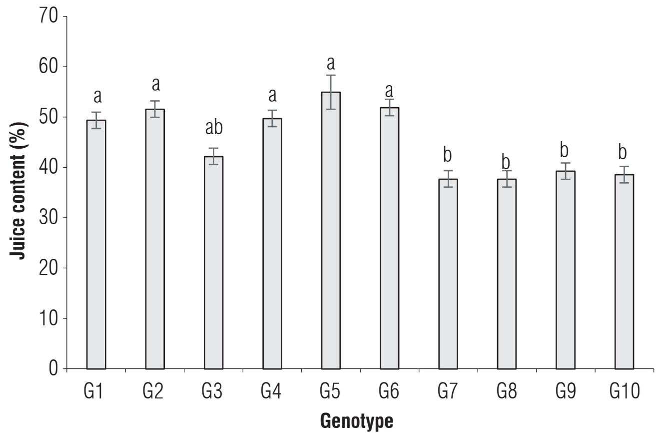

FIGURE 4. Mean percentage of juice content of ten blackberry genotypes assessed in Silvania, Cundinamarca. Each value represents the mean ± SE of juice released for ten berries per replicate with four replicates over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

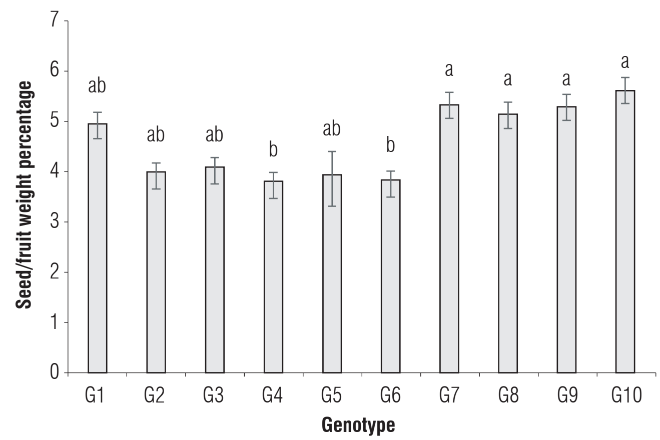

FIGURE 5. Seed/fruit weight percentage of ten blackberry genotypes assessed in Silvania, Cundinamarca. Each value represents the mean ± SE of ten berries replicated over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

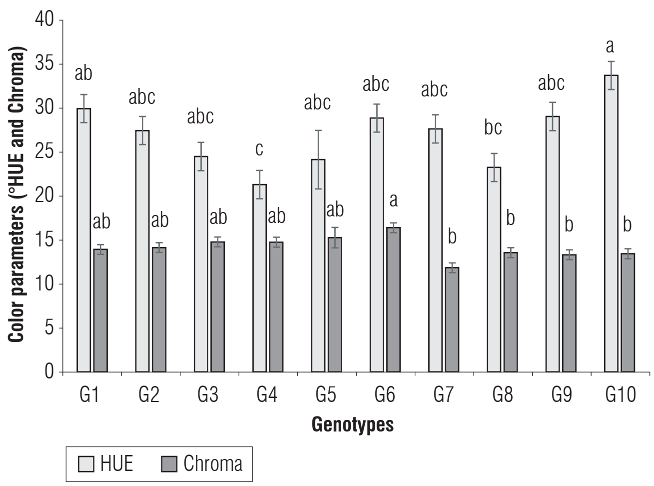

FIGURE 6. HUE and chroma values of ten blackberry genotypes cultivated in Silvania, Cundinamarca. Each value represents the mean ± SE of ten fruits replicated over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

Color

Fruit ripening and color of blackberry fruits are associated with better customer acceptance. Consumers look for fully dark but shiny red-purple drupelets, but not completely red as this means unripe fruit. Based on the results, it can be proposed that the color of all genotypes corresponds to a red color but with different grades of saturation. Figure 6 shows that genotype 4 had significant differences compared to genotype 10, highlighting its stronger red-purple color according to the HUE angle values found. This color is related to the presence of anthocyanins and according to these results it seems that genotype 4 shows a higher anthocyanin content (Zielinski *et al*., 2015).

Genotype 6 achieved the most intense color compared to genotypes 8, 10, 9, and 7 which reported the lowest color intensity when interpreting chroma values. Genotype 4 could be considered a good option for breeding since it has a good balance between the red-purple color and intensity. According to these color parameters, most of the genotypes show a red-purple color when they are ripe, although small differences exist among them. However, there were no genotypes with marked differences in color, like 'Natchez', 'Osage' or 'Ouachita', which are characterized by having a black color when ripe.

Chemical parameters of fruits

pH, soluble solids content and titratable acidity

The highest pH values (2.8) were observed in genotypes 7, 4, and 2 establishing markedly significant differences from genotypes 9, 1, and 10 (Fig. 7). The pH parameter showed important differences among genotypes and can be considered a good indicator. Nevertheless, pH values found in all the genotypes were lower than those reported for genotypes Ouachita (3.2), Natchez (3.1), Navaho (3.2), and Osage (3.6) (Perkins-Veazie *et al*., 2000; Clark *et al*., 2019).

The SSC is one of the most common fruit maturity indices and is a very important variable in the food industry as well as a good indicator of consumer appraisal (Abiodun and Akinoso, 2014). Significant differences between genotypes 7 and 10 were found, as the first one achieved the highest SSC (7.55), while genotype 10 only obtained a value of 5.68 °Brix. According to these results, most of these genotypes were harvested in ripeness stage 4 according to NTC 4106. It is also important to point out that genotypes 7, 8 and 3

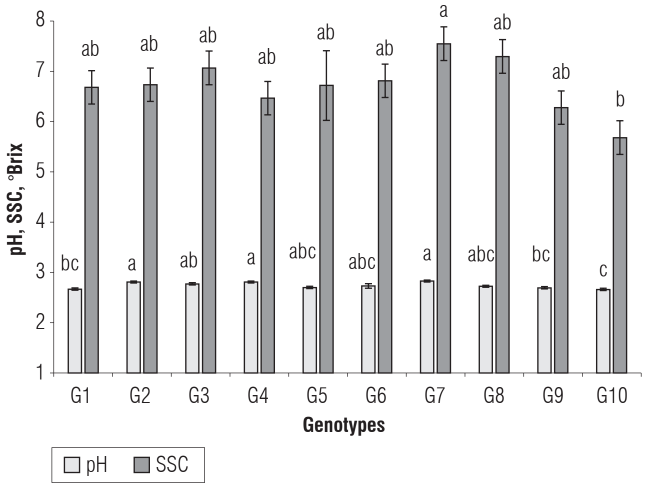

FIGURE 7. pH and soluble solids content (SSC) of ten genotypes assessed in Silvania, Cundinamarca. Each value represents the mean ± SE of the pH and the SSC values of juice obtained from ten berries per replicate, with four replicates over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

reach SSC values as high as those achieved in popular genotypes such as 'Brazos', 'Caingangue', 'Choctaw', 'Comanche', 'Guarani', 'Tupy', and 'Xavante', according to Santos Guedes *et al.* (2013). On the other hand, these values are lower than those reported for genotypes Natchez, Osage, Ouachita, Prime-Ark® 45, Prime-Ark®Traveler, Cancaska, and Chester (Threlfall *et al.*, 2016). Joo *et al.* (2011) report SSC values between 8 and 11%. Thus, genotype 8 can be recommended for a plant breeding program because of its red color and high total SSC.

Regarding total acidity, genotype 4 yielded the lowest value (2.48%) compared to the highest acid contents in genotypes 7, 8, 1 and 5 (3.1-3.24%); but there were no significant differences among them. These values reached by all genotypes under study were higher than those reported by genotypes Brazos, Caingangue, Cherokee, Choctaw, Comanche, Ebano, Guarani, Tupy, and Xavante (Santos Guedes *et al.*, 2013). Genotypes Natchez, Osage, Ouachita, Prime-Ark® 45, and Prime-Ark® Traveler are characterized by their acidity values lower than 1.4%. Thus, it is necessary to continue looking for genotypes with less acid fruits and that are appropriate for Colombian conditions. Despite the fact that acid fruits have a longer shelf life since acidity acts as protection against microorganisms, this advantage is not so important for the industry which searches for fruits that combine low acidity and high sugar content in order to reduce sugar consumption and, therefore, the processing cost.

Flavor is also an interesting fruit quality characteristic and is composed basically of aroma, astringency, acidity and sweetness qualities (Clark and Finn, 2008). Total acidity and SSC are important fruit quality attributes that determine the taste and processability of fruits (Vrhovsek *et al.*, 2008). Markets prefer a balance of sweetness and acidity, as high acidity with high soluble solids content is associated with a full and intense flavor, whereas lower acidity results in a "flat" flavor. Also, high acidity is related to longer anthocyanin stability (Clark and Finn, 2011). Then, if genotypes show high acidity, their SSC should also be high, otherwise other genotypes with lower acidity must be found.

Maturity index

The impact of fruit ripeness in blackberries is very important as it establishes fruit shelf life and consumer appeal. The sweetest blackberries are obtained when fruits are completely ripe, but the fruit is also softer at this stage. It is necessary to look for the most appropriate ripening stage to harvest the fruit, not only to satisfy consumer preferences, but also to reach a longer shelf life. More than SSC or acidity, the ratio between these two attributes is a better maturity indicator and is called the maturity index (MI). In Colombia, the standard maturity index for blackberry harvests ranges from 2.2 to 3.1 (Icontec, 1997). This variable was discriminative, showing significant differences between genotypes 4, 2, and 7 (2.69, 2.57, and 2.56, respectively), while genotype 10 had the lowest score of 1.98 (Fig. 8), which is not appropriate for consumers, since it has the most pronounced acid taste.

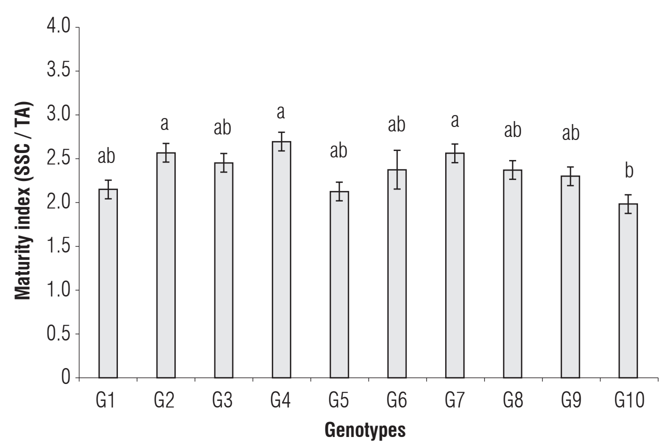

FIGURE 8. SSC/Total acidity ratio (maturity index) for ten blackberry genotypes cultivated in Silvania, Cundinamarca. Each value represents the mean ± SE of the SSC/Total acidity ratio of the juice of ten berries per replicate, with four replicates over three harvest dates per genotype. Means followed by the same letter within a single column bar are not significantly different ($\alpha = 0.05$).

Clustering genotypes

To classify the ten assessed genotypes into homogeneous groups based on the variables fruit weight, soluble solids content, acidity, pH, juice content, seed/fruit weight percentage, equatorial diameter, firmness, and color (°HUE), we performed a principal component analysis (PCA). The first three components explained 81.62% of the total data variability. The first component reported 39.76% whereas the second one showed 26.68% and the third one 15.18%. Moreover, the first component was associated with the variables seed/fruit weight percentage, firmness, and color (°HUE); the second component was related to the equatorial diameter, and finally, the third component was related to pH and acidity, as seen in Table 2.

TABLE 2. Variables and their relation to the first three principal components (PC).

Variables	PC1	PC 2	PC 3
Fruit weight	-.380456	0.347499	0.261560
Soluble solids content	-.273206	0.369684	0.011704
Acidity	0.176598	0.330511	0.609547
pH	-.360983	0.031391	-.532782
Juice content	-.212779	-.453601	0.457970
Seed/fruit weight percentage	0.430697	0.309257	-.250258
Equatorial diameter	-.013141	0.569760	0.004723
Firmness	0.456069	-.005135	0.049697
Color (°HUE)	0.424465	-.079420	-.034471

Figure 9 shows a dendrogram with the classification of these 10 genotypes in the dendrogram (Fig. 9), five groups were formed by cluster analysis.

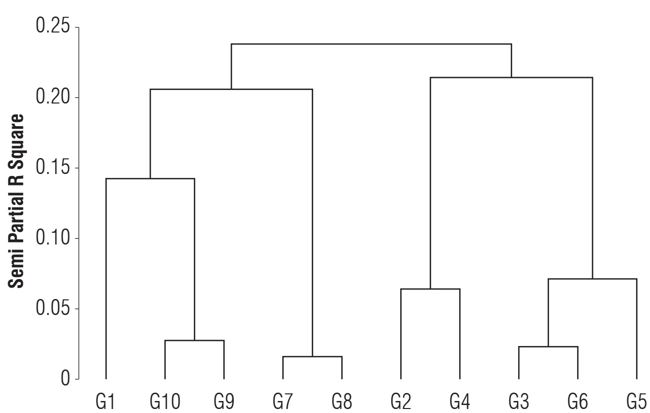

FIGURE 9. Dendrogram of the 10 evaluated genotypes of blackberry.

Table 3 lists the means for each group of each of the variables; the groups are comprised as follows:

Group 1, consisting of two genotypes (G7 and G8), is characterized as the group with the highest mean soluble solids content value (°Brix) (7.5), compared with the other groups. Fruit weight, acidity, pH, seed/fruit weight percentage, equatorial diameter and firmness were higher than the general mean of all the genotypes.

Group 2, consisting of the three genotypes (G3, G5 and G6) was characterized by having the highest juice content value (49.8%), as well as the highest fruit weight value (5.7 g). The content of soluble solids, acidity and equatorial diameter exceeded the general average for all genotypes.

TABLE 3. Averages for each group for each of the variables in the analysis.

Variables	Group 1	Group 2	Group 3	Group 4	Group 5	General
Fruit weight (g)	5.3	5.7	4.7	4.8	4.5	5.1
Soluble solids content (°Brix)	7.5	6.8	5.9	6.6	6.7	6.7
Acidity (%)	3.1	3.1	2.9	2.6	3.2	3.0
pH	2.8	2.7	2.7	2.8	2.7	2.7
Juice content (%)	37.9	49.8	38.9	50.7	49.4	45.4
Seed/fruit weight percentage	5.3	4.0	5.5	3.9	5.0	4.6
Equatorial diameter (mm)	21.0	20.2	20.2	19.1	19.1	20.0
Firmness	3.3	2.5	3.5	2.8	4.2	3.1
Color (°HUE)	25.4	25.9	31.5	24.4	29.9	27.0

Group 3, constituted two genotypes (G9 and G10) and showed the highest average values for color (31.5°HUE) and seed/fruit weight percentage (5.5%) for all the groups. The equatorial diameter (20.2 mm) and firmness (3.5) values exceeded the general average.

Group 4 included two genotypes (G2 and G4) and stood out by having the highest mean value in terms of juice content (50.7%). The other variables had lower mean values compared to the average of the other genotypes, except for pH, which exceeded the corresponding general average (2.8).

Finally, group 5 comprised the genotype G1, characterized by the highest acidity (3.2) and firmness (4.2) values of all the groups, and surpassed the general average for juice and seed/fruit weight percentage and color (°HUE).

Analyzing the results independently from each of the three harvest times it can be concluded that to evaluate new materials it is important to carry out long term studies. In such research it would be important to meticulously monitor edaphoclimatic conditions, since these drastically affect the behavior and performance of the crop, and as well, the crop needs adaptation time. This agrees with what

Clark *et al*. (2019) report, who found an increase in yield, soluble solids content, and weight in an evaluation period of three years after the crop was established. Also, Conway (2018) reports that warm and dry climates produce less acid and sweeter fruits, but when temperatures are higher than 30°C the flavor and sweetness content are reduced, especially in humid climates. The results achieved in this research pointed out that equatorial diameter, fruit length, juice content, SSC and acidity have low values during the rainy season, whereas pH reached the highest values in this season. In addition, the maturity index showed the highest values in the dry season, supporting the proposal that dry seasons produce the best performance for all blackberry genotypes. Regarding firmness, the values increased with time, reaching the highest values in the third harvest.

Thus, to attain a deeper knowledge of these genotypes and obtain more reliability for them to be recommended as part of a breeding program, longer evaluation periods should be considered.

Conclusions

According to the overall results, genotypes 8, 4 and 6 are recommended for a breeding program based on various desirable characteristics. Genotype 8 is outstanding for traits like size, firmness, SSC and red-purple color; genotype 4 is interesting for its low seed/fruit weight percentage, red color, low acidity, pH, and high MI; and genotype 6 is outstanding for its high juice content, low seed/fruit weight percentage, and color intensity. Despite the low number of genotypes evaluated (only 10), these showed significant differences that allowed classifying them into five different groups.

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Characterization of chlorogenic acids (CGA) and nine isomers in an F_2 population derived from *Coffea arabica* L.

Caracterización de ácidos clorogénicos (ACG) y nueve isómeros en una población F_2 derivada de *Coffea arabica* L.

Iván Loaiza-Campiño[1]*, Andrés Villegas-Hincapié[2], Victoria Arana[3], and Húver Posada[4]

ABSTRACT

Chlorogenic acids (CGA) and their isomers have been associated with sensory attributes of the coffee beverage such as acidity, astringency, and bitterness. They have been linked to coffee rust resistance and acknowledged as bioactive compounds due to their antioxidant power with benefits for human health. The total chlorogenic acids (TCGA) and nine isomers of three groups, caffeoylquinic acid or CQA (5-CQA, 4-CQA, 3-CQA), dicaffeoylquinic acid or diCQA (3,4-diCQA; 3,5-diCQA, 4,5-diCQA) and feruloylquinic acid or FQA (5-FQA, 4-FQA, 3-FQA) were determined in an F_2 population of *Coffea arabica* from the crossbreed (Bourbon x Maragogype) x Timor Hybrid. TCGA contents were quantified by UV-VIS spectrophotometry and High-Resolution Liquid Chromatography - HPLC. The group of caffeoylquinic acids (CQA) represented 82% of the TCGA. From the diCQA, 4,5-diCQA showed lower contents, whereas the highest isomer was 3,5-diCQA. Results per quartile for TCGA-UV and for every isomer showed statistical differences among group averages per isomer. The population behaved as a parental Maragogype according to contents of 5-CQA, 3,5-diCQA, and TCGA-UV. TCGA contents were higher in the parental GQ956 derived from the Timor hybrid 832-1, with resistance to coffee rust. From the three groups, the first characteristic of parental Bourbon showed a higher concentration of diCQA and FQA; the second one showed a lower concentration of TCGA and CQA isomers and the third group higher TCGA and 5-CQA concentrations. This research allowed establishing the basis for plant selection in the F_2 generation of *C. arabica* due to the TCGA content and isomers derived from CQA, diCQA, and FQA.

Key words: distribution, caffeoylquinic acids, introgression, coffee quality, Timor hybrid, plant breeding.

RESUMEN

Los ácidos clorogénicos (ACG) y sus isómeros han sido asociados a los atributos en la bebida del café especialmente la acidez, astringencia y el amargo. Estos compuestos han sido reportados como relacionados a la resistencia a la roya del café y reconocidos como compuestos bioactivos en la salud humana por su capacidad antioxidante. Se determinó la distribución de ácidos clorogénicos totales (ACGT) y nueve isómeros pertenecientes a tres grupos, los ácidos cafeoilquínicos o CQA (5-CQA, 4-CQA y 3-CQA), los ácidos dicafeoilquínicos o diCQA (3,4-diCQA; 3,5-diCQA y 4,5-diCQA) y los ácidos feruloilquínicos o FQA (5-FQA, 4-FQA y 3-FQA) en una población F_2 de *Coffea arabica* proveniente del cruce de (Bourbon x Marapagogype) x Híbrido de Timor. Se cuantificó el contenido de ACGT mediante espectrofotometría UV-VIS y cromatografía líquida de alta resolución - HPLC. El grupo de los ácidos cafeoilquínicos (CQA) representó el 82% de los ACGT. De los diCQA, el 4,5-diCQA mostró los menores contenidos, mientras que el isómero mayoritario fue el 3,5-diCQA. Los resultados por cuartil para ACGT-UV y cada isómero indicaron diferencias estadísticas entre los promedios de los grupos por cada isómero. La población se comportó como el padre Maragogype según los contenidos de 5-CQA, 3,5-diCQA, y los ACGT-UV. Los contenidos de ACGT fueron mayores en el parental GQ956 derivado del híbrido de Timor 832-1, cuya característica principal es la resistencia a roya. Se formaron tres grupos de plantas de acuerdo a los isómeros analizados. El grupo uno fue característico del parental Bourbon al presentar mayor concentración de diCQA y FQA; el grupo dos presentó menor concentración de ACGT y de isómeros del CQA; y el grupo tres estuvo caracterizado por presentar mayor concentración de ACGT y 5-CQA. Este trabajo permitió establecer las bases para la selección de plantas en una generación F_2 de *C. arabica* por el contenido total de ácidos clorogénicos y los isómeros derivados de CQA, diCQA y FQA.

Palabras clave: distribución, ácidos cafeolquínicos, introgresión, calidad de café, híbrido de Timor, fitomejoramiento.

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[1] Research group in plant and microbial genetics and biotechnology (GEBIUT), Distance Education Institute, Universidad del Tolima, Ibague (Colombia).
[2] Project Coordinator, Local Partners Foundation, Manizales (Colombia).
[3] Chemistry program, Basic Sciences Faculty, Universidad del Atlántico, Puerto Colombia (Colombia).
[4] Director of coordinating offices and other territories, National Federation of Coffee Growers, Bogota (Colombia).
* Corresponding author: idloaizac@ut.edu.co

Introduction

The world culture of *Coffea arabica* L. has been performed mainly with traditional varieties such as Typica and Bourbon either by mutation or selection. Another important group of varieties is that with resistance to coffee rust, which has derived from the crossbreed between the traditional varieties of *C. arabica* L. and the Timor hybrid (Van Der Vossen *et al*., 2015).

The conventional method of coffee genetic improvement has been determined by the method of selection by pedigree (Van Der Vossen *et al*., 2015). After an original crossbreed, the seeds of the F_1 plants advance to a new generation F_2, which offers the first opportunity to practice a selection of only those plants that meet the desired requirements. In the development of coffee varieties, there has been a focus to select the best plants and upgrade them to F_3 or F_4 generations, placing emphasis on inheritable variables such as production and resistance to diseases (coffee rust), and assessing the quality in the last generations of the selection (Van Der Vossen, 2009).

The quality in a cup of coffee depends on the contents of chemical compounds present in the green coffee, which at the same time are influenced by factors such as species, variety, environment, crop condition, and harvest and post-harvest processes, among others (Bertrand *et al*., 2008). There are studies related to the determination of chemical compounds associated with quality that show the appearance of contrasting levels among species and at the intra-specific level (Herrera and Lambot, 2018).

Among the studied compounds, there are chlorogenic acids (CGA) and their isomers, which have been associated with sensory attributes of the beverage, especially acidity, astringency, and bitterness (Mazzafera and Melo, 2004).

CGA have been associated with coffee rust resistance (Guerra-Guimarães *et al*., 2015) and acknowledged as bioactive compounds due to their antioxidant power. These compounds have benefits on human health causing different biological effects such as free radical capture, metal chelation, enzymatic activity modulation and signal transduction way alteration (Cheng *et al*., 2016).

CGA determination in green coffee is mainly performed through analytic techniques of Ultraviolet-Visible Spectrophotometry (UV-VIS) for total concentration and High-Resolution Liquid Chromatography (HPLC) form isomer measure (Brighenti *et al*., 2017). These techniques have been used to identify the presence of chemical compounds in different plant organs, obtain the diversity of *Coffea* species, and characterize different coffee varieties (Etienne *et al*., 2018).

For CGA quantification in coffee varieties, Guerrero *et al*. (2001) found that 4,5-caffeoylquinic acid was present in all the material of Colombian varieties: varieties from *C. arabica*, three accessions of Timor hybrid, the first generation (F_1) of a crossbreed from *C. canephora* x Timor hybrid and three accessions of *C. canephora*. Therefore, it can be used to separate these two varieties from the rest of the genotypes.

Bertrand *et al*. (2008), studied the content of chlorogenic acids in four introgressed lines and the Caturra variety. Their results showed differences among genotypes for most of the studied chlorogenic acids, with the exception of 3-CQA and 3-FQA, whereas regarding the chlorogenic acids the highest effect was found in 3,4-diCQA.

The objective of the current study was to determine the concentration of chlorogenic acids and nine isomers in an F_2 population of *Coffea arabica*.

Materials and methods

Population of work and sampling

An F_2 population formed by 143 plants from the crossbreed of Maragogype parentals, Bourbon resistant to *Ceratocystis fimbriata* and Timor Hybrid (GQ956 - CIFC 832-1) was used. These plants were sown in the Station Naranjal in the municipality of Chinchina (4°59' N, 75°39' W, and altitude of 1,400 m a.s.l.).

Samples were taken during the peak week of the main harvest (September-November). Coffee cherries were collected from each of the plants and then they were washed and sun-dried until their mass reached a verified humidity from 10 to 12%. Once dried, green coffee was obtained by the dry milling process and ground in a cryogenic mill at 14000 rpm.

Extraction

The extraction of chlorogenic acids in green coffee was carried out following the methodology proposed by Bicchi *et al.* (1995). The study was carried out in two phases:

Phase 1

Consisted in the selection of the best analytic method for total chlorogenic acids (TCGA) using two methodologies:

Visible Ultra-Violet (UV-VIS) and High-Resolution Liquid Chromatography (HPLC).

A *t*-test was used for the comparison of the means of both techniques and to determine if there were statistical differences in the quantification of TCGA.

The following formula was applied for the *t*-test:

$$t = \frac{\overline{X}_{ACT\text{-}UV} - \overline{X}_{ACT\text{-}HPLC}}{\sigma\sqrt{\frac{1}{N_1}+\frac{1}{N_2}}} \quad (1)$$

where $\sigma = \sqrt{\frac{N_1 s_1^2 + N_2 s_2^2}{N_1 + N_2 - 2}}$

$\overline{X}_{TCGA\text{-}UV}$: Mean of chlorogenic acids by UV-VIS.

$\overline{X}_{TCGA\text{-}HPLC}$: Mean of chlorogenic acids by HPLC.

N: Number of population individuals.

S^2: Variance of each of the population individuals.

If the total chlorogenic acids quantified by both techniques did not show statistical differences, then the contents of isomers 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, 3-FQA, 4-FQA, and 5-FQA were compared, which were quantified by HPLC through the proportionality of each isomer concerning the total. This procedure was carried out following the methodology of Bastos De Maria and Alvez (2004) and using the methodologies validated in stage 1.

Phase 2

Consisted in the application of the method selected in phase 1 and the determination of isomers in the F_2 population (143 plants) and the respective parentals.

Results and discussion

The results of the *t*-test are presented in Table 1, which showed that the determination of TCGA by both techniques is the same and either of both technologies can be used. Since UV-VIS is the one with the lower cost, this method is the best option to determine TCGAs in an analytic form.

TCGA determined by UV showed an average content of 4% in the population, with a minimum and maximum 2.78% and 5.05%, respectively, while TCGA by HPLC showed an average content of 3.96% in the population, with a minimum and maximum of 2.62% and 4.93%, respectively.

TABLE 1. *T*-test for the mean of total chlorogenic acids quantified by UV-VIS and HPLC, with different variance.

Variables	HPLC	UV-VIS
Mean	3.96	4.00
Variance	0.18	0.21
Observations	143	143
Mean difference hypothesis	0	
GL	289	
t-statistic	0.81	
t critical two-tail	1.97	

Table 2 presents the contents for each one of the nine isomers tested by HPLC. Values ranged between 3.67% for 5-CQA and 0.01 for 3-FQA, in which isomer 5-CQA has the highest concentration and 3-FQA is the one with the lowest. CQA showed higher contents than diCQA and FQA, which is similar to the results obtained by Cheng *et al*. (2018).

Six isomers showed a normal distribution: three isomers of the CQA (5-CQA, 4-CQA, and 3-CQA), two isomers of the diCQA (3,5-diCQA and 4,5-diCQA) and one of the FQA (5-FQA). Isomers 3,4-diCQA, 3-FQA and 4-FQA were not adjusted to a normal distribution. The TCGA by HPLC was not adjusted to a normal distribution. The results of the frequency analysis for five isomers and the TCGA-UV (normally adjusted) are shown in Figure 1.

TABLE 2. Minimum and maximum values observed, mean, standard deviation and lowest and upper limits for the content of nine isomers of the family of chlorogenic acids expressed in dry matter percentages in an F_2 population.

Isomer Group	Isomer	Minimum	Maximum	Mean	Standard deviation	LL (95%)	UL (95%)
TOTAL CQA	UV	2.78	5.06	4.00	0.44	3.93	4.08
	HPLC	2.62	4.94	3.96	0.42	3.89	4.03
CQA	5-CQA	1.77	3.67	2.66	0.35	2.60	2.71
	3-CQA	0.13	0.42	0.25	0.04	0.24	0.26
	4-CQA	0.22	0.58	0.35	0.05	0.34	0.36
	TCQA	2.17	4.35	3.26	0.38	3.19	3.32
diCQA	3,4-diCQA	0.05	0.21	0.12	0.04	0.11	0.13
	3,5-diCQA	0.11	0.35	0.23	0.05	0.22	0.24
	4,5-diCQA	0.06	0.16	0.10	0.02	0.10	0.11
	TdiCQA	0.26	0.70	0.46	0.09	0.44	0.47
FQA	5-FQA	0.11	0.34	0.19	0.04	0.18	0.20
	4-FQA	0.00	0.08	0.04	0.01	0.04	0.05
	3-FQA	0.00	0.05	0.01	0.01	0.01	0.02
	TFQA	0.14	0.45	0.25	0.05	0.24	0.26

LL= lowest limit; UL= upper limit.

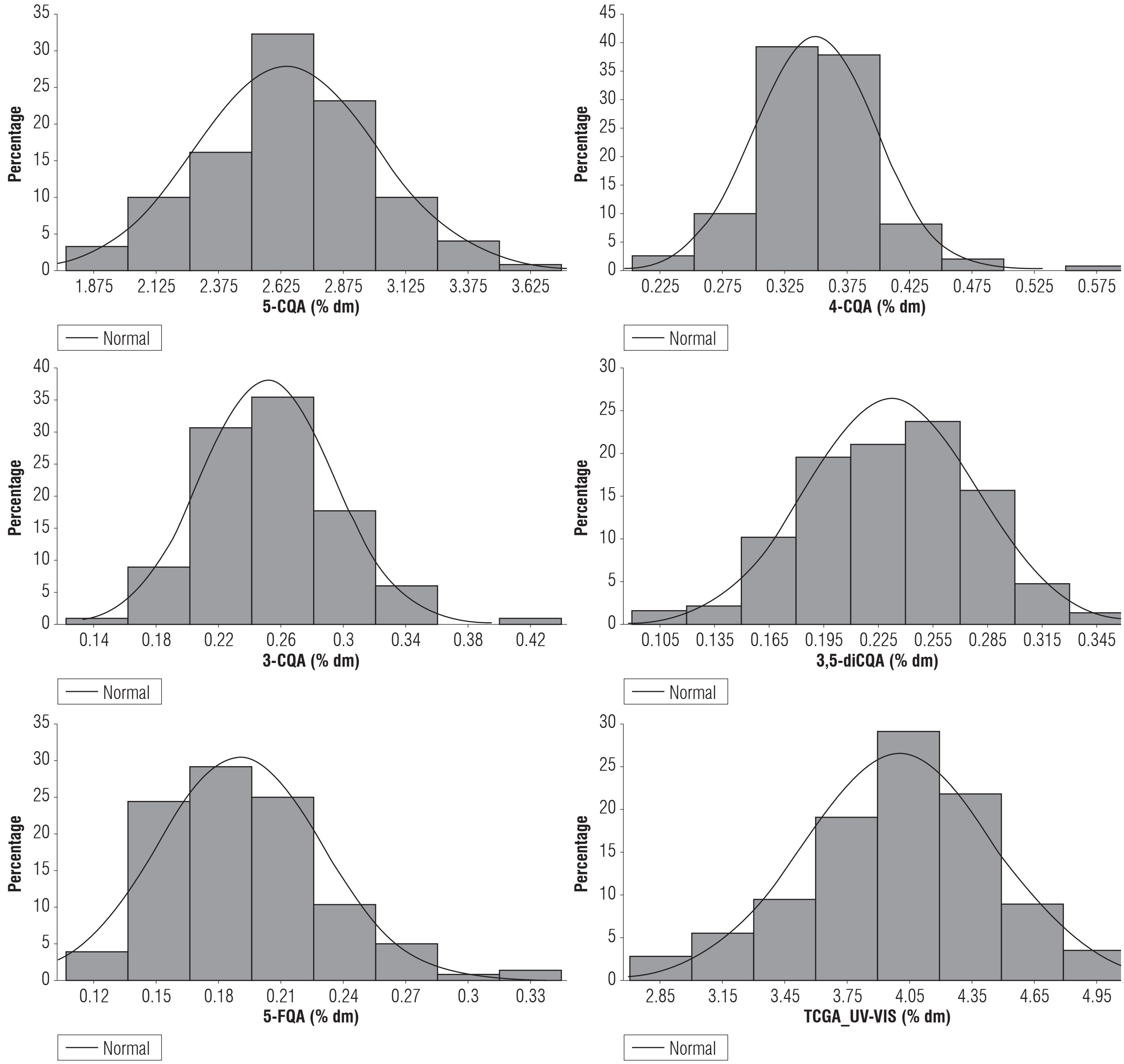

FIGURE 1. Frequency distribution for five isomers and total chlorogenic acids quantified by UV-VIS (TCGA-VIS).

Isomers of the CQA group had a concentration of 3.27%; the highest content was found for 5-CQA with 2.67% and the minimum for 3-CQA with 0.13%, whereas diCQA had a value of 0.45%. The isomer that showed the highest content was 3,5-diCQA with a 0.35%, whereas the lowest value was found for 3,4-diCQA with 0.05%.

Caffeoylquinic acids (CQA)

The content of 4-CQA and 3-CQA represented between 13% and 9% of the content of 5-CQA. 5-CQA was the isomer with the highest average content, being statistically different from the other isomers. This behavior was reported by Guerrero *et al.* (2001) and Perrone *et al.* (2008). The isomer 4-CQA is the second isomer in importance and 3-CQA is the third isomer in abundance, even though their content does not differ descriptively from 3,5-diCQA.

The behavior for the content of group CQA matches one of the majoritarian isomers reported in studies of *C. arabica* varieties by Scholz *et al.* (2016) and Barbosa *et al.* (2019).

Dicaffeoylquinic acids (diCQA)

From the diCQA group, isomer 3,5-diCQA showed the highest contents, which differs from that reported by other authors in commercial lines who found 4,5-diCQA and 3,4-diCQA as the isomers with the highest contents. The behavior of isomers found in our study for this group was the following: 3,5-diCQA > 3,4-diCQA > 4,5-diCQA.

However, Guerrero *et al.* (2001) report in an early population (F_1), low levels for 4,5-diCQA compared to 3,5-diCQA, which has the highest contents within these groups. This is because an F_1 population presents dominant characteristics in the expression of chemical compounds.

Feruloylquinic acids (FQA)

Isomer 5-FQA presented an average content of 0.19%, it is the isomer with the highest content within the FQA group, and it was superior to the content of isomers 3,4-diCQA and 4,5-diCQA. This behavior was reported by Bertrand *et al.* (2008) in four materials introgressed by Timor Hybrid. The minimum and maximum values for this isomer were 0.15 and 0.34%, respectively. For isomers 3-FQA and 4-FQA, no content was detected in the 38% of tested plants.

FQA presented a relationship of proportionality lower than 2% before total chlorogenic acids were quantified by UV-VIS (TCGA-UV). This proportion was reported by Ky *et al.* (2001) about biochemical diversity in the *Coffea arabica* L genus.

Total Chlorogenic Acid (TCGA)

Table 3 shows the descriptive statistics for the variable proportion of chlorogenic acids quantified by HPLC. The percentage participation of the CQA group (3-, 4- and 5-CQA) was 82% of the TCGA with an average content of 3.27%. Farah and Donangelo (2006) report contents for CQA of 83%, similar to those found in this study.

In the CQA group, 5-CQA was the isomer that descriptively represented the highest percentage participation with 66.27% of TCGA, which matches what was reported by Cheng *et al.* (2016). These authors showed that 5-CQA represents 66% of chlorogenic acids. The minimum and maximum values of proportionality for this isomer were 58.77 and 76.31%, respectively. Fifty percent of the tested materials are above 66.15% of 5-CQA with a standard deviation of 3.46%.

Isomer 4-CQA represented 8.77% of the TCGA-UV and is the second isomer in terms of percentage of participation. The minimum and maximum values of proportionality for this isomer were 5.41 and 17.28%, respectively. Fifty percent of the materials tested are above 8.67% and the other 50% is under this value with a standard deviation of 1.22%.

Isomer 3-CQA represented descriptively the 6.34% of the TCGA-UV, and it is the third isomer in the percentage of participation. Fifty percent of the materials tested are under 6.20%. The behavior of isomers the CQA group was the same as reported by Bertrand *et al.* (2008), where 5-CQA > 4-CQA > 3-CQA.

The diCQA group showed an average content of 0.45%, which represented 11% of the TCGA content, whereas isomer 3,5-diCQA represented 51.11% of the diCQA content.

The FQA group had an average content of 0.24%, representing el 6% of total chlorogenic acids. The isomer 5-FQA

TABLE 3. Minimum and maximum values observed, mean for the variable proportion of chlorogenic acids quantified by HPLC.

Proportion (%)	Minimum	Maximum	1st Quartile	3rd Quartile	Mean
TCQA	0.74	0.96	0.79	0.84	0.81
TdiCQA	0.07	0.16	0.10	0.13	0.11
TFQA	0.04	0.11	0.06	0.07	0.06
5-CQA	0.59	0.76	0.64	0.68	0.66
3-CQA	0.04	0.13	0.05	0.07	0.06
4-CQA	0.05	0.17	0.08	0.09	0.09
3,4-diCQA	0.01	0.05	0.02	0.04	0.03
3,5-diCQA	0.03	0.08	0.05	0.07	0.06
4,5-diCQA	0.01	0.04	0.02	0.03	0.03
5-FQA	0.03	0.08	0.04	0.05	0.05
4-FQA	0.00	0.02	0.01	0.01	0.01
3-FQA	0.00	0.01	0.00	0.01	0.00

represented descriptively 4.77% of the TCGA-UV, a value that matches the contents reported by Guerrero *et al*. (2001) for *Coffea canephora* materials with 4.76%. This is the last isomer of the five tested (5-CQA, 4-CQA, 3-CQA, 3,5-diCQA, and 5-FQA). The minimum and maximum values of proportionality for this isomer were 3.13% and 8.32% respectively.

The proportionality of the isomers was as follows: 5-CQA > 4-CQA > 3-CQA > 3,5-diCQA > 5-FQA > 3,4-diCQA > 4,5-diCQA > 4-FQA > 3-FQA. However, diCQA and FQA showed differences. It was found that the higher isomer of diCQA was 3,5-diCQA and not 4,5-diCQA. This order is different from the one reported by Perrone *et al*. (2008). They reported that in two varieties of the *Coffea arabica* species (Mundo Novo and Catuai Vermelho) and in a variety of *Coffea canephora* (Conillon) the proportionality 5-CQA > 4-CQA > 3-CQA > 4,5-diCQA > 3,4-diCQA > 3,5-diCQA > 5-FQA > 4-FQA > 3-FQA was established.

Quartiles for TCGA-UV

The value of the first group Q1 was 3.4%, which indicates an average content of approximately 25% of the 143 F_2 plants, with 32 plants in this quartile; Q2 was 3.9%, and the number of plants was 37; Q3 was 4.16% and the number of plants was 37, and Q4 was 4.53%, and the number of plants in this quartile was 37.

Quartiles for isomer 5-CQA

The average content of approximately 25% of the F_2 population, belonging to the first group or Q1 was 2.2%, where the number of plants in this quartile was 32; Q2 was 2.55% and the number of plants was 36; in the third quartile (Q3), the average was 2.75% and the number of plants was 37. The average value in Q4 was 3.09%, and the number of plants classified in this quartile was 38.

Quartiles for isomer 4-CQA

The value for Q1 was 0.29%, and the number of plants for this quartile was 32; Q2 had 0.33% as average, and the number of plants in this quartile was 36; Q3 had a value of 0.36% and the number of plants was 38. Q4 was composed of 37 plants and an average value of 0.41%.

Quartiles for isomer 3-CQA

The Q1 value indicated that approximately 25% of the population had an average content of 0.2% and 32 plants were classified in this quartile. The average value was 0.23% in Q2 with 36 plants; the average value of Q3 was 0.26% and the number of plants was 38; finally, the value for Q4 was 0.3% with 37 plants.

Quartiles for el isomer 3,5-diCQA

Q1 was 0.17% and the number of plants classified in this quartile was 34; the value of Q2 was 0.21%, with 36 plants located in this quartile; the value of Q3 was 0.25% with 36 plants located in this group; and the average value of the population in Q4 was 0.29%, with 37 plants.

Quartiles for isomer 5-FQA

The average value of Q1 was 0.14% with 32 plants; Q2 was 0.17% with 35 plants classified in this group; Q3 had an average content of 0.2% and 39 plants; finally, the average value for the population of Q4 was 0.24% with 37 plants classified.

The Duncan comparison tests showed significant differences between the average values of every inter-quartile range for each of the tested isomers as well as the content of total chlorogenic acids by UV. Inter-quartile averages, Duncan comparison and the number of plants per range are shown in Table 4.

Table 5 shows the average contents of chlorogenic acids for the three groups and five isomers of the family of chlorogenic acids, quantified in three reference materials of the subject population. In all isomers, it was possible to observe a difference between the highest isomer (5-CQA) and the other isomers. It is set that the group of CQA is highest, followed by the diCQA and FQA isomer groups.

TABLE 4. Average for the Chlorogenic Acids variable expressed in percentage of dry matter per plant in every quartile and for each of the tested isomers.

Isomer	Q1	Plants	Q2	Plants	Q3	Plants	Q4	Plants
5-CQA	2.2 D	32	2.55 C	36	2.75 B	37	3.09 A	38
4-CQA	0.29 D	32	0.33 C	36	0.36 B	38	0.41 A	37
3-CQA	0.20 D	32	0.23 C	36	0.26 B	38	0.30 A	37
3,5-diCQA	0.17 D	34	0.21 C	36	0.25 B	36	0.29 A	37
5-FQA	0.14 D	32	0.17 C	35	0.2 B	39	0.24 A	37
TCGA-UV	3.40 D	32	3.9 C	37	4.16 B	37	4.53 A	37

Different letters show average differences among isomers according to the Duncan test at 5%.

TABLE 5. Average contents (% dm) of chlorogenic acids for the three groups and nine isomers of the family of chlorogenic acids, analyzed in three reference materials and the average value of an F_2 population.

Group	Compound	Parental			Mean F_2
		Maragogype	Bourbon	GQ956	
CQA	5-CQA	1.88	1.76	2.26	2.67
	4-CQA	0.28	0.29	0.35	0.35
	3-CQA	0.21	0.23	0.27	0.25
diCQA	3,5-diCQA	0.22	0.22	0.27	0.23
FQA	5-FQA	0.16	0.12	0.14	0.19
TCGA	TCGA-UV	3.07	2.91	3.62	4.00
	TCGA-HPLC	2.97	2.84	3.54	3.96

Only 27 of the 143 plants and in one of the three reference materials (Maragogipe), the grouping of the CQA coincided with the grouping of the TCGA-UV. The low coincidence in the grouped plants shows the variation for each of these isomers and the independence of the behavior of each of the plants in the population.

The quartiles of the TCGA-UV and that of the 5-CQA isomer coincided in 94 of the 143 F_2 plants and the three reference materials evaluated.

In 42 of the 143 plants, the grouping of isomers 3,5-diCQA matched that of the TCGA-UV. Groupings of TCGA-UV and isomer 5-FQA matched in 54 of the 143 F_2 plants and in two out of the three reference materials tested.

The plants classified in the first quartile or Q1 show the lowest contents found in approximately 25% of the population. The plants found in the first quartile show a better quality cup, so they must undergo evaluations of cupping panels (plants 199, 225, 251 and 300). In Q2, just three plants complied with the group and those plants were 220, 269 and 294, which determines a high variability. Q3 shows plants that exceed the average content of CGA; these plants are 147, 175, 178, 181, 183, 219, 227, 243, 253 and 290; in quartile Q4, plant 198 was the only one whose isomers classified it totally in this quartile, showing the highest contents of CGA.

Plant 198 showed that the five isomers were found in the first quartile exhibiting low contents, while plant 199 showed that the five isomers were found in Q4. This indicates that despite being planted in the same location and plot, next to one another, under the same environmental conditions and with the same agronomic handling, there is a clear difference considering that this is the offspring that shows higher segregation of hereditary characters.

Isomer 5-CQA has a higher content in the GQ956 material with 2.26%, whereas 4-CQA has a content of 0.35 in the population. This content was equal to the one found in the material GQ956 (0.35), coming from the Timor Hybrid. In contrast, the lower material was found in Maragogype, with 0.28% (Fig. 2). Isomer 3-CQA content was 0.25 in the population and it was similar to the one found in material GQ956 (0.27) and Bourbon (0.23). In isomer 3,5-diCQA (Fig. 3), the highest content was found in the reference material GQ956 with 0.27%; in contrast, the lowest content was found in Bourbon and Maragogipe type materials both with 0.22%.

Isomer 5-FQA shows content far from the reference materials concerning the population. The average of the population was 0.19, while the average of the closest reference material was 0.16, which corresponds to Maragogype (Fig. 4). The content of TCGA-UV of the population had a value of 4%, and the reference material GQ956 had the closest content with 3.62%.

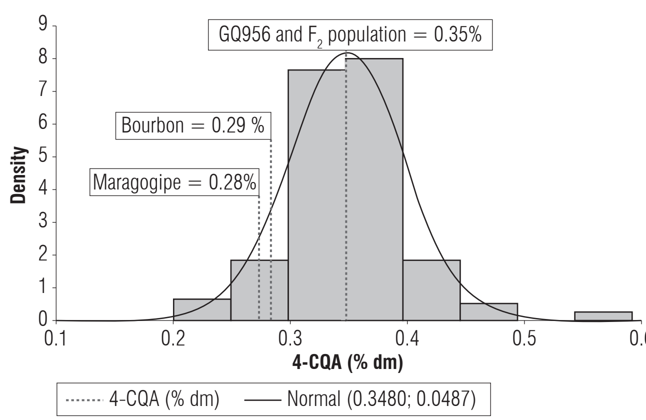

FIGURE 2. Distribution of isomer 4-CQA of the population concerning the reference parentals.

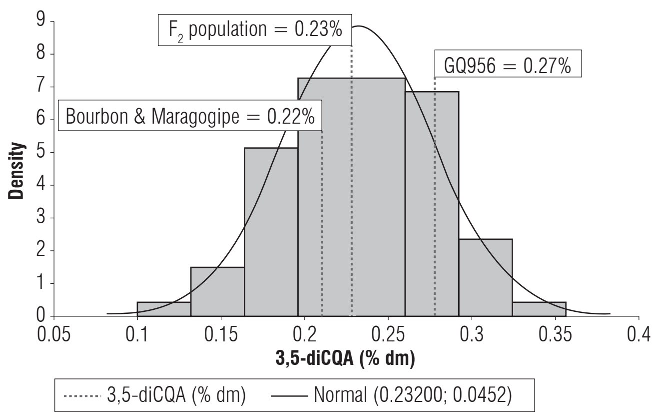

FIGURE 3. Distribution of isomer 3,5-diCQA of the population concerning the reference parentals.

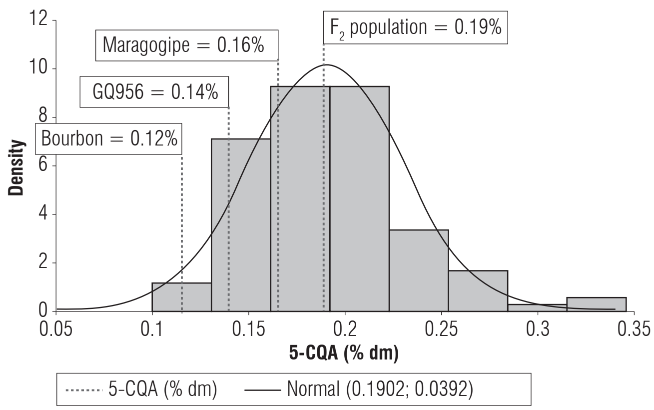

FIGURE 4. Distribution of isomer 5-FQA of the population concerning the reference parentals.

With the ascending hierarchic classification, three groups were found as presented in Figure 5. The first group (Bourbon group) was formed by 86 plants, characterized by the genotype Bourbon as parental and typified for presenting a higher concentration of diCQA and FQA; group two (Timor hybrid - Resistance to Rust) was formed by 42 plants, characterized by the parentals Timor Hybrid and Maragogype and presented a lower concentration of total CQA and the isomers 3, 4 and 5-CQA; group 3 was formed by 18 plants, characterized for presenting higher concentration of total CGA and a higher concentration of 5-CQA. The plants that made up of each group are presented in Table 6.

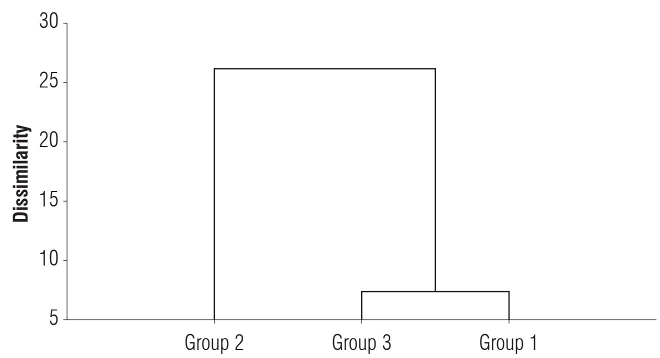

FIGURE 5. Groups formed by the content of 9 isomers of chlorogenic acids in an F_2 population and their parentals.

TABLE 6. Average values (%dm) of each class of the 9 isomer contents of the chlorogenic acids and TCGA by HPLC for the F_2 population and the parentals.

	Group		
Compound	**1**	**2**	**3**
CGA-HPLC	4.07	3.43	4.58
5-CQA	2.73	2.23	3.21
3-CQA	0.26	0.24	0.26
4-CQA	0.36	0.32	0.37
3,4-diCQA	0.13	0.11	0.13
3,5-diCQA	0.24	0.22	0.24
4,5-diCQA	0.10	0.10	0.11
5-FQA	0.20	0.16	0.20
4-FQA	0.03	0.04	0.05
3-FQA	0.02	0.01	0.02
Plants	147, 148, 149, 150, 151, 153, 158, 159, 162, 163, 165, 166, 167, 172, 173, 174, 175, 176, 178, 180, 181, 183, 185, 186, 189, 190, 192, 193, 197, 201, 202, 203, 204, 205, 209, 211, 212, 214, 215, 216, 217, 218, 219, 220, 224, 226, 227, 231, 233, 235, 237, 239, 240, 242, 243, 244, 246, 247, 252, 253, 254, 257, 259, 260, 261, 263, 265, 266, 267, 268, 269, 270, 272, 273, 275, 276, 279, 283, 285, 286, 287, 288, 290, 294, 298, Bourbon	152, 155, 169, 170, 184, 187, 194, 199, 200, 206, 210, 213, 222, 225, 232, 234, 236, 238, 245, 249, 250, 251, 255, 256, 258, 262, 264, 271, 274, 277, 278, 282, 284, 289, 292, 293, 296, 297, 299, 300, GQ956, Maragogype	154, 156, 157, 160, 164, 168, 171, 177, 188, 198, 221, 223, 228, 229, 241, 248, 281, 291

Conclusions

The contents of TCGA by the HPLC or UV methodologies are statistically equal; however, since UV-VIS has a lower cost, it is the best option for the determination of TCGA. The variability of diCQA and FQA makes it necessary to implement the HPLC technique.

The group of caffeoylquinic acids (CQA) represented 82% of the total chlorogenic acids (TCGA). Of the diCQA, 4,5-diCQA showed the lowest contents, the highest isomer was 3,5-diCQA; the proportionality of the isomers was in the order 5-CQA> 4-CQA> 3-CQA> 3,5-diCQA> 5-FQA> 3,4-diCQA> 4,5-diCQA> 4-FQA> 3-FQA, which allows an indirect estimate of each of the isomers.

The results per quartile for TCGA-UV and each isomer indicated statistical differences between groups for each isomer. The population behaved like parental Maragogype according to the contents of 5-CQA, 3,5-diCQA, and TCGA. The contents were higher in the GQ956 material derived from the Timor hybrid 832-1 whose main characteristic is the resistance to rust.

Three groups of plants were formed according to the analyzed isomers, with the Bourbon parent as the main one. The first group was characterized by a higher concentration of diCQA and FQA, whereas the second group had a lower concentration of total CQA and the CQA isomers and the third group was characterized for having a higher concentration of total CGA and a higher concentration of 5-CQA.

This work allowed establishing the bases for the selection of plants in an F_2 population by the total content of chlorogenic acids and isomers derived from CQA, diCQA, and FQA.

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Reuse of agricultural waste as an alternative substrate in the production of eggplant (*Solanum melongena* L.) seedlings

Reutilización de residuos agrícolas como sustrato alternativo en la producción de plántulas de berenjena (*Solanum melongena* L.)

Karoline Matiello Almeida[1], Paola Alfonsa Vieira Lo Monaco[2], Marcelo Rodrigo Krause[1], Dandara Lyone Silva de Oliveira[3], Lorena Aparecida Merlo Meneghelli[1], Caroline Merlo Meneghelli[1]*, and Louise Pinto Guisolfi[1]

ABSTRACT

Seedling formation is one of the most important phases for the eggplant crop cycle. One of the decisive factors for obtaining quality seedlings and the consequent increase in productivity is the type of substrate used. The objective of this research was to evaluate the growth of eggplant seedlings grown in alternative substrates with increasing levels of "moinha" (residue from the coffee dry milling process) replacing the commercial substrate. The experiment was carried out in a completely randomized design, with six treatments and ten replicates. The treatments were: T1: commercial substrate (control); T2: 0% moinha (MO) + 40% burnt rice husk (BRH) + 15% coconut fiber (CF) + 5% eggshell (ES) + 40% commercial substrate (CS); T3: 10% MO + 40% BRH + 15% CF + 5% ES+ 30% CS; T4: 20% MO + 40% BRH + 15% CF+ 5% ES + 20% CS ; T5: 30% MO + 40% BRH + 15% CF + 5% ES + 10% CS; T6: 40% MO + 40% BRH + 15% CF + 5% ES + 0% CS. The electrical conductivity of the substrates and the seedling total dry mass, plant height and stem diameter were evaluated. It is recommended to use the substrate containing 20% MO + 40% BRH + 15% CF + 5% ES + 20% CS.

Key words: growth, moinha, *Solanum melongena,* seedling quality, horticulture.

RESUMEN

La formación de plántulas es una de las fases más importantes para el ciclo de cultivo de la berenjena. El tipo de sustrato utilizado es uno de los factores decisivos para obtener plántulas de calidad y el consiguiente aumento de la productividad. El objetivo de este estudio fue evaluar el crecimiento de las plántulas de berenjena cultivadas en sustratos alternativos con niveles crecientes de "moinha" (residuo del proceso de molienda en seco de café), en sustitución del sustrato comercial. El experimento se realizó en un diseño completamente al azar, con seis tratamientos y diez repeticiones. Los tratamientos fueron: T1: sustrato comercial (control); T2: 0% de moinha (MO) + 40% de cáscara de arroz quemada (CAQ) + 15% de fibra de coco (FC) + 5% de cáscara de huevo (CH) + 40% de sustrato comercial (SC); T3: 10% MO + 40% CAQ + 15% FC + 5% CH + 30% SC; T4: 20% MO + 40% CAQ + 15% FC + 5% CH + 20% SC; T5: 30% MO + 40% CAQ + 15% FC + 5% CH + 10% SC; T6: 40% MO + 40% CAQ + 15% FC + 5% CH + 0% SC. Se evaluó la conductividad eléctrica de los sustratos y la masa seca total de las plántulas, la altura de la planta y el diámetro del tallo. Se recomienda utilizar el sustrato que contiene 20% de MO + 40% de CAQ + 15% de FC + 5% de CH + 20% de SC.

Palabras clave: crecimiento, moinha, *Solanum melongena*, plántulas de calidad, horticultura.

Introduction

In the production of quality vegetables, seedling formation is one of the most important phases for the crop cycle (Zaccheo *et al*., 2013), and the type of substrate used for growing them is one of the crucial factors for obtaining high-quality seedlings and, therefore, high-yield plants.

The substrate should provide an adequate supply of air and water to the root system, besides physically supporting plant growth (Júnior *et al*., 2014). In addition, growth media or substrates must be free of plant pathogens, easy to handle, long-lasting, highly available and of low cost (Fernandes *et al.,* 2006; Mesquita *et al*., 2012).

Several studies have been developed to reuse agricultural and agro-industrial residues in the composition of substrates to minimize the environmental impact that would be caused by inadequate disposal, and reduce production costs by replacing commercial substrates (Krause *et al*., 2017; Almeida *et al*., 2018; Guisolfi *et al*., 2018; Meneghelli *et al*., 2018). The reuse of residues to compose substrates

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1 Department of Plant Science, Federal University of Viçosa, Viçosa (Brazil).
2 Department of Plant Science, Federal Institute of Education, Science and Technology of Espírito Santo, Santa Teresa campus, Santa Teresa (Brazil).
3 Department of Plant Science, Darcy Ribeiro North Fluminense State University, Campos dos Goytacazes (Brazil).
* Corresponding author: carol.merlo@hotmail.com

in eggplant seedling production has been investigated by several authors (Costa *et al*., 2012; Bardiviesso *et al*., 2014; Ferreira *et al*., 2014).

Agricultural wastes, such as coir (or coconut fiber), burnt rice husk, eggshell, and the dry residue of coffee beans, also known as "moinha", are highly available and potential alternative materials to compose substrates for the production of eggplant seedlings.

Coconut coir dust has a predominance of medium and fine particle size fractions, which makes it an adequate substrate when looking for high porosity and presence of micropores. These are important factors to obtain good aeration and water-holding capacity (Zorzeto *et al*., 2014). Coconut fiber is a renewable and highly biodegradable substrate with improved mechanical properties and low cost (Machado *et al*., 2014). Therefore, its high availability, low cost, and physical-chemical properties make coconut fiber an adequate alternative material for the composition of substrates.

Burnt rice husk has a high water-holding capacity and fast and efficient drainage. These characteristics provide good oxygenation to the roots, well-aerated conditions, resistance to decomposition, relative structure stability, low density, and pH close to neutrality (Soares *et al*., 2012). Due to the aforementioned properties, rice husk has become intensively used as a substrate for plant growth.

Eggshells are composed of organic and inorganic substances. Calcium carbonate ($CaCO_3$) is the main component in the inorganic phase of hen eggshells (Medeiros and Alves, 2014) and they can be used as one of the nutritional materials for the formulation of a substrate.

Moinha is a residue generated during the processing of coffee beans. It is composed of plant residues, such as leaves, branches, inflorescences, and poorly formed coffee beans, which, when dried together with the coffee beans, are burned and released from the dryer. When chemically analyzed, this material was found to contain a high content of organic matter as well as phosphorus, potassium, and nitrogen (Meneghelli *et al*., 2016). In addition to its potential use as a fertilizer, the authors found a high electrical conductivity in this residue, which is why they verified that concentrations greater than 10% of moinha in the substrate provided lower values in the analyzed variables in Conilon coffee seedlings. In this sense, different concentrations of moinha in the composition of substrates for different crops should be investigated, considering the hypothesis that this residue is an important substrate material, but in an adequate proportion that requires experimentation.

It is believed that partial or even total replacement of commercial substrates by the combination of different agricultural residues may be a sustainable alternative for the production of eggplant seedlings since all of them have appropriate characteristics to seedling germination and development. In addition, it reduces costs when compared to commercial growing media (Mesquita *et al*., 2012; Ferreira *et al*., 2014). However, it is necessary to know the exact proportions of moinha and commercial substrate in the composition of the alternative substrate, which will provide high-quality eggplant seedlings.

The objective of this research was to evaluate the growth of eggplant seedlings grown in alternative substrates with increasing levels of moinha, replacing the commercial substrate.

Material and methods

The experiment was carried out at the plant nursery of the Federal Institute of Espirito Santo, Campus Santa Teresa, located in the municipality of Santa Teresa, Espirito Santo State (18°48' S, 40°40' W, 130 m a.s.l.), in Brazil. According to Köppen and Geiger (1928), the climate of the region, is Cwa (subtropical dry-winter climate), with an average annual temperature of 24.6°C and average annual precipitation ranging from 700 to 1200 mm. The temperature and relative humidity of the air during the experimental period ranged from 19.9 to 38.2°C and 47.5 to 69.5%, respectively. The plant nursery was covered with a shading screen, which provided a reduction of solar radiation by 50%.

The residues used in the alternative substrate composition for the production of eggplant seedlings were: residues from the coffee dry milling process (moinha), eggshells, dry coconut fiber (coir), and burnt rice husk. Eggshells were collected from local restaurants and dried in the sun for 3 d. Subsequently, they were crushed and ground into a fine powder. Burnt rice husk and coir were donated by the company Fibria, located in Aracruz-ES, Brazil. Moinha was collected from a private property (Sítio da Saudade), located near the experimental area in Santa Teresa, Espirito Santo, Brazil. Before being used, moinha was sieved in a stainless-steel sieve with a 4 mm mesh.

The chemical and physical-chemical characterization of the residues used in the composition of the substrates was carried out at the Soil and Solid Waste Laboratory of the

Agricultural Engineering Department of the Federal University of Viçosa. The physical-chemical analysis consisted of the determination of the electrical conductivity (EC), through a laboratory conductivity meter (model P613, Shanghai Yoke Instrument Co. Ltd., Shangai, China). The chemical analysis consisted in the determination of the pH, using a bench pH meter (model HMCDB-150, Highmed Solutions in Measurement Technology Ltd- ME Tatuape, São Paulo, Brazil) and the quantification of organic matter (OM), total nitrogen (TN), phosphorus (P) and potassium (K), according to the methodology described by de Matos (2015).

The chemical and physical-chemical attributes (EC) of coconut fiber, moinha, eggshells and burnt rice husk used in the experiment are shown in Table 1.

TABLE 1. Chemical and physical chemical attributes (EC) of eggshells (ES), coconut fiber (CF), moinha (MO) and burnt rice husk (BRH) used in the experiment.

Residues	pH[(a)]	EC	OC_{ro}	OC_T	OM	N_T	P	K	Ca
		$dS\ m^{-1}$	$g\ kg^{-1}$						
ES	9.37	0.37	2.8	3.6	6.2	0.87	0.08	0.06	31.9
CF	7.15	0.09	57.1	74.1	127.7	0.66	0.05	0.14	0.0
MO	5.60	6.49	45.3	58.9	101.5	3.7	0.14	0.71	0.0
BRH	5.9	1.15	31.5	40.9	70.5	0.59	0.08	0.03	0.0

pH[(a)]: potential of hydrogen in water; EC: electrical conductivity; OC_{ro}: readily oxidizable organic carbon; OC_T: total organic carbon; OM: organic matter; N_T: total nitrogen; P: phosphorus; K: potassium; Ca: calcium.

A completely randomized experimental design (CRD) was used, with six treatments and ten replicates. Each experimental unit consisted of 20 seedlings, totaling 1,200 seedlings used in the experiment. Six plants were considered useful for each experimental unit.

The treatments: T1: commercial substrate (control); T2: 0% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshells + 40% commercial substrate; T3: 10% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshell + 30% commercial substrate; T4: 20% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshell + 20% commercial substrate; T5: 30% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshell + 10% commercial substrate; T6: 40% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshell + 0% commercial substrate were evaluated.

Chemical attributes of the commercial Bioplant® substrate, obtained by Paixão *et al.* (2012) are shown in Table 2.

TABLE 2. Chemical analysis of the commercial substrate Bioplant®.

pH[(a)]	N_T	P	K	Ca	OC_T	OM
	$g\ kg^{-1}$					
5.62	0.62	1.55	0.44	1.84	21.00	52.21

pH[(a)] in $CaCl_2$($CaCl_2$ pH); N_T: total nitrogen; P: phosphorus; K: potassium; Ca: calcium; OC_T: total organic carbon; OM: organic matter.

Seeds of eggplant (*Solanum melongena* L.) cultivar Embu were sown in a 200-cell Styrofoam propagation tray, placing two seeds per cell. The seedling production system was arranged in suspended trays, placed in masonry raised beds. Seedlings were manually irrigated twice a day, once in the morning and once in the afternoon without fertilizer application. Fifteen days after sowing (DAS) thinning was performed leaving only one seedling per cell, the most vigorous one.

The variables plant height, stem diameter and total dry mass were evaluated at 31 DAS. Plant height was obtained using a millimeter ruler, measuring from the stem base to the apical bud that originated the last leaf. The stem diameter was measured using a precision digital caliper (Zaas Precision). To obtain the total dry mass, the roots were carefully washed in running water, over a sieve. Afterward, the seedlings were packed in paper bags and placed in a forced air circulation drying oven (model WGL-30B, Westtune, Suzhou Beiyin medical equipment Co., Ltd., Zhejiang, China). The seedlings were maintained at 65°C until a constant weight was achieved. Subsequently, the materials were weighed in an electronic precision scale (1 g) (reference LS5, Scientific Mars, São Paulo, Brazil). In addition to the biometric and gravimetric analysis, the electrical conductivity of the substrates was determined using a conductivity meter (model P613, Shanghai Yoke Instrument Co., Ltd, Shangai, China) following the methodology proposed by de Matos (2015).

All the evaluated variables were submitted to the tests of normality (Lilliefors) and homoscedasticity (Bartlett), to validate and assess their variance. Due to the qualitative difference between the commercial substrate treatment (T1) and the other substrates (T2 to T6), the analysis of variance for qualitative treatments was performed only considering the contrast between T1 and the other treatments in the decomposition of the sums of squares of the treatments. In addition, for the comparisons between treatments T2, T3, T4, T5, and T6, related to the moinha level, their adjustments were adopted in regression models using the orthogonal polynomial method. For all procedures, an "α" equal to 0.05 was adopted. All the statistical analyzes were performed through the SAEG 9.1 software (2007).

Results and discussion

For the variables stem diameter (SD), plant height (PH), and total dry mass (TDM), superiority ($P<0.05$) was observed for the group of treatments containing 0, 10, 20, 30 and 40% of moinha, in comparison to the commercial substrate treatment (Tab. 3). This result may be associated with the greater amount of nutrients contained in this residue, especially nitrogen. Nitrogen can favor leaf expansion and provide greater plant growth (Eichler *et al*., 2008). Increases in shoot dry mass are expected due to the application of nitrogen doses, mainly because this nutrient contributes to vegetative growth, leaf expansion, and stem growth rate (Aleman and Chaves, 2016) Thus, the higher content of nitrogen in the treatment group containing moinha may have provided greater vegetative growth in the seedlings.

The use of eggshells and coconut fiber may have positively contributed to seedling development, even because no moinha was added (0% moinha) in T1. The eggshell mineralized in a short period of time could have contributed to the availability of nutrients. Studying the use of eggshell as a soil acidity corrective, Lo Monaco *et al*. (2015) concluded that in a period of 2 weeks, the residue added to the soil in the form of powder was mineralized and favored the increase of pH in the soil. Likewise, Niezer and Silveira (2014), using eggshell to recompose the pH of the soil, found a decrease in acidity after 3 weeks of incubation of the soil with the residue. Although it has the lowest organic matter content compared to the other residues used, eggshells provide nutrients, especially calcium (Tab. 1), in greater amounts than the commercial substrate (Paixão *et al*., 2012). Calcium is extremely important in the primary growth phase of plants, since it is involved in the construction of cell walls, in addition to other essential processes such as photosynthesis, cell division, cytoplasmic movements, and increased cell volume (Malavolta *et al*., 1997). Moreover, calcium plays an important role in ion absorption and when in deficit it affects root growth points (Vitti *et al*., 2006). Coconut fiber may have contributed to the physical quality of the substrate, providing adequate aeration and water retention. Granulated coconut fiber shows a predominance of intermediate and fine fractions, which may be appropriate when seeking high porosity and presence of micropores, which are responsible for good aeration and water retention in the environment (Zorzeto *et al*., 2014).

In highly weathered tropical and subtropical soils, organic matter is of great importance for crop nutrient supply, cation retention, toxic and micronutrient complexation, structure stability, infiltration and retention, aeration, and microbial activity, constituting a fundamental component of its productive capacity (Bayer and Mielniczuk, 1999). Organic matter added to soil favors numerous microbiological processes related to mineralization and nutrient release to plants, nitrogen fixation (symbiotic to non-symbiotic), and decomposition of organic waste. It also improves the physical qualities of the soil such as structure development and aggregate stability, which are beneficial for plant growth and development (Bento, 1997). In addition to physical factors, the increase in organic matter content promotes improvements in chemical attributes such as increased pH and base saturation, as well as aluminum complexation and soil solution precipitation (Mello and Vitti, 2002).

Regarding the variable electrical conductivity (EC), there was no difference ($P<0.05$) between the two groups of contrasted treatments.

TABLE 3. Significance levels, contrast estimators and *P* value between the conventional substrate treatment and those with different levels of moinha in the composition.

	Treatments							
	Conventional	**0%**	**10%**	**20%**	**30%**	**40%**	**Estimator**	***P* value**
				Moinha level				
Contrast coefficients	5	-1	-1	-1	-1	-1	-	-
				Stem diameter (mm)				
Means of the treatment	1.67	1.57	1.88	1.99	2.05	1.87	-1.00	0.0189
				Plant height (cm)				
Means of the treatment	4.00	4.00	5.09	6.04	5.78	4.48	-5.38	0.0082
				Total dry mass (mg)				
Means of the treatment	213.75	298.63	270.25	492.0	454.63	376.63	-823.38	0.0034
				Electrical conductivity (μS cm^{-1})				
Means of the treatment	48.75	45.00	55.00	55.00	31.25	83.75	-26.25	0.4986

When the different moinha levels were evaluated (polynomial regression), second-degree effects ($P<0.05$) were observed for the variables electrical conductivity (EC), stem diameter (SD) and plant height (PH). For the total dry mass (TDM), no adjustment was observed in regression models ($P<0.05$).

It was observed that, in general, increasing percentages of moinha in the substrate composition led to an increase of the salinity level in the substrate (greater electrical conductivity) (Fig. 1). This result was already expected since moinha showed higher electrical conductivity than the other residues (Tab. 1). The high concentration of salts is a stress factor for plants, because it reduces the osmotic potential and causes the action of ions on the protoplasm (Harter *et al*., 2014). Water is osmotically retained in the saline solution, so that the increased concentration of salts makes it less and less available to plants (Ribeiro *et al*., 2001).

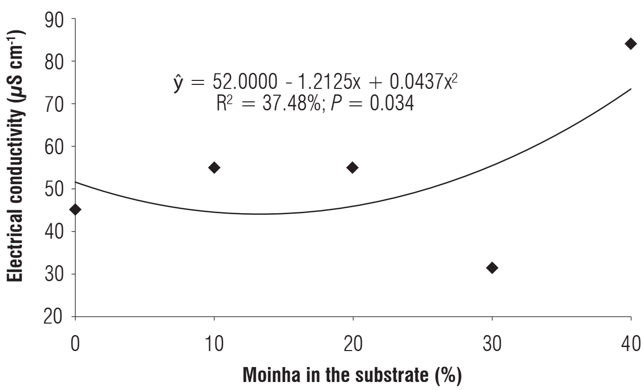

FIGURE 1. Electrical conductivity as a function of increasing percentages of moinha in the composition of the substrates.

The stem diameter is a key variable to evaluate the survival potential and growth of seedlings (Souza *et al*., 2006). These authors emphasize that within the same species, plants with large stem diameters have higher survival rates because they have the capacity to generate and develop roots. In this sense, it can be observed that the substrate containing 26.3% moinha provided the highest stem diameter (2.05 mm), with a decrease in the variable above this percentage (Fig. 2). However, the stem diameter values obtained at doses higher than 26.3% were still greater when compared to the treatment without the addition of moinha in the substrate (0% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshell + 40% commercial substrate), demonstrating the beneficial effect of this residue on substrate composition.

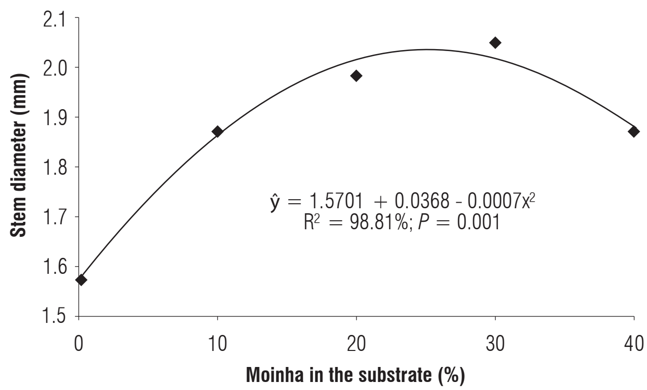

FIGURE 2. Stem diameter of eggplant seedlings as a function of increasing percentages of moinha in the composition of substrates.

The smaller amount of nutrients present in the substrate, due to the absence of moinha, may have been the reason for having a smaller stem diameter in T2. In the highest concentrations of moinha, the decrease in this variable can be attributed to the higher salinity of this residue, which impairs plant growth and development, as mentioned earlier. A similar result was obtained by Almeida *et al*. (2018), who verified a decrease in the stem diameter of cucumber seedlings with the increase of the residue in the alternative substrate.

The plant height increased significantly with the gradual replacement of the commercial substrate by moinha up to the percentage of 21.80%, providing a maximum height of 5.94 cm (Fig. 3). Above this percentage, there is a decrease in the plant height values. However, similarly to what occurred for the stem diameter, the plant height values obtained at higher moinha concentrations were still greater than those registered for the treatment in which no moinha was added (T2).

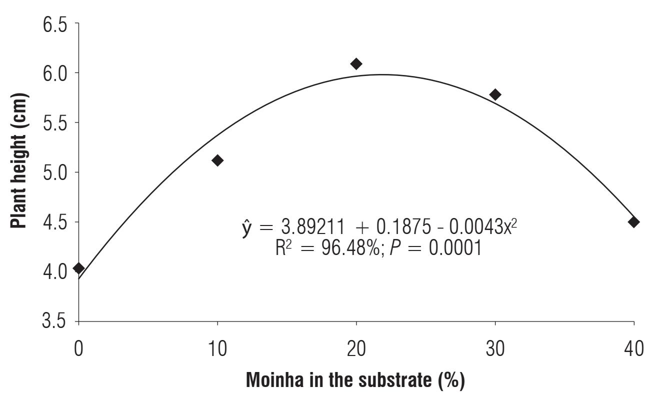

FIGURE 3. Plant height of eggplant seedlings as a function of increasing percentages of moinha in the composition of the substrates.

In general, it was verified that the highest values obtained for the assessed variables were those in which around 20% moinha was added in the composition of the substrates. When moinha proportion increased above this percentage, all variables showed decreasing values. It is believed that this result could be associated with the higher salinity levels achieved with increasing moinha percentages in the substrate, evidenced by the greater electrical conductivities (Fig. 1). The high concentration of salts is a stress factor for plants, as it reduces the osmotic potential and provides the action of ions on the protoplasm (Harter *et al*., 2014). The water is osmotically retained in the saline solution, making it less and less available to the plants, and, consequently, interfering negatively in the growth and productivity of the crop.

Studying the use of agricultural residues in the composition of substrates for the production of tomato seedlings, Krause *et al*. (2017) found that the highest values obtained from the analyzed variables were those in which around 15 to 32% of moinha was used in the composition substrates. With the increase in this proportion, all variables showed decreasing values. The authors also believe that this fact may be associated with higher salinity in the substrate, evidenced by the greater electrical conductivity in the residue. Likewise, Meneghelli *et al.* (2016), studied moinha as an alternative substrate in conilon coffee seedlings and also found a decrease in the analyzed growth variables and concluded that the high salinity of the residue may have favored this result.

The use of about 20% of moinha in the substrate composition promoted the best development of eggplant seedlings, probably because at this proportion the negative effects of salinity did not overcome the beneficial effects of the nutrients, especially nitrogen, present in higher amounts (Tab. 1).

Conclusion

The moinha can be used to partially replace the commercial substrate for eggplant seedling production. We recommend using the substrate containing 20% moinha + 40% burnt rice husk + 15% coconut fiber + 5% eggshell and 20% commercial substrate.

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Effect of water deficit on some physiological and biochemical responses of the yellow diploid potato (*Solanum tuberosum* L. Group Phureja)

Efecto del déficit hídrico sobre algunas respuestas fisiológicas y bioquímicas en papa amarilla diploide (*Solanum tuberosum* L. Group Phureja)

Wilmar Ariza[1], Luis Ernesto Rodríguez[1], Darwin Moreno-Echeverry[1], Carlos Arturo Guerrero[2], and Liz Patricia Moreno[1]*

ABSTRACT

Water availability is one of the main limitations of potato yields due to the high sensitivity of this crop to water deficit. The objective of this study was to determine the effect of water deficit on some physiological and biochemical responses in yellow diploid potato plants (*Solanum tuberosum* L. Group Phureja) of the cultivars Criolla Colombia, Criolla Dorada and Criolla Ocarina. Plants at tuber initiation were subjected to two treatments: continuous irrigation and water deficit imposed by withholding water at tuber initiation for 17 d. The results showed that plants under water deficit increased chlorophyll concentration, malondialdehyde and proline content. However, these plants showed a decrease in stomatal conductance, leaf area, total dry mass and exhibited a higher root/shoot ratio in all potato cultivars. In addition, all the cultivars also showed a decrease in yield, which was associated with sensitivity to water stress. Although the high content of proline and high root/shoot ratio may be associated with tolerance to water deficit, this association was not observed in these cultivars, probably due to the high reduction of stomatal conductance, which limited the production of photoassimilates, plant growth, and, therefore, the yield.

Key words: leaf area, membrane stability, malondialdehyde, proline.

RESUMEN

La disponibilidad hídrica es uno de las principales limitantes del rendimiento en papa debido a la alta sensibilidad de este cultivo al déficit hídrico. El objetivo de este estudio fue determinar el efecto del déficit hídrico sobre algunas respuestas fisiológicas y bioquímicas en plantas de papa amarilla diploide (*Solanum tuberosum* L. Group Phureja) de los cultivares Criolla Colombia, Criolla Dorada y Criolla Ocarina. Las plantas al inicio de la tuberización fueron sometidas a dos tratamientos: riego continuo y déficit hídrico por suspensión de riego al inicio de la tuberización durante 17 días. Los resultados mostraron que las plantas con déficit hídrico aumentaron la concentración de clorofila y el contenido de malondialdehído y prolina. Sin embargo, estas plantas también mostraron una disminución en la conductancia estomática, el área foliar y la masa seca total, y presentaron una mayor relación raíz/parte aérea en todos los cultivares. Además, todos los cultivares mostraron una disminución en el rendimiento, que se asoció con su sensibilidad al déficit hídrico. Aunque el alto contenido de prolina y la alta relación raíz/parte aérea pueden estar asociados con la tolerancia al déficit hídrico, esta asociación no se observó en estos cultivares, probablemente debido a la alta reducción de la conductancia estomática, que limitó la producción de fotoasimilados, el crecimiento de la planta y por tanto el rendimiento.

Palabras clave: área foliar, estabilidad de membranas, malondialdehído, prolina.

Introduction

The potato (*Solanum tuberosum* L.) is the fourth most important crop in planted areas worldwide and the third most important food for human consumption after rice and wheat (Devaux *et al*., 2014; Hardigan *et al*., 2017; Kammoun *et al*., 2018). Potatoes are among the plants that are susceptible to water deficit causing a decrease in both yield and quality of tubers (Monneveux *et al*., 2013). The effect of water deficit on the yield of this crop depends on the stage of plant development at which it occurs and also on the duration and severity (Jefferies, 1995).

Tuber initiation is one of the most sensitive stages to water deficit, since the development of the plant is affected, as well as the supply of photoassimilates to the tuber

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[1] Departamento de Agronomía, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Bogota (Colombia).
[2] Departamento de Ciencias Fisiológicas, Facultad de Medicina, Universidad Nacional de Colombia, Bogota (Colombia).
* Corresponding author: lpmorenof@unal.edu.co

(Dallas-Costa *et al*., 1997). Water deficit in the potato (*S. tuberosum* L.) reduces stomatal conductance (g_s), resulting in a decrease in the photosynthetic rate and an increase of reactive oxygen species (ROS) (Shi *et al*., 2015). ROS cause the degradation of macromolecules such as chlorophylls, proteins, nucleic acids and lipids (Mahmud *et al*., 2015). In addition, increasing ROS can produce photoinhibition affecting the functionality of photosystems. This is observed in potatoes under severe stress conditions (Li *et al*., 2017). Another target of ROS is the cell membranes due to lipid peroxidation that modifies their permeability (Lima *et al*., 2002). The modifications already mentioned cause a decrease in the growth of the plant since cell expansion processes are limited by the decline in water content and the loss of turgor pressure (Rolando *et al*., 2015; Kesiime *et al*., 2016). One of the first responses to water deficit is osmotic adjustment due to the accumulation of osmotically active molecules that allow plants to absorb water and maintain cellular turgor pressure (Singh *et al*., 2000). In potatoes, the increase in proline accumulation is associated with osmoregulation under water deficit (Mahmud *et al*., 2015).

The yellow diploid potato (*S. tuberosum* L. Group Phureja) is widely cultivated in the Andes from western Venezuela to central Bolivia with an important center of diversity in southern Colombia and northern Ecuador (Ghislain *et al*., 2006). A great variability in response to water deficit is found in diploid varieties (Coleman, 2008; Anithakumari *et al*., 2012; Cabello *et al*., 2012). The sensitivity of yellow potato to water deficit limits its distribution to areas with optimal levels of precipitation and irrigation, and this reduces the potential area of cultivation. New diploid yellow potato cultivars have been recently developed in Colombia with high levels of tolerance to the pathogen *Phytophtora infestans* (Mont.) de Bary and with better nutritional characteristics regarding levels of iron, zinc and proteins, such as Criolla Ocarina (UN 64) and Criolla Dorada (UN 04) (Peña *et al*., 2015). The objective of this research was to determine the effect of water deficit on some physiological and biochemical responses of the cultivar (cv.) Criolla Colombia (Colombia), the most commercially important cultivar nowadays, as well on the new cultivars Criolla Dorada (Dorada) and Criolla Ocarina (Ocarina).

Materials and methods

Plant material and growth conditions

This research was carried out in 2016 under greenhouse conditions at Facultad de Ciencias Agrarias, Universidad Nacional of Colombia, at 2,600 m a.s.l. Potato tubers of cultivars *S. tuberosum* L. Group Phureja, Colombia, Dorada and Ocarina, were planted in black plastic bags that contained 7 kg of soil. Plants were irrigated every day from the time of planting. During the experiment, daily records of temperature and relative humidity (average temperature of 19.7°C and an average relative humidity of 68.1%) were registered with a weather station (EL-USB-2, China).

Plants of the three cultivars were subjected to two treatments: well-watered (WW) maintaining a volumetric soil water content (VSWC) of 30% and water-deficit (WD) by withholding water for 17 d from 48 d after sowing, at tuber initiation. After the stress period, the plants were re-watered for recovery until the end of the crop cycle. A completely randomized design (CRD) with three replications was used. The measurements of the variables were taken at 5, 8, 11, 14 and 17 d after treatment (DAT) from completely expanded leaves of the upper third of the plant. For biochemical parameters, three technical replicates were used.

Field sampling and processing

Volumetric soil water content and leaf water status

The VSWC was measured at dawn with a time domain reflectometer (TDR-300, USA) at 20 cm depth. The leaf water potential (Ψ_w) was measured at dawn with a Scholander pressure chamber (PMS Model 615, CA, USA). Relative water content (RWC) was determined according to Anithakumari *et al*. (2012). At each point, leaves of the upper third of the plant were sampled and their fresh weight (FW) immediately recorded. Subsequently, the turgid weight (TW) was recorded after overnight rehydration at 4°C. For dry weight (DW) determination, samples were dried to constant weight at 75°C. Relative water content was calculated with the following equation:

$$\text{RWC (\%)} = (\text{FW-DW / TW-DW}) \text{ X } 100 \qquad (1)$$

Stomatal conductance and chlorophyll a fluorescence

The stomatal conductance (g_s) was measured with a porometer (SC-1, Decagon Device, USA) from 9.00 am to 11.00 am. The chlorophyll a fluorescence (Fv/Fm) was measured in dark-adapted leaves for 30 min using a MINI-PAM modulated fluorometer (Walz®, GmbH Effeltrich, Germany). The chlorophyll molecules were excited for 0.80 s with 1,500 μmol m^{-2} s^{-1} of actinic light. The parameter of maximum quantum yield of photosystem II (Fv/Fm) was registered. Fv/Fm is a ratio that indicates the quantum efficiency of photosystem II (PSII).

Chlorophyll concentration

Chlorophyll concentration (Chl_{SPAD}) was measured in leaves using a portable chlorophyll meter (SPAD-502 model, Konica Minolta, Sakai, Osaka, Japan) from 8.00 am to 11.00 am.

Malondialdehyde and proline content

The leaf samples were ground to a fine powder in liquid nitrogen and were stored at -80°C until the determinations were performed. Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction (Wang *et al*., 2013). The leaf sample (0.4 g) was homogenized in 2 ml of 10% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4126 xg for 30 min. Then, 4 ml of TCA (10%) containing 0.5% (w/v) of TBA were added to 1 ml of the supernatant. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The extracts were centrifuged at 4126 xg for 15 min, and the absorbance was measured at 450, 532 and 600 nm. The concentration of MDA was calculated by applying the formula proposed by Wang *et al*. (2013), CMDA (μmol ml^{-1}) = 6.45 X (A532 - A600) - 0.56 X A450. Proline content was determined based on the proline reaction with ninhydrin. For the proline determination, a 1:1:1 solution of proline, ninhydrin and glacial acetic acid was incubated at 97°C for 1 h. The reaction was arrested in an ice bath, the chromophore was extracted with toluene, and its absorbance was measured at 520 nm. The proline content is determined using a standard curve and is expressed as μmol proline g^{-1} fresh weight (FW) (Bates *et al*., 1973)

Growth parameters, dry-mass partitioning and yield

The leaf area (LA) was determined using a portable leaf area meter (LICOR Li 3100C, USA). The plants were individually separated into roots, stems and leaves and dried at 70°C until the weight became constant. The dry-mass (D-M) partitioning and the root/shoot ratio (R/S) were determined. These measurements were taken at the end of the stress treatment (17 DAT). The tuber yield was determined as tuber fresh weight per plant at the end of the crop cycle. The decrease in yield in plants under WD was calculated in comparison to the yield of WW plants.

Data analysis

The effects of irrigation treatments and time on the physiological parameters and yield were assessed by an analysis of variance (ANOVA) with repeated measures over time using a PROC MIXED procedure (Littell *et al*., 1998) of SAS 9.4 software (SAS institute). A one-way ANOVA was carried out to determine the effect of the treatments per day. The comparison of the means was performed with a Tukey multiple range test (P<0.05).

Results and discussion

Volumetric soil water content and leaf water status

VSWC remained close to 30% in WW treatments; these values were similar to those reported for well-watered plants under semi-controlled conditions (Banik *et al*., 2016). In the WD treatment, VSWC decreased significantly for all cultivars from 5 DAT (47%) to 17 DAT (72%) (Fig. 1). In this study, the irrigation suspension led to a high drop in the VSWC from 5 DAT to 17 DAT, which indicated that the plants had water deficit.

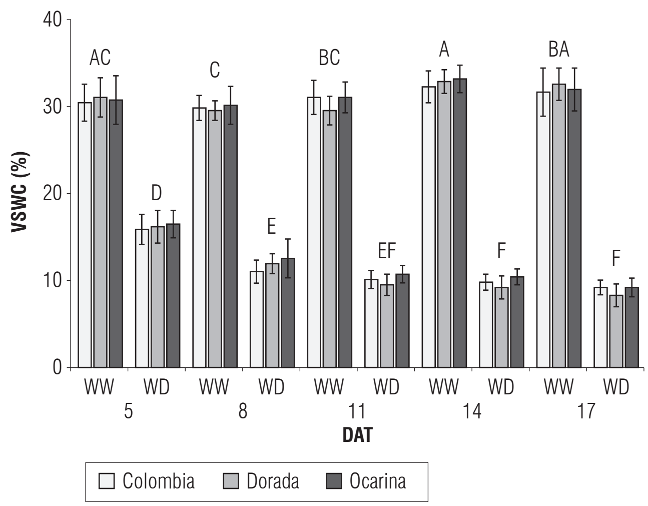

FIGURE 1. Volumetric soil water content (VSWC) in three yellow diploid potato cultivars. WW: well-watered, WD: water-deficit, DAT: days after treatment. Values represent means ± SD, n=6. Capital letters indicate the differences between treatments in time. Means denoted by the same letter do not significantly differ at P<0.05 according to the Tukey test.

The Ψ_w in plants subjected to WD was significantly lower (-0.57 MPa to -1.74 MPa) than in WW plants (-0.20 MPa to -0.24 MPa). In plants with WD at 8 DAT the lowest Ψ_w was observed in the cv. Colombia (-0.86 MPa) compared with cv. Dorada and Ocarina (-0.71 MPa). At 11 DAT water potential of the cv. Colombia was similar to cv. Ocarina (-1.31 MPa) but lower than in the cv. Dorada (-1.18 MPa). At 14 and 17 DAT there were no differences in Ψ_w between cultivars (Fig. 2A). RWC in plants subjected to WD (81% - 57%) was significantly lower than in WW plants (90%) without differences between cultivars.

In plants with WD, the RWC decreased at 5 DAT (81%), at 8 DAT (70%), at 11 DAT (64%), and at 17 DAT (57%) in comparison to irrigated plants. From 5 DAT, both Ψ_w and RWC gradually decreased in plants under WD (Fig. 2B). Both the RWC and the Ψ_w are variables that indicate the hydric state of the plant as well as the level of stress that it

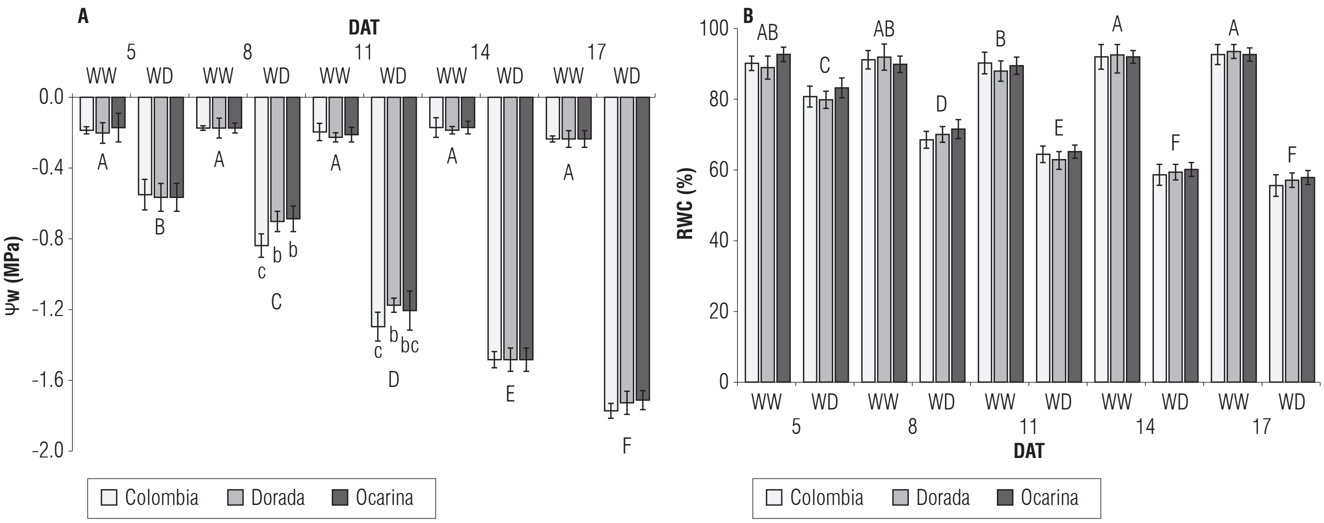

FIGURE 2. Changes in (A) leaf water potential (Ψ_w) and (B) relative water content (RWC) in three yellow diploid potato cultivars. WW: well-watered, WD: water-deficit, DAT: days after treatment. Values represent means ± SD, n=6. Capital letters indicate the differences between treatments in time. Lowercase letters indicate the differences per day. Means denoted by the same letter do not significantly differ at $P<0.05$ according to the Tukey test.

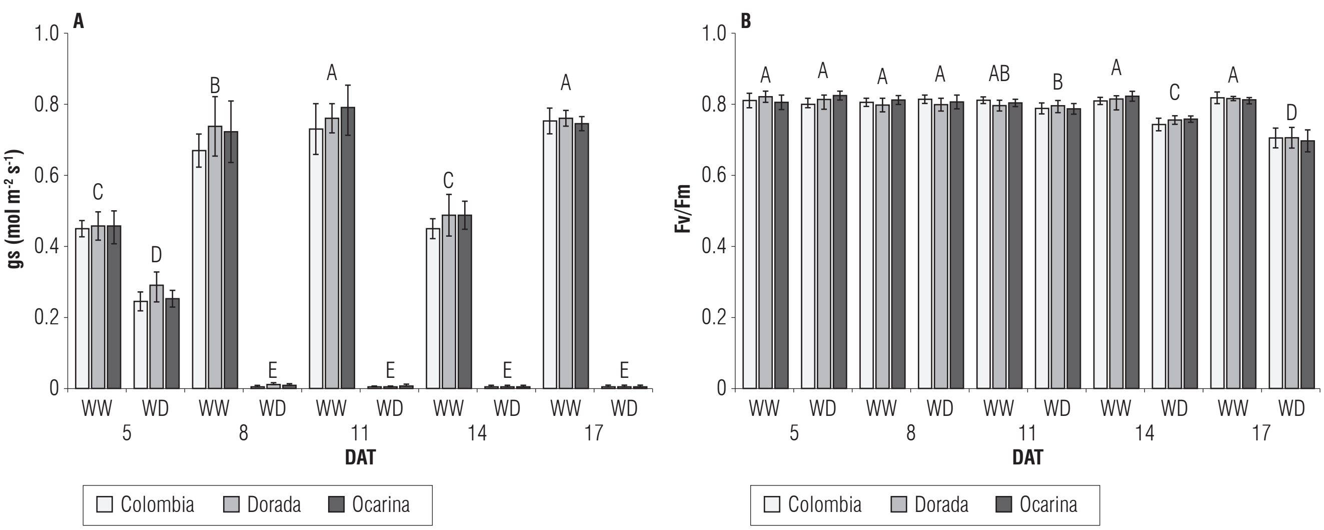

FIGURE 3. Changes in (A) stomatal conductance (g_s) and (B) chlorophyll a fluorescence (Fv/Fm) in three yellow diploid potato cultivars. WW: well-watered, WD: water-deficit, DAT: days after treatment. Values represent means ± SD, n=6. Capital letters indicate the differences between treatments in time. Means denoted by the same letter do not significantly differ at $P<0.05$ according to the Tukey test.

shows (Hsiao, 1973; Soltys-Kalina *et al*., 2016). Plants of all cultivars subjected to WD showed a reduction in both the RWC and Ψ_w. Based on the decrease in the RWC compared with well-watered plants, and according to the criteria established by Hsiao (1973), the three cultivars showed a mild water deficit at 5 DAT, while the stress was severe after 8 DAT. The RWC presented a pattern of decrease similar to that of the drop observed in the VSWC, while the Ψ_w showed a greater decrease, mainly at 17 DAT. These data suggest that the cultivars developed mechanisms to retain or absorb water. These mechanisms include the synthesis of different osmolytes such as proline.

Stomatal conductance and Chlorophyll a fluorescence

Stomatal conductance was significantly lower in plants under WD (0.265 mol m^{-2} s^{-1} - 0.008 mol m^{-2} s^{-1}) compared with WW plants (0.456 mol $m^{-2}s^{-1}$ - 0.761 mol m^{-2} s^{-1}). The highest decrease in g_s in plants subjected to WD was at 5 DAT (0.265 mol m^{-2} s^{-1}) and at 8 DAT (0.012 mol $m^{-2}s^{-1}$). From 8 DAT to 17 DAT, g_s did not show significant differences in plants under WD (Fig. 3A). Due to the decrease of VSWC in the first days of irrigation suspension, the plants showed a high decrease in g_s, indicating that g_s in these cultivars is very sensitive to the reduction in water availability (Stiller *et al*., 2008; Timothy *et al*., 2018). The observed stomatal closure was related to an isohydric behavior as a strategy to avoid losing water under conditions of water deficit as has already been described for tetraploid potato cultivars (Liu *et al*., 2005). The stomatal closure observed also suggests an early stomatal limitation of photosynthesis in the three cultivars. Decreases in stomatal conductance cause an imbalance between light harvesting, electron transport and carbon assimilation, which leads to the production of ROS resulting in damage to PSII.

Fv/Fm was higher than 0.8 in WW plants and in plants subjected to WD until 11 DAT. In plants under WD, Fv/Fm values were lower than 0.8 at 14 DAT (0.75) and 17 (0.70) (Fig. 3A). Neither g_s nor Fv/Fm showed differences between cultivars (Fig. 3B). As a consequence of the stomatal closure induced by water deficit, the intercellular CO_2 concentration decreases causing an imbalance between the phases of photosynthesis and an increase in ROS that leads to photoinhibition (Lima *et al*., 2002; Rudack *et al*., 2017; Timothy *et al*., 2018). An indicator of the functionality of the photosynthetic apparatus is the quantum efficiency of photosystem II, determined by the Fv/Fm ratio (Lu and Zhang, 1998). Here, an Fv/Fm value of 0.7 was found at 17 DAT, suggesting the presence of mild PSII damage, since in plants with values higher than 0.75 there is an absence of damage in the PSII (Van der Mescht *et al*., 1999). The presence of Fv/Fm values higher than 0.8 at 8 DAT and 14 DAT, when severe stress and a high reduction in g_s are observed, suggests that these plants present early defense mechanisms to deal with oxidative stress caused by water deficit, which prevents or diminishes the damage of the PSII (Mane *et al*., 2008).

Chlorophyll concentration

Chl_{SPAD} was significantly higher in plants subjected to WD (46 SPAD Units - 57 SPAD Units) than in WW plants (41 SPAD Units - 43 SPAD Units) throughout the experimental period, without differences between cultivars (Fig. 4). Another factor that affects the photosynthetic capacity of plants is the content of the chlorophylls, since they are essential in the capture of light. An increase in Chl_{SPAD} was found in plants under water deficit in all the cultivars. These results contrast with what was reported in other diploid and tetraploid cultivars in which a reduction in the chlorophyll content under water deficit is found (Anithakumari *et al*., 2012). The increase in Chl_{SPAD} was observed from 5 DAT and was related to the severity of the stress and the reduction in the growth of the leaf. The increase in Chl_{SPAD} is also observed in tetraploid cultivars of potato under water deficit conditions (Ramírez *et al*., 2014; Rolando *et al*., 2015). Therefore, the increase in the content of chlorophylls observed is associated with the susceptibility of these cultivars to water deficit (Ramírez *et al*., 2014).

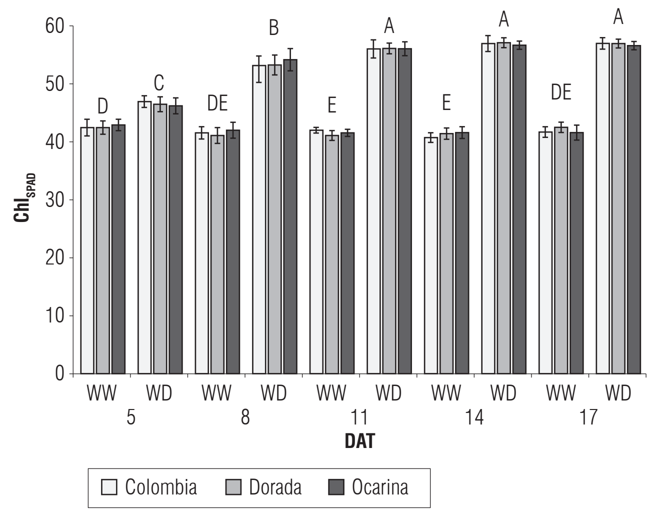

FIGURE 4. Chlorophyll concentration (Chl_{SPAD}) in three yellow diploid potato cultivars. WW: well-watered, WD: water-deficit, DAT: days after treatment. Values represent means ± SD, n=6. Capital letters indicate the differences between treatments in time. Means denoted by the same letter do not significantly differ at $P<0.05$ according to the Tukey test.

Malondialdehyde and proline content

MDA content in plants under WD increased significantly from 8 DAT (1.32 µmol g^{-1} FW - 1.46 µmol g^{-1} FW) to 17 DAT (3.05 µmol g^{-1} FW - 3.24 µmol g^{-1} FW) in comparison to WW plants (1.03 µmol g^{-1} FW - 1.13 µmol g^{-1} FW) (Tab. 1). Another effect of water deficit is cell membrane damage due to an increase in ROS (Shi *et al*., 2015). In potatoes, the increment in MDA under water deficit conditions is considered an indicator of the loss of membrane stability caused by lipid peroxidation (Li *et al*., 2017; Kammoun *et al*., 2018). There was an increase in MDA content in the three cultivars under WD. The data suggests an increase in oxidative stress that alters cellular metabolism, reducing plant performance in these cultivars (Anithakumari *et al*., 2012).

The proline content in plants subjected to WD also showed an increase at 8 DAT (1268 µg g^{-1} FW) and 17 DAT (1868 µg g^{-1} FW), reaching values 55 and 73 times higher than in WW plants (Tab. 1). Neither MDA nor proline content showed differences between cultivars. One of the first responses of the plant to water deficit is the synthesis of osmolytes in order to make an osmotic adjustment and absorb water (Shao *et al*., 2009). We found that the three cultivars under WD showed an increase in the proline content compared with WW plants. The data suggested that the synthesis of proline in the three cultivars contributed

TABLE 1. Malondialdehyde (MDA) and proline content in three yellow diploid potato cultivars.

DAT	Treatment	Cultivars	MDA μmol g^{-1} FW	Proline μg g^{-1} FW
8	WW	Colombia	1.06 ± 0.05	23.5 ± 4.1
		Dorada	1.13 ± 0.15	22.3 ± 4.8
		Ocarina	1.11 ± 0.11	22.4 ± 2.1
		Means	1.10 C	22.8 C
	WD	Colombia	1.46 ± 0.05	1181.8 ± 121.6
		Dorada	1.32 ± 0.12	1366.5 ± 85.8
		Ocarina	1.35 ± 0.18	1256.1 ± 107.6
		Means	1.38 B	1268.1 B
17	WW	Colombia	1.03 ± 0.15	26.5 ± 2.3
		Dorada	109 ± 009	23.3 ± 1.7
		Ocarina	1.10 ± 0.27	26.5 ± 2.8
		Means	1.08 C	25.4 C
	WD	Colombia	3.09 ± 0.05	1830.1 ± 111.1
		Dorada	3.05 ± 0.06	1833.1 ± 84.7
		Ocarina	3.24 ± 0.14	1943.4 ± 101.7
		Means	3.12 A	1868.9 A

WW: well-watered, WD: water-deficit, DAT: days after treatment, Treatment, FW: fresh weight. Values represent means ± SD, n=6. Capital letters indicate the differences between treatments in time. Means denoted by the same letter do not significantly differ at $P<0.05$ according to the Tukey test.

to the maintenance of water status during the water deficit period, preventing the RWC from being lower than 57%, even though the VSWC was low (9%). In potatoes, the presence of osmoregulation due to the increase in proline accumulation under water deficit is known (Teixeira and Pereira, 2007). The data also suggest that the increase in proline is one of the protection mechanisms of these cultivars in response to water deficit, and may partly explain the lack of damage observed in photosystem II (Mane *et al*., 2008).

Growth parameters, dry-mass partitioning and yield

The plants under WD at 17 DAT showed a significant decrease in LA (1426 cm^2) and in the total dry mass (TDM) (53 g) compared with WW plants (Figs. 5A-B). The plants with WD showed a higher fraction of D-M partitioning to the roots (35% - 40%) and a lower fraction of D-M partitioning to the leaves (17% - 22%) and the tubers (13% - 12%) in comparison to WW plants (Fig. 5C). The R/S ratio was significantly higher in plants subjected to WD (0.81 - 0.69) compared with WW plants (0.38 - 0.50) (Fig. 5D). The yield decreased significantly under WD for the cultivars Colombia (37%), Dorada (45%) and Ocarina (41%) compared to WW plants (Tab. 2). There were no differences between cultivars in the LA, DTM, D-M partitioning and yield (Fig. 5, Tab. 2). Water deficit in potatoes reduces the growth and yield (Jefferies, 1993; Lahlou *et al*., 2003; Kammoun *et al*., 2018). The high reduction of VSWC affects the water intake generating a decrease in the RWC and a loss of turgor pressure that limits the cellular elongation process (Rolando *et al*., 2015). Besides the high decrease of g_s observed after 8 DAT, the water deficit causes an increase in the resistance to the diffusion of CO_2 inside the leaf, reducing the production of photoassimilates required for growth (Dallas-Costa *et al*., 1997).

Yield in potatoes is considered as an indicator of tolerance to water deficit (Tourneux *et al*., 2003; Timothy *et al*., 2018). Here, a decrease in yield in plants under WD in the three cultivars was observed (Tab. 2). This reduction in yield was attributed mainly to the high sensitivity of g_s to the water deficit that limits the production of photoassimilates and the growth. Other parameter associated with a decrease in yield under water deficit is the D-M partitioning (Jefferies, 1993). In the evaluated cultivars, water deficit caused an increase in the D-M partitioning to the roots, generating a higher R/S ratio. Although the increase in the R/S ratio is a defense mechanism to cope with water deficit, it is considered as an indicator of susceptibility to water deficit of these cultivars since the partitioning of photoassimilates into tubers decreased (Jefferies, 1993; Tourneux *et al*., 2003).

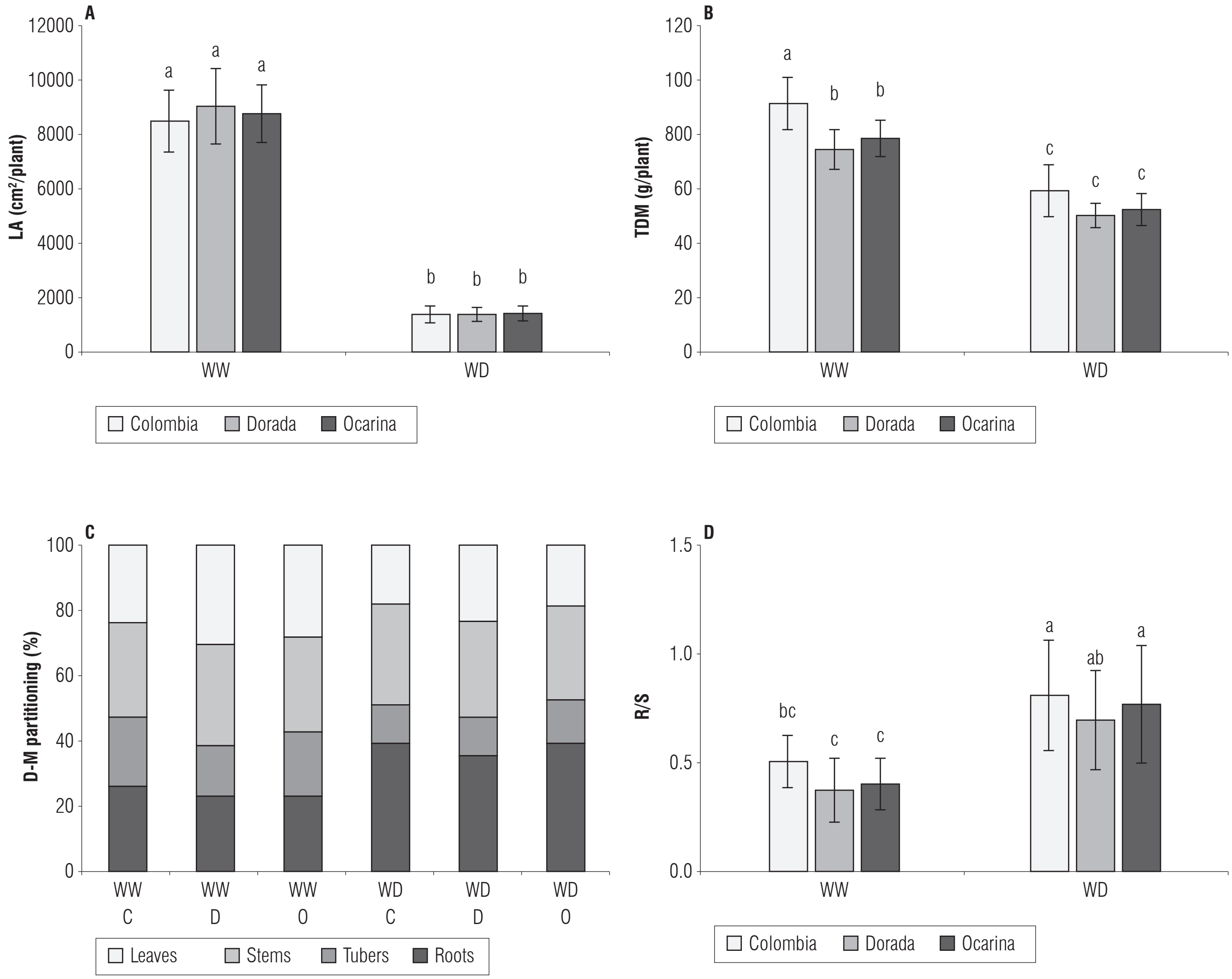

FIGURE 5. Changes in (A) leaf area (LA), (B) total dry mass (TDM), (C) dry-mass (D-M) partitioning and (D) root/shoot ratio (R/S) in three yellow diploid potato cultivars. WW: well-watered, WD: water-deficit, O: Ocarina, D: Dorada, C: Colombia. Values represent means ± SE, n=12. Means denoted by the same letter do not significantly differ at $P<0.05$ according to the Tukey test.

TABLE 2. Yield and reduction in yield (RY) in three yellow diploid potato cultivars.

Cultivars	Yield (g/plant)		RY (%)
	WW	WD	
Colombia	600 ± 59	378 ± 42	37
Dorada	613 ± 53	339 ± 32	45
Ocarina	624 ± 64	366 ± 31	41

WW: well-watered, WD: water-deficit. Values represent means ± SE, n=12. Means denoted by the same letter do not significantly differ at $P<0.05$ according to the Tukey test.

In conclusion, the drop in yield observed in the three cultivars may have also been related to the severity of the stress and to the defense mechanisms that the plants exhibited, such as the increase in proline. In this research, a high production of proline in all cultivars was observed in WD conditions. Proline synthesis requires both carbon skeletons and nitrogen that are also needed for plant growth. The development of defense mechanisms may be associated with tolerance to water deficit; however, this association was not observed in these cultivars due to the reduction of g_s and a limited production of photoassimilates. Although the three cultivars evaluated in this research showed sensitivity to water deficit, according to the percentage of reduction in yield compared to WW plants, the most sensitive to the water deficit imposed was cv. Dorada and the least sensitive was cv. Colombia.

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Chlorophyll *a* fluorescence and development of zucchini plants under nitrogen and silicon fertilization

Fluorescencia de la clorofila *a* y desarrollo de plantas de calabacín bajo fertilización con nitrógeno y silicio

Jackson de Mesquita Alves[1], Alex Serafim de Lima[2], Francisco Romário Andrade Figueiredo[3]*, Toshik Iarley da Silva[4], Lourival Ferreira Cavalcante[5], Francisco de Oliveira Mesquita[6], Evandro Franklin de Mesquita[2], and Cesenildo de Figueiredo Suassuna[1]

ABSTRACT

Zucchini (*Cucurbita pepo* L.) has a great economic and productive potential in the semi-arid region of Brazil, due to the wide acceptance by consumers and quick financial return. The nitrate (NO_3^-) assimilation process in C_3 plants such as zucchini is related to photorespiration, and Si accumulated near the stomata reduces the transpiration rate, making the photosynthesis cycle more efficient. The objective of this study was to evaluate the interaction between nitrogen and silicon fertilization on growth, chlorophyll index, and chlorophyll *a* fluorescence of zucchini plants. The treatments were distributed in a split-plot scheme in a randomized block design with three replicates. The plot was arranged by silicon levels (0 and 6 g/plant) and the subplots constituted by five nitrogen levels (30, 60, 90, 120 and 150 kg ha^{-1}). Leaf, stem and total dry masses, chlorophyll *a*, chlorophyll *b*, total chlorophyll, chlorophyll *a*/*b* ratio and chlorophyll *a* fluorescence were evaluated. The highest dry matter productions in zucchini were obtained in treatments without Si. Si and N application together positively influences the chlorophyll *a*/*b* ratio of zucchini plants. The interaction between Si and N positively influences the maximum fluorescence, variable fluorescence and quantum yield of photosystem II of zucchini plants.

Key words: *Cucurbita pepo* L., fertilizers, photosynthetic efficiency.

RESUMEN

El calabacín (*Cucurbita pepo* L.) presenta un gran potencial económico y productivo en la región semiárida de Brasil, debido a su amplia aceptación por los consumidores y su rápido rendimiento financiero., El proceso de asimilación de nitrato (NO_3^-) en plantas C_3 como el calabacín estaría relacionado en parte con la fotorrespiración, y el Si acumulado cerca de los estomas reduce la tasa de transpiración, haciendo que el ciclo de fotosíntesis sea más eficiente. Por lo tanto, el objetivo de este estudio fue evaluar la interacción entre el nitrógeno y la fertilización con silicio en el crecimiento, el índice de clorofila y la fluorescencia de la clorofila *a* en plantas de calabacín. Los tratamientos se distribuyeron en un esquema de parcelas divididas en un diseño de bloques al azar con tres repeticiones. La parcela se formó por niveles de silicio (0 y 6 g/planta) y las subparcelas constituidas por cinco niveles de nitrógeno (30, 60, 90, 120 y 150 kg ha^{-1}), totalizando 30 parcelas experimentales. Se evaluaron masas de hojas, tallos y secas totales, clorofila *a*, clorofila *b*, clorofila total, clorofila *a*/*b* y fluorescencia de la clorofila *a*. Las mayores producciones de materia seca de calabacín se obtuvieron en tratamientos sin Si. La aplicación de Si y N juntos influye positivamente en la proporción de clorofila *a*/*b* de las plantas de calabacín. La interacción entre Si y N influye positivamente en la fluorescencia máxima, la fluorescencia variable y el rendimiento cuántico del fotosistema II de plantas de calabacín.

Palabras clave: *Cucurbita pepo* L., fertilizantes, eficiencia fotosintética.

Introduction

Italian zucchini (*Cucurbita pepo* L. - Cucurbitaceae) is a vegetable-like fruit with an erect bushy growing habit, despite having an herbaceous stem. It is one of the most popular and appreciated vegetables in Brazilian cuisine (Nakada-Freitas, 2014; Azambuja *et al*., 2015). Zucchini is a summer squash that develops in autumn and spring,

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[1] Federal University of Campina Grande, Pombal, Paraiba (Brazil).
[2] State University of Paraíba, Catole do Rocha, Paraiba (Brazil).
[3] Federal Rural University of the Semi-arid, Mossoro, Rio Grande do Norte (Brazil).
[4] Federal University of Viçosa, Viçosa, Minas Gerais (Brazil).
[5] Federal University of Paraíba, Areia, Paraiba (Brazil).
[6] National Institute of the Semi-arid, Campina Grande, Paraiba (Brazil).
* Corresponding author: romarioagroecologia@yahoo.com.br

and also during the winter in places with a warmer climate (Filgueira, 2012).

The effects of nutrition and fertilization management practices on growth, development, and yield of zucchini, as well as other crops, has been one of the most studied agricultural aspects (Domingos *et al*., 2015). Nitrogen (N), which is responsible for several functions in plants and is directly linked to crop yield, is the main topic for most researchers (Oliveira *et al*., 2014).

Nitrogen is the second most required nutrient by plants (Viciedo *et al*., 2017). It is an essential macro-element that participates in the synthesis of amino acids, nucleic acids, secondary metabolites, and components of the chlorophyll molecule. In addition, this nutrient stimulates the photosynthetic capacity of leaves by increasing the content of stromal and thylakoid proteins in chloroplasts (Akram *et al*., 2011). However, over-fertilization with nitrogen has negative effects, such as disordered plant growth, susceptibility to pathogens and insects, nutrient deficiency due to competition with other nutrients, auto shading, and increased production costs (Porto *et al*., 2012; Xu *et al*., 2012).

Rational nitrogen fertilization practices are of great importance, being essential the search for methods that boost the beneficial effects of this nutrient. Therefore, nitrogen application combined with silicon (Si) may provide higher yields (Artigiani *et al*., 2014), making it a viable alternative. Adequate Si supply may induce growth, resistance to biotic and abiotic stresses, and increase the photosynthetic capacity (Ferraz *et al*., 2014). In addition, Si application provides a better utilization of some nutrients by plants (Watanabe *et al*., 2001).

Chlorophyll *a* fluorescence can be a good indicator of the plant's physiological state. The absorbed light energy excites the chlorophyll molecule and the disposing of its available energy occurs through three alternative pathways which compete for the excitation energy (photochemical dissipation, fluorescence or non-photochemical dissipation). Thus, changes in one of these processes, as in photosynthesis for example, shall affect the other ones (Cendrero-Mateo *et al*., 2016).

Plants spend about 25% of their total energy on NO_3^- assimilation (Rubio-Asensio *et al*., 2015). In C_3 plants, this process would be partly coupled with photorespiration, which stimulates the transport of chloroplasts malate to the cytosol for Nicotinamide Adenine Dinucleotide Hydride (NADH) formation (Bloom, 2015). NADH is used by nitrate reductase to reduce NO_3^- to NO_2^-, NO_3^- assimilation process and would be related in part to photorespiration. In this regard, Si accumulated near the stomata reduces the rate of transpiration (Oliveira and Castro, 2002), providing a direct relationship between transpiration and chlorophyll fluorescence and Si and N, making the photosynthesis process more efficient.

Chlorophyll fluorescence parameters are widely used in physiological studies since they are obtained by a non-destructive method that allows the qualitative and quantitative analysis of the absorption and use of light energy by the photosynthetic apparatus (Oliosi *et al*., 2017). Therefore, the aim of this study was to evaluate the interaction between nitrogen and silicon fertilization on growth, chlorophyll index and chlorophyll *a* fluorescence of zucchini plants.

Materials and methods

The experiment was carried out under field conditions in the agroecology sector of the Agrarian and Exact Sciences Department (DAE) of the State University of Paraiba (UEPB), Campus IV, in the municipality of Catole do Rocha, Paraiba, Brazil (6º20'38" S, 37º44'48" W, 275 m a.s.l.).

Zucchini seeds cv. Caserta were directly sown in planting pits, spaced at 1 × 1 m. After digging the pits of 30 × 30 × 30 cm dimension, 2 L of tanned cattle manure were added (Filgueira *et al*., 2012).

The soil of the experimental area was classified as eutrophic Fluvic Neosol, with a sandy loam texture (Embrapa, 2013). Before the installation of the experiment, soil samples were collected at a depth of 0-20 cm for physical and fertility analysis (Tab. 1) and the cattle manure chemical analysis was also performed (Tab. 2), according to the methodology proposed by Embrapa (2013). The soil was prepared by plowing sequenced by harrowing.

The treatments were distributed in a split-plot scheme in a randomized complete block design, with three replicates, and one plant per replicate. The plot was arranged by silicon levels (0 and 6 g/plant) and the subplots were five nitrogen levels (30, 60, 90, 120, and 150 kg ha^{-1}). The nitrogen source used was urea (45% N). The silicon levels were based on an estimate of 60 kg ha^{-1}. The product Bugram Protect®, which contains 100% pure silicon dioxide, pH 6.8 and 0.9% moisture, was used as a silicon source. The N doses were equally divided and applied to cover the soil twice, first at 15 days after planting (DAP) and then at 30 DAP. Foliar fertilization with Si was also divided into two applications,

TABLE 1. Chemical and physical characteristics of the Eutrophic Fluvic Neosol used in the experiment.

Chemical characteristics (Fertility)												
pH	Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	S	H+Al	CEC	PBS	OC	OM	N	AP
	$cmol_c dm^{-3}$						$cmol_c kg^{-1}$	%				$mg dm^{-3}$
6.7	1.49	0.54	0.10	1.72	3.85	0.00	3.85	100%	0.67	1.2	0.07	16.83

Physical characteristics									
Sand	Silt	Clay	EC	GD	PD	TP	FC	PWP	AW
$g kg^{-1}$			$g dm^{-3}$			(%)	$g kg^{-1}$		
640.0	206.0	154.0	Sandy loam	1.54	2.68	42.54	146.9	76.60	70.3

CEC: Cation exchange capacity; PBS: Percent base saturation; OC: Organic carbon; OM: Organic matter; AP: Assimilable phosphorus ; EC: Exchangeable cations; GD: Global density; PD: Particle density; TP: Total porosity; FC: Field capacity; PWP: Permanent wilting point; AW: Available water.

TABLE 2. Chemical characteristics of the cattle manure used in Catole do Rocha, 2019.

N	P	K^{+}	Ca^{2+}	Mg^{2+}	Na^{+}	Zn^{2+}	Cu^{2+}	Fe^{2+}	Mn^{2+}	SOM	OC	C/N
$g kg^{-1}$						$mg kg^{-1}$				$g kg^{-1}$		
12.76	2.57	16.79	15.55	4.02	5.59	60	22	8550	325	396.0	229.7	18:1

SOM: Soil organic matter; OC: Organic carbon; C/N: carbon-to-nitrogen ratio.

the first at 14 DAP and the second at 28 DAP. Thus, 540 g of silicon dioxide was diluted in 30 L of water in the first application and 540 g was diluted in 40 L of water in the second application. The silicon solutions were uniformly applied on the plants, using a 10 L capacity backpack sprayer, totaling 1.080 kg of silicon dioxide.

Micro-irrigation was performed through a drip irrigation system, spaced at 0.2 m and with a flow rate of 1.7 L per hour, at a service pressure of 147 KPa, using 16 mm drip tapes. The water was supplied through an Amazon well near the experiment site, and it was classified as moderately saline (1.2 dS m^{-1}) (Ayers and Westcot, 1999).

The chlorophyll *a* fluorescence trials were performed at 35 DAP using a modulated fluorometer (Model OS-30p, Hudson, USA). Foliar tweezers were placed for 30 min before the readings to adapt the leaves to the dark. The initial fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence ($F_v = F_m - F_0$) and quantum yield of photosystem II (F_v/F_m) were evaluated. Chlorophyll *a*, *b* and total contents were estimated by the non-destructive method using a portable chlorophyll meter (ClorofiLOG®, model CFL 1030, Porto Alegre, Brazil), and expressed as Falker Chlorophyll Index (FCI). For leaf, stem and total dry mass estimation, each part of the plant was packed in Kraft paper bags and dried in a forced-air circulation oven at 65°C until reaching constant weight. Subsequently, the material was weighed in a precision analytical balance (0.001 g), and the results expressed in g/plant.

The data were submitted to an analysis of variance by the F test at 5% probability. Polynomial regression analysis for the nitrogen factor and the Tukey test for the silicon factor were performed in case of significance. The statistical program R was used (R Core Team, 2018).

Results and discussion

Leaf dry mass (LDM), stem dry mass (SDM) and total dry mass (TDM) of zucchini were significant at 1% of probability in the interaction between the studied factors, nitrogen (N) and silicon (Si) (Tabs. 3 and 4).

For LDM, an inverse behavior was observed in the treatments with and without Si, respectively. The highest increase in LDM was found in the treatments without Si and at the maximum N dose (150 kg ha^{-1}), which corresponds to 338.6 g/plant. However, the LDM in the presence of Si reached its best value in the order of 265.4 g/plant, at the estimated dose of 87 kg ha^{-1} (Fig. 1A).

The values for SDM adjusted to a quadratic effect, both in the presence and absence of Si. The highest increase was registered at the N dose of 79.8 kg ha^{-1} in the treatments without Si, which corresponds to a 21.5% increase when compared to treatments under Si application, with values in the order of 33.3 and 27.4 g/plant, respectively (Fig. 1B).

For TDM, the results were similar to those of LDM. The best performance was obtained at the N dose of 150 kg ha^{-1} in the treatments without Si, corresponding to 355.5 g/plant, which may be related to the maximum increase observed in LDM (Fig. 1C).

TABLE 3. Summary of the analysis of variance by the mean-square values, from leaf dry mass (LDM), stem dry mass (SDM) and total dry mass (TDM) of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization in Catole do Rocha, 2019.

SV	DF	Mean square		
		LDM	SDM	TDM
Block	1	68.10^{ns}	0.85^{ns}	67.20^{ns}
Si	2	3853.30*	126.12**	5373.70*
Error a	2	180.10	0.10	174.00
N	4	4571.50**	213.17**	4090.60**
Si x N	4	11825.70**	132.69**	10190.10**
Error b	16	239.10	1.15	256.80
CV 1 (%)		5.60	1.19	4.95
CV 2 (%)		6.45	3.99	6.01

SV: Source of variation; DF: Degrees of freedom; CV: Coefficient of variation; ns: Not significant; ** and *: Significant effect by the F test at the level of 1 and 5% probability, respectively.

TABLE 4. Mean values for leaf dry mass (LDM), stem dry mass (SDM) and total dry mass (TDM) of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization in Catole do Rocha, 2019.

Parameter	Silicon (g/plant)	Nitrogen (kg ha^{-1})				
		30	60	90	120	150
LDM	0	242 a	250 b	191 b	217 a	355 a
	6	185 b	277 a	257 a	227 a	195 b
SDM	0	26 a	31 a	46 a	25 b	18 b
	6	21 b	26 b	27 b	27 a	23 a
TDM	0	268 a	280 a	236 b	241 a	372 a
	6	206 b	303 a	283 a	254 a	217 b

Means followed by equal letters in the same column do not differ statistically from each other by the Tukey test at 5% probability.

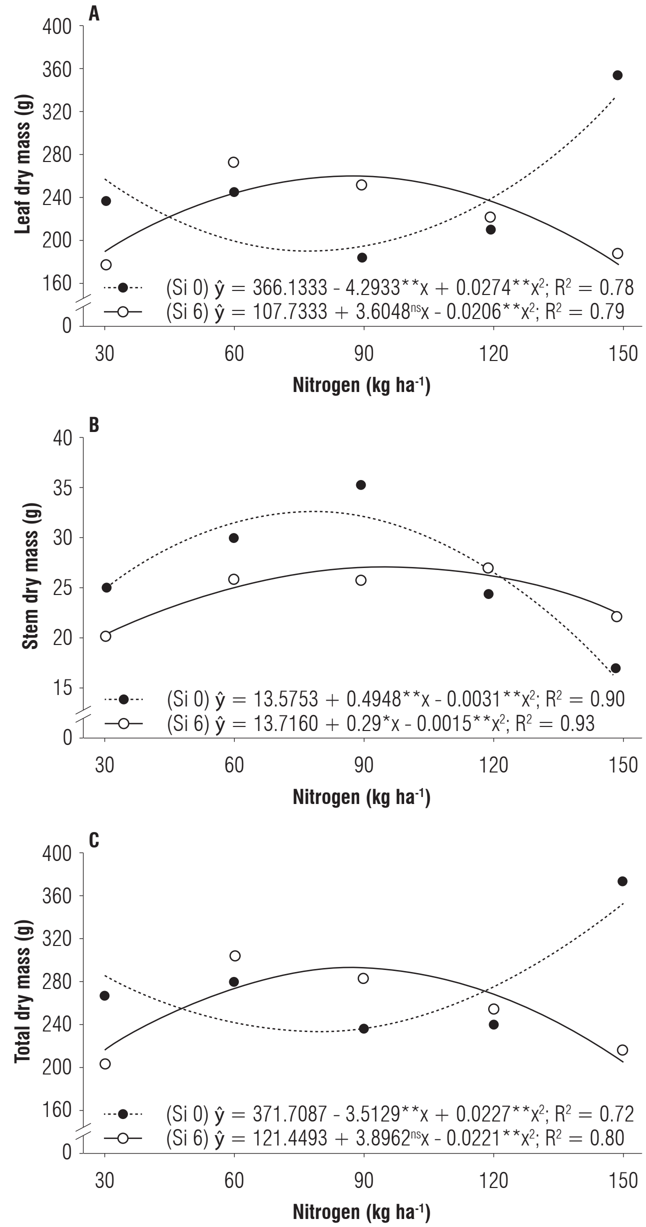

FIGURE 1. (A) Leaf dry mass, (B) stem dry mass, and (C) total dry mass of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization.

The positive values obtained from nitrogen fertilization and the different morphological parameters may be due to the fact that this nutrient provides increases in the rate of carbohydrates conversion into protein and the size of the cells. This may lead to greater leaf expansion and higher growth rates, which directly influences the production of dry matter. Yasari *et al.* (2009) and Rodrigues *et al.* (2014) also registered positive results for dry mass production with increased N supply in paracress plants (*Acmella oleracea* L.), in which a linear increase was verified, with the highest efficiency registered at 112.5 kg ha^{-1} of N.

The decrease in dry mass of silicon-applied plants may be associated with the fact that silicon deposited on shoots is condensed by transpiration and subsequently transformed into amorphous silicon located in the cell wall of epidermal and vascular tissues, causing reduced water loss (Alsaeedi *et al.*, 2019).

According to the results of the ANOVA, it was observed that there was a significant effect of the nitrogen doses on the chlorophyll contents and chlorophyll *a* fluorescence parameters. For the silicon factor, it was observed that there was a significant difference only for the fluorescence variables, except for the variable fluorescence (F_v). However, the interaction between the factors significantly affected all variables analyzed (Tab. 5).

Chl *a* and Chl *b* values presented a quadratic effect both in the treatments with and without Si, with the highest index

TABLE 5. Summary of the analysis of variance by the mean-square values, for chlorophyll *a* (Cl*a*), chlorophyll *b* (Cl*b*), total chlorophyll (tCl), chlorophyll *a*/*b* ratio (Cl*a*/Cl*b*), initial fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v), and quantum yield of photosystem II (F_v/F_m) of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization in Catole do Rocha, 2019.

SV	DF	Mean square			
		Cl*a*	**Cl*b***	**tCl**	**Cl*a*/Cl*b***
Block	1	0.070^{ns}	0.050^{ns}	1.339^{ns}	0.006^{ns}
Si	2	0.551^{ns}	0.075^{ns}	1.254^{ns}	0.0002^{ns}
Error a	2	0.113	0.007	0.636	0.001
N	4	12.617**	3.654**	9.905**	0.035**
Si x N	4	11.510**	2.167**	41.764**	0.026**
Error b	16	0.168	0.034	0.727	0.004
CV 1 (%)		1.01	0.72	1.80	1.14
CV 2 (%)		1.23	1.58	1.93	2.16
		F_0	F_m	F_v	F_v/F_m
Block	1	11.000^{ns}	59.000^{ns}	65.100^{ns}	0.000001^{ns}
Si	2	77724.000**	69216.000**	246.530^{ns}	0.019**
Error a	2	8.000	8.000	27.230	0.000034
N	4	3637.000**	3072.000**	921.800**	0.022**
Si x N	4	363.000**	86.000**	784.370**	0.0004**
Error b	16	44.000	18.000	65.580	0.00004
CV 1 (%)		1.31	0.99	6.56	0.80
CV 2 (%)		3.15	1.45	10.17	0.81

SV: Source of variation; DF: Degrees of freedom; CV: Coefficient of variation; ns: Not significant; ** and *: Significant effect by the F test at the level of 1 and 5% probability, respectively.

TABLE 6. Mean values for chlorophyll *a* (Cl*a*), chlorophyll *b* (Cl*b*), total chlorophyll (tCl), chlorophyll *a*/*b* ratio, initial fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v), and quantum yield of photosystem II (F_v/F_m) of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization in Catole do Rocha, 2019.

Parameters	Silicon (g/plant)	Nitrogen (kg ha^{-1})				
		30	**60**	**90**	**120**	**150**
Cl*a*	0	32.83 b	29.61 b	35.31 a	34.86 a	34.21 a
	6	34.09 a	32.58 a	34.43 b	30.40 b	33.97 a
Cl*b*	0	11.68 b	10.02 b	12.21 b	12.61 a	11.89 b
	6	12.02 a	10.71 a	12.60 a	10.38 b	12.20 a
tCl	0	45.86 a	40.13 b	42.72 b	46.38 a	46.99 a
	6	42.47 b	45.63 a	47.39 a	39.76 b	44.79 b
Chlorophyll *a*/*b* ratio	0	2.81 a	2.96 a	2.89 a	2.76 b	2.88 a
	6	2.84 a	3.04 a	2.73 b	2.93 a	2.78 a
F_0	0	150.67 b	162.67 b	189.00 b	158.00 b	139.00 b
	6	236.33 a	254.33 a	315.33 a	260.00 a	242.33 a
F_m	0	235.67 b	249.33 b	281.00 b	226.67 b	219.00 b
	6	341.67 a	348.00 a	366.67 a	324.33 a	311.33 a
F_v	0	85.00 b	86.67 a	92.00 a	68.67 a	80.00
	6	105.33 a	93.67 a	51.33 b	64.33 a	69.00
F_v/F_m	0	0.67	0.72	0.79	0.73	0.62 a
	6	0.71	0.76	0.84	0.78	0.69 a

Means followed by equal letters in the same column and line do not differ statistically from each other by the Tukey test at 5% probability.

found at 146.1 kg ha^{-1} of N without Si application, evidencing a 5.6% increase compared to the treatment with Si. This behavior in the Si-containing treatments demonstrates FCI values for Chl *a* and Chl *b* in the order of 35.4 and 33.4, respectively (Fig. 2A).

For Chl *b*, a similar behavior was observed, with the highest increase in the treatments without Si and under the N dose of 137.7 kg ha^{-1}, with an FCI of 12.8. Under Si application, the N dose that provided the highest efficiency was 93.8 kg ha^{-1} (Fig. 2B). Since N is one of the main components of chlorophyll molecules, an adequate supply of this nutrient maximizes the indices of this photosynthetic pigment (Wang *et al*., 2014).

The total chlorophyll index increased linearly from the N dose of 51.7 kg ha^{-1}, in the treatments without Si application. Under Si fertilization, the best performance of plants for this variable was found at 103.0 kg ha^{-1} of N, with a 46.8 FCI (Fig. 2C). However, it can be observed that, for the chlorophyll *a*/*b* ratio, the Si-containing treatments presented the best results, with the greatest increase at 82.3 kg ha^{-1} of N (Fig. 2D).

Corroborating this statement, Ávila *et al*. (2010) report a leaf chlorophyll synthesis increase in silicon-fertilized rice plants (*Oryza sativa* L.), especially at high doses of nitrogen. Contrary to this, Rodrigues *et al*. (2016) state that increased potassium silicate doses provide increases in chlorophyll *a* levels in tomato plants (*Solanum lycopersicum* L.) under high and low light conditions. However, the increase in chlorophyll *a*/*b* ratio may be associated with the greater synthesis of chlorophyll *a*, which is an indication that the plant was not experiencing stress.

It was observed that F_0 and F_m showed a similar behavior, adjusting to a quadratic effect, and the best results were obtained in the treatments under Si fertilization, with values in the order of 288.5 and 355.2 /quantum at the N doses of 91.9 and 72.3 kg ha^{-1}, respectively (Figs. 3A and B). This increase in F_0 indicates that in the aforementioned N dose there was a reduction in the capacity of excitation energy transfer to the photosystem II (SÁ *et al*., 2015). F_m refers to the state in which the reaction centers of photosystem II reach their maximum capacity (Silva *et al*., 2014). Silicon accumulated near the stomata reduces leaf transpiration rates (Oliveira and Castro, 2002), a fact that may have benefited

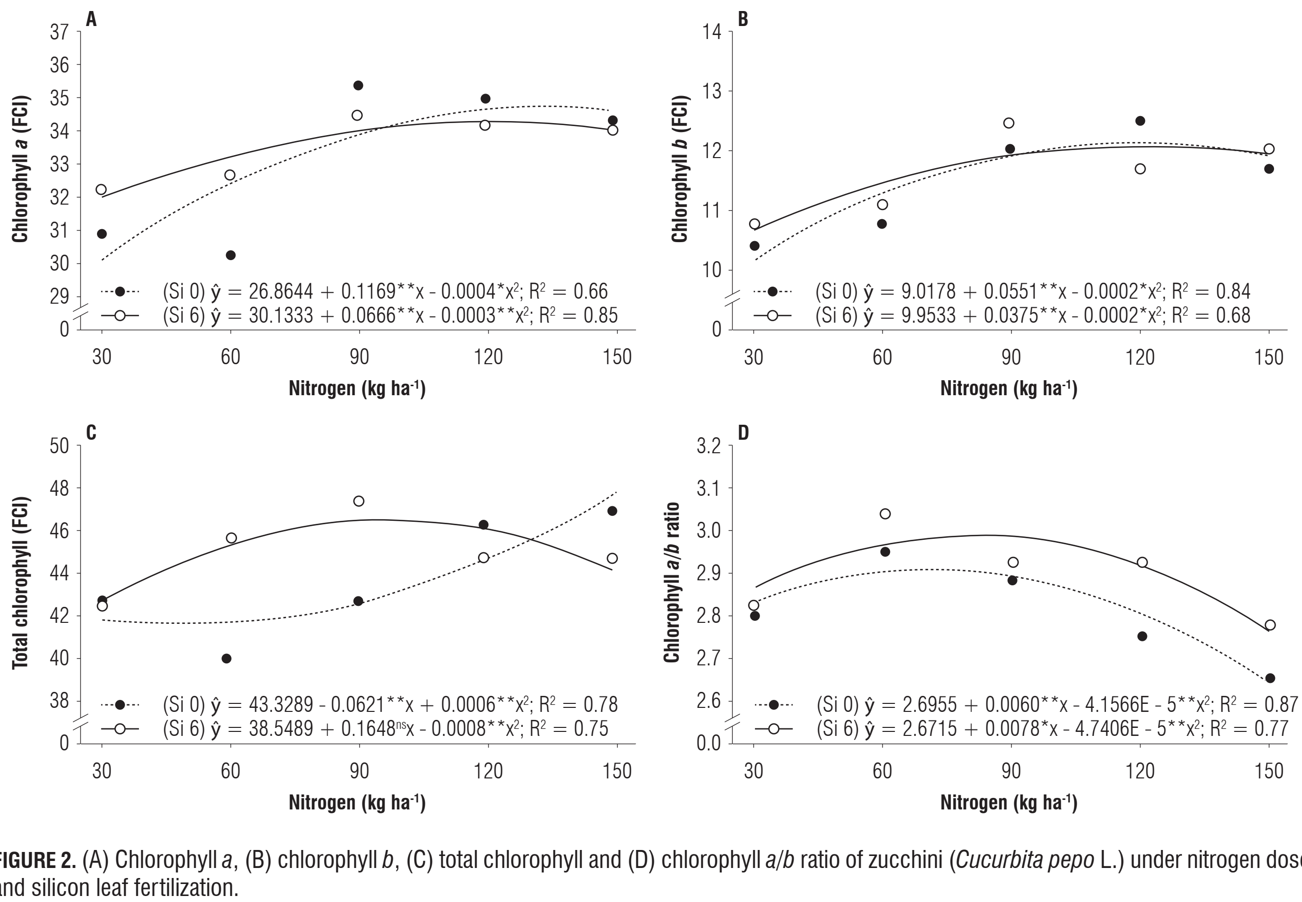

FIGURE 2. (A) Chlorophyll *a*, (B) chlorophyll *b*, (C) total chlorophyll and (D) chlorophyll *a*/*b* ratio of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization.

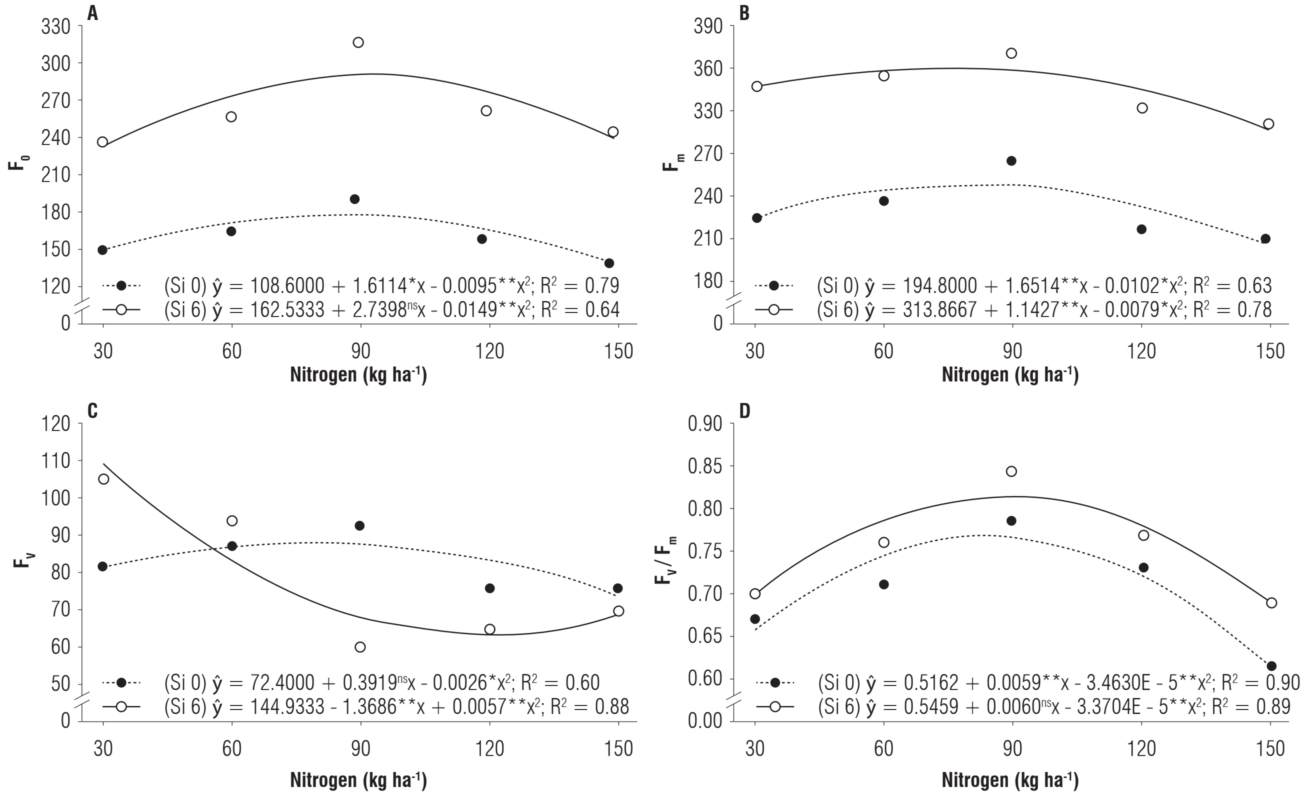

FIGURE 3. (A) Initial fluorescence (F_0), (B) maximum fluorescence (F_m), (C) variable fluorescence (F_v), and (D) quantum yield of photosystem II (F_v/F_m) of zucchini (*Cucurbita pepo* L.) under nitrogen doses and silicon leaf fertilization.

nitrate reductase in the photorespiration process, having increased chlorophyll indices in chloroplasts (Bloom, 2015).

The F_v in the treatments with Si showed the best result at the N dose of 30 kg ha^{-1}, with a value of 109.0 quantum electrons (Fig. 3C). For the F_v/F_m variable, the N dose of 89.0 kg ha^{-1} under Si application provided the highest value (0.81 quantum electrons), a 6.2% increase compared to treatments without Si (76 quantum electrons) (Fig. 3D).

Variable fluorescence refers to active potential energy in photosystem II, which is directly influenced by the maximum fluorescence (Sá *et al.*, 2018). According to these same authors, these increases in fluorescence may be related to the higher content of chlorophyll in the reaction centers as a function of the N doses. Based on the F_v/F_m results, it can be stated that the N combined with Si induced a better energy stability compared to the treatments that did not contain Si (0.81), which is within those indicated as normal (between 0.75 and 0.85) in non-stressed plants (Neves *et al.*, 2019).

Conclusions

The highest dry matter productions in zucchini plants were obtained in treatments without Si. The application of Si and N together positively influences the chlorophyll *a*/*b* ratio of zucchini plants. The interaction between Si and N positively influences the maximum fluorescence, variable fluorescence and quantum yield of photosystem II of zucchini plants.

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Effect of aqueous extracts of *Brachiaria decumbens* on the development of ornamental pepper

Efecto de extractos acuosos de *Brachiaria decumbens* en el desarrollo de pimiento ornamental

Marcelo Rodrigo Krause[1], Lorena Aparecida Merlo Meneghelli[1], Caroline Merlo Meneghelli[1]*, Hediberto Nei Matiello[2], and Mayana Ferreira Nascimento[1]

ABSTRACT

The use of natural plant growth promoters can be an effective, sustainable, and low-cost alternative to reduce input costs for the production of ornamental pepper. Thus, the objective of this study was to evaluate the effect of *Brachiaria decumbens* aqueous extract on the growth of ornamental pepper (*Capsicum frutescens*). The experiment was carried out in the Plant Nursery sector of the Federal Institute of Espirito Santo (IFES), located in the municipality of Santa Teresa, in the Serrana region of the Espirito Santo State (Brazil). The study was conducted using a randomized block design (RBD) with four replicates in a 4×2 factorial scheme with four doses of the *Brachiaria decumbens* extract (0, 50, 75 and 100%) and two application methods (edaphic application or foliar sprays). The number of days to flowering, plant height, leaf number, fruit number, leaf area, fruit, root, shoot, and total dry mass were determined. According to the results, the variables number of fruits and root dry mass had no significant effect on the interaction, with the best results found with the edaphic application when compared to the foliar sprays. When considering interaction, the application of 100% of the extract reduced the number of days to flowering for both edaphic application and foliar sprays. For growth variables, this same dose promoted the best results for most of the variables. Based on the results, the edaphic application of *B. decumbens* at 100% dosage is recommended to reduce the number of days to flowering and promote better development of ornamental pepper.

Key words: *Capsicum frutescens,* plant life cycle reduction, flowering, early plant development.

RESUMEN

El uso de promotores naturales de crecimiento de plantas puede ser una alternativa efectiva, sostenible y económica, que reduce los costos de insumos para la producción de pimentón ornamental. Por lo tanto, el objetivo de este estudio fue evaluar el efecto del extracto acuoso de *Brachiaria decumbens* sobre el crecimiento del pimentón ornamental (*Capsicum frutescens*). El experimento se llevó a cabo en el sector de viveros de plantas del Instituto Federal de Espírito Santo (IFES), ubicado en el municipio de Santa Teresa, en la región de Serrana del estado de Espírito Santo (Brasil). Se utilizó un diseño de bloques al azar con cuatro repeticiones en un esquema factorial 4×2, con cuatro dosis del extracto de *Brachiaria decumbens* (0, 50, 75 y 100%) y dos métodos de aplicación (aplicación edáfica o spray foliar). El número de días para la floración, altura de planta, número de hojas, número de frutos, área foliar, y masa seca de frutos, raíz, brote y total se determinaron. Según los resultados, las variables número de frutos y masa seca de la raíz no mostraron interacción, encontrando los mejores resultados con la aplicación edáfica en comparación con la aplicación foliar. Al considerar la interacción, la aplicación del 100% del extracto redujo el número de días de floración tanto para la aplicación foliar como para el edáfica. Para las variables de crecimiento, esta misma dosis promovió los mejores resultados para la mayoría de las variables. Basándose en los resultados, se recomienda la dosis del 100% de extracto de *B. decumbens* aplicado por el método edáfico para reducir el número de días para la floración y promover un mejor desarrollo del pimentón ornamental.

Palabras clave: *Capsicum frutescens*, reducción del ciclo de vida de la planta, floración, desarrollo temprano de la planta.

Introduction

The production of flowers and ornamental plants represents one of the fastest-growing sectors in Brazilian agriculture. This market is based on periodic releases of new cultivars with formats that express new patterns of colors, contrasts or sizes. In this context, pepper plants stand out for having a dual-purpose, culinary or ornamental application, due to their small size, erect and colorful fruits and leaves that can present different colors and sizes (Nascimento *et al*., 2013). Furthermore, pepper plants are easy to grow and have a long post-production shelf life, fruit and leaf durability, in

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[1] Department of Plant Science, Federal University of Viçosa, Viçosa (Brazil).
[2] Department of Plant Science, Federal Institute of Education, Science and Technology of Espírito Santo, Santa Teresa campus, Santa Teresa (Brazil).
* Corresponding author: carol.merlo@hotmail.com

addition to the continuous production of fruits (Neitzke *et al*., 2016). All these favorable attributes increase the internal and external market demand for ornamental peppers. In Brazil, cultivation is being carried out by small, medium and large producers, adjusting to the models of family agriculture and integration of large companies with small producers (do Rêgo and do Rêgo, 2016).

Plants can produce, store and release a wide range of allelopathic secondary metabolites during their life cycle (Schandry and Becker, 2019). These secondary allelopathic chemicals are synthesized by plants and released into the environment to promote or inhibit the development of other plants (Oliveira *et al*., 2018).

Brachiaria decumbens is of great importance in the Brazilian cattle industry, due to its high potential for forage production and adaptability to acid soils with low fertility (Monteiro *et al*., 2016). It is believed that substances produced through the metabolism of *B. decumbens* plants and released by dissolution in water may influence the development of pepper plants, due to the presence of mineral nutrients, amino acids and organic acids, carbohydrates or growth regulators. Younger leaves, especially the ones in intense cell division, are the main production sites of compounds that contribute to plant growth (Taiz and Zeiger, 2013).

There is no consensus regarding the effect of *Brachiaria* on plant development. The application of *B. decumbens* extract inhibited the growth of watercress (*Lepidium sativum*), lettuce (*Lactuca sativa*), timothy (*Phleum pratense*) and ryegrass (*Lolium multiflorum*) seedlings, being the substance (6R, 9S)-3-oxo-α-ionol, isolated from the extract, one of the possible responsible for this effect (Kobayashi and Kato-Noguchi, 2015). On the other hand, *B. brizantha* extract promoted increased germination of *Stylosanthes macrocephala* (Rodrigues *et al*., 2012). Therefore, it is believed that *Brachiaria* has both inhibitory compounds and favors plant development, depending on the sensitivity of plants to these extracts and the part of the plant (root or shoot).

The pepper crop has been little studied in Brazil, especially regarding organic fertilization and natural plant growth promoters (Weckner *et al*., 2018). Therefore, the use of *B. decumbens* extracts by small producers can be a viable and economical alternative, which may reduce input costs. Thus, the study aimed to evaluate the effect of *Brachiaria decumbens* aqueous extract on the growth of ornamental pepper.

Materials and methods

The study was conducted in the plant nursery sector of the Federal Institute of Espírito Santo (IFES), located in the municipality of Santa Teresa, in the Serrana region of the Espirito Santo State, with an altitude of 160 m a.s.l., latitude 19°48'20" S and longitude 40°40'32" W. According to Köppen's classification, the region presents a Cwb climate type (temperate oceanic climates/tropical altitude climate), with an average annual temperature of 24.6°C and annual precipitation ranging between 700 and 1,200 mm. The plant nursery where the experiment was carried out has a 50% shading screen.

A randomized block design (RBD) with four replicates in a 4x2 factorial scheme was used, with four doses of *B. decumbens* extract (0, 50, 75 and 100%) and two application methods (edaphic application or foliar sprays). The block was used to control the effect of seedling size. The experimental unit consisted of a pot containing one plant, with a total of 32 experimental units.

Capsicum frutescens plants were propagated by seeds and germinated in expanded polystyrene trays with 200 cells, each one with a volume of 18 cm^3. Two seeds were sown per cell. Following germination, plants were thinned to one plant per cell.

When the seedlings had two pairs of leaves, they were transplanted to 1.5 L polyethylene pots. The substrate used in the pots was a mixture of the commercial substrate Plantmax® and coffee straw, at a 2:1 ratio. In the substrate mixture the chemical fertilizers simple superphosphate and potassium chloride were added at doses of 2.5 g L^{-1} and 0.8 g L^{-1}, respectively.

Sprinkler irrigation was performed daily in order to reach the field capacity of the substrate in all treatments. The pepper plants were fertilized during the experiment with the mineral fertilizer NPK 04-30-10, and the fertilizer was previously diluted in water and applied through fertigation.

To prepare the aqueous extract, *B. decumbens* young leaves were grounded with water in a 1:2 ratio (w/w) in an industrial blender (LC6, Skymsen, Brusque, Brazil). The obtained mixture was sieved and then applied to the plants. Thus, 0% represents the control (water) and 100% represents the undiluted extract.

A sample of *B. decumbens* young leaves was chopped and dried in a forced air circulation oven (SL-102/1152, SOLAB, Piracicaba, Brazil) at 70°C for 72 h. After dried, the sample was sent to the Laboratory of Agronomic and Environmental Analysis-FULLIN, Linhares-ES, Brazil, for the determination of macro-and micronutrient concentration.

The application of the aqueous extract started at 21 d after transplanting (DAT), in which four applications were performed fortnightly. For the foliar sprays, each leaf was sprayed until runoff using a sprayer directed to both leaf surfaces. The edaphic application was performed by pouring 250 ml of the aqueous extract per pot.

The commercial stage of pepper plants for sale was defined when fruit color was purplish or reddish in more than 50% of their surface. At this stage, plant height (PH), leaf number (LN), fruit number (FN), leaf area (LA), fruit dry mass (FDM), root dry mass (RDM) shoot dry mass (SDM) and total dry mass (TDM) were determined. The number of days to flowering (DTF), i.e. the period between transplanting and flowering, was also determined.

Afterward, a metal cylinder was used to cut leaf circles that were weighed in an analytical balance (AUW220, Shimadzu, Barueri, Brazil) and the leaf area was indirectly estimated through a rule of three. To obtain the FDM, RDM, SDM, and TDM, the shoot and root of each plant were cut, washed and dried in a forced-air circulation oven at 65ºC until reaching constant weight. After dried, the plant material was weighed in an analytical balance. The TDM comprises the fruit, leaf, root and stem dry masses.

Data were subjected to tests of normality (Lilliefors) and homocedasticity (Bartlett), in order to verify the Analysis of variance assumptions. The data that did not meet the assumptions were transformed into logarithm or square root. The variables that met the assumptions were submitted to Analysis of variance. For the “application method” factor the result of the analysis of variance was conclusive (only two levels for each one). For the “extract doses” factor, in the case of its significant effect, the decomposition of its degrees of freedom in regression by orthogonal polynomials was performed. For all procedures, a $P<0.05$ was adopted.

All statistical analyzes were performed using the Assistat 7.7 (Silva and Azevedo, 2016) and Sisvar 5.6 (Ferreira, 2011) software.

Results and discussion

According to the results of the analysis of *B. decumbens* leaves used to prepare the aqueous extract, it can be observed that the leaves show high contents of nutrients, mainly nitrogen and potassium, thus, contributing to the development of pepper plants (Tab. 1).

TABLE 1. Nutritional composition of the *Brachiaria decumbens* leaves used in the aqueous extract.

Macronutrients (g kg^{-1})						Micronutrients (mg kg^{-1})				
N	**P**	**K**	**Ca**	**Mg**	**S**	**Fe**	**Zn**	**Cu**	**Mn**	**B**
30.38	2.87	26.88	3.68	2.81	3.07	88.00	45.00	8.00	212.00	14.00

There was a significant interaction ($P<0.05$) between the application method and the *B. decumbens* extract doses for the variables number of days to flowering, plant height, leaf number, leaf area, fruit dry mass, shoot dry mass and total dry mass, which means that the behavior of the levels of one factor was different when the levels of the other factor changed.

There was no significant effect of the interaction on the number of fruits and root dry mass. In this case, the *Brachiaria* extract doses and the application methods act independently for the fruit number and root dry mass.

Regarding the isolated effect of the application methods, it was observed that the pepper plants presented higher fruit number and root dry mass values with edaphic applications of *B. decumbens* extract (Tab. 2). Pepper fruits are one of the features that most attract the consumers’ attention at the time of purchase (Neitzke *et al*., 2010), so it is of interest that the plants present a great number of fruits.

TABLE 2. Fruit number and root dry mass of *Capsicum frutescens* as a function of edaphic application or foliar sprays of *Brachiaria decumbens* aqueous extract.

	Application method		*P* Value
	Edaphic	**Foliar**	
Fruit number	7.81 a	4.08 b	0.0019
Root dry mass (g)	5.82 a	2.66 b	< 0.0001

Means followed by different letters differ from each other according to the F test ($P<0.05$).

The superior result observed for the edaphic application, a fertigation-like method, may be associated with the amount of nutrients and compounds absorbed by the plants. When evaluating the performance of second harvest corn subjected to different nitrogen doses, Biscaro *et al*. (2011) observed that the fertilization via edaphic application is more efficient than the foliar sprays, even when lower doses are applied. Although the absorption of foliar sprays of *B. decumbens* extract is faster and more efficient, the exclusive use of this method is not the most indicated since the amount absorbed is not sufficient for the adequate development of pepper plants.

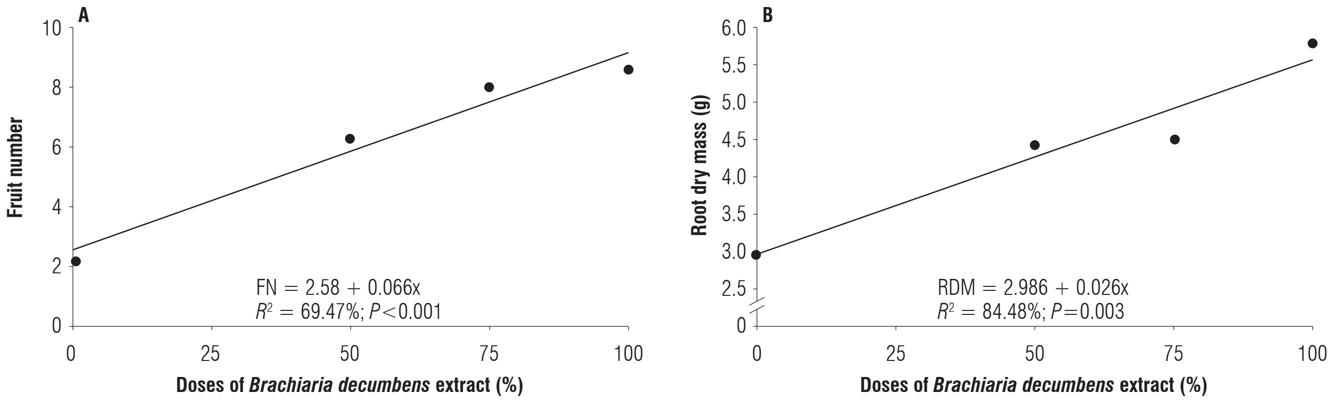

FIGURE 1. (A) Fruit number and (B) root dry mass of ornamental pepper subjected to different doses of *Brachiaria decumbens* aqueous extract. FN: fruit number, RDM: root dry mass.

Regarding the isolated effect of the *B. decumbens* extract doses, the best results for fruit number (9.18) and root dry mass (5.59 g) were obtained at 100% (Fig. 1A-B).

The best performance of pepper plants regarding the number of fruits and root dry mass observed at the concentration of 100% probably occurred due to the amount of nutrients and chemical compounds present in the *B. decumbens* extract, showing that lower doses were not enough to promote such expressive results. When applying *Pinus* extract on the initial development stages of soy plants, Faria *et al*. (2009) reported that the greatest dose (2 kg L^{-1}) provided higher hypocotyl and radicle length, emphasizing the importance of using larger doses.

For the variables days for flowering, plant height, leaf number, leaf area, fruit dry mass, shoot dry mass, and total dry mass, the interaction between the factors application method and doses of extract was significant ($P \leq 0.05$) and the obtained results are shown in Figures 2A-H.

When considering the interaction between the application method and the *B. decumbens* extract doses, the edaphic application showed a linear behavior (Fig. 2A-H). The foliar sprays for the variables number of days to flowering and shoot dry mass showed a linear behavior; for the variable leaf area, there was quadratic behavior, and for the other variables there was no adjustment to the different polynomials (Fig. 2A-H).

Figure 2A shows that the number of days to flowering (DTF) decreased as the doses of *B. decumbens* extract increased regardless of the application method. The best results of DTF (50.01 d) were registered when edaphic applications of *B. decumbens* extract at 100% were performed, with flowering occurring 50 d after transplanting into the pot. On the other hand, the treatment containing 0% (control) of *B. decumbens* aqueous extract presented the worst results, registering 108.42 and 132.2 d to flowering, when the aqueous extract was applied by edaphic and foliar sprays, respectively. The variable days to flowering (DTF) is a measure of precocity and as such, it is of interest that it exhibits a reduction (do Rêgo *et al*., 2012). Oliveira (2012), studying the use of biofertilizers in the cultivation of *Capsicum baccatum* var. *pendulum*, obtained a reduction of 6 d for flowering when applying organic yeast composed of water, fresh cattle manure, green leaves, sugarcane candy, MB-4 rock powder, and yeast starter.

Regarding the variable plant height (PH), the foliar application of *B. decumbens* aqueous extract did not show a significant statistical difference as a function of the doses (Fig. 2B). With edaphic applications, plant height increased with the augmentation of the extract doses, obtaining the highest value (51.76 cm) at 100% of *B. decumbens* aqueous extract. The higher efficiency of the edaphic application may be related to the greater absorption capacity of the roots when compared to leaves. The proper height of an ornamental pepper is determined by the size of its pot (Veiling, 2019). Small-sized pepper varieties are desirable for potting. Peppers that exceed the required size for potting can be cultivated in functional gardens, destined to spice, medicinal and aromatic plants (Neitzke *et al*., 2010).

The best results obtained with increased doses of the extract may be related to the macro and micronutrients available in the *B. decumbens* leaves (Tab. 1) as well as growth-promoting compounds such as auxins. Once available in liquid form and in favorable amounts, the active compounds act rapidly in the plant metabolism, improving plant growth and development (Weckner *et al*., 2018). According to Araújo *et al*. (2007), organic fertilization with fresh cattle

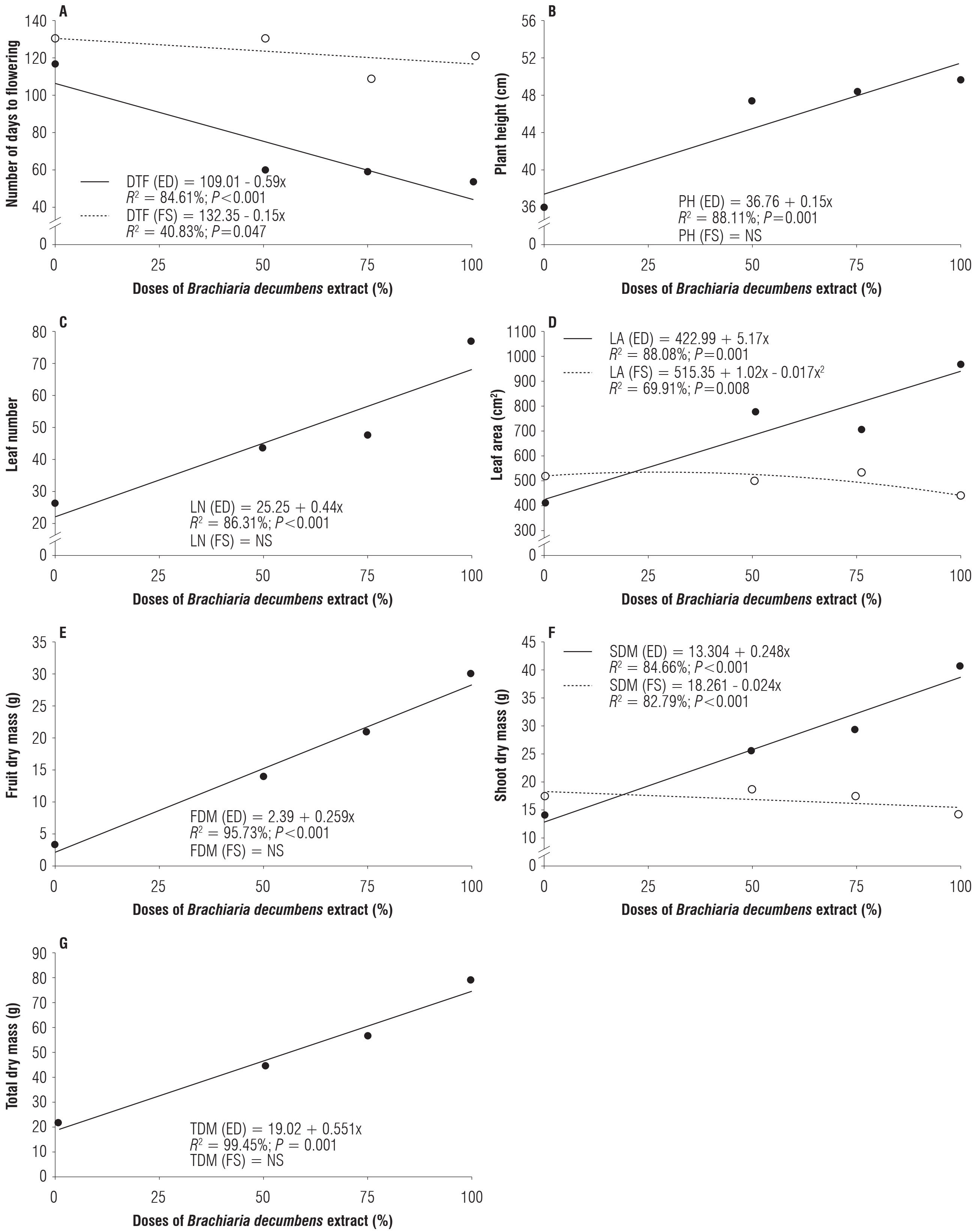

FIGURE 2. (A) Number of days to flowering, (B) plant height, (C) leaf number, (D) leaf area, (E) fruit dry mass, (F) shoot dry mass, and (G) total dry mass in function of edaphic application or foliar spray of *Brachiaria decumbens* aqueous extract. ED: edaphic application, FS: foliar spray, NS: not significant, DTF: number of days to flowering, PH: plant height, LN: leaf number, LA: leaf area, FDM: fruit dry mass, SDM: shoot dry mass, TDM: total dry mass.

manure, applied solo or associated with organic matter, is a great alternative to promote the growth of sweet pepper (*Capsicum annum*).

Edaphic applications of increasing doses of the *Brachiaria* aqueous extract promoted an increase in the leaf number (Fig. 2C). At the 100% dose, the pepper plants showed 69.25 leaves, an increase of 174.26% when compared to the 0% dose. When applied via foliar spray, there was no significant difference between the treatments. The leaves are an important feature of ornamental peppers. A high number of leaves provides dense foliage and the green leaves contrast with the diversity of fruits color, which makes ornamental peppers more appealing.

The high potassium content (Tab. 1) in 100% of *Brachiaria* aqueous extract probably influenced the increase in leaf number, since potassium fertilization is essential to photosynthesis process. Corroborating the results found in the present study, Barcelos *et al.* (2015) registered increased chlorophyll content in pepper pout plants with the augment of potassium doses.

It can be observed that edaphic applications showed better results when compared to the foliar sprays (Fig. 2D). When the edaphic application of the *B. decumbens* extract was performed, the leaf area increased with increasing doses. This aqueous extract at 100% was considered the best dose, providing 939.99 cm^2 of leaf area. On the other hand, for the foliar sprays, the highest LA value (531.14 cm^2) was obtained at a dose of 30%, and then it decreased with increasing doses. According to Larcher (2000) and Hachmann *et al.* (2014), the larger the leaf area, the greater the active photosynthetic surface available and, therefore, the greater photoassimilate production available for fruit development.

When the edaphic application of the extract was performed, increased fruit dry mass (FDM) was observed as the doses increased (Fig. 2E). The best result was obtained at 100%, with 28.29 g of fruit dry mass, whereas 2.65 g was registered for plants that were not treated with *B. decumbens* extract. Therefore, the application of *Brachiaria* extract at 100% dosage promoted, approximately, a 1,000% increase for this variable. Plants sprayed with the extract did not show any significant statistical difference for the fruit dry mass. The fruit dry mass is an important factor for pepper growers since fruits can be used to produce paprika powder.

Again, such results may be associated with the presence of potassium in *B. decumbens* leaves (Tab. 1), a key nutrient for fruit development. The essential mineral nutrients present in fertilizers have many functions in plant metabolism. When available in optimum concentrations, potassium exerts essential activity in the synthesis of proteins, carbohydrates, sugars, organic acids, and other compounds, being fundamental to fruit production and quality (Gonçalves *et al.*, 2008). When evaluating the effect of biofertilizer doses (0 to 120 m^3 ha^{-1}) on two sweet pepper cultivars (Amanda and Rubia), Sediyama *et al.* (2014) verified an increase in productivity as the doses increased, mainly due to a nutritional state improvement, provided especially by higher leaf contents of N, B, and Zn. According to Oliveira (2012), different types of biofertilizers promoted a significant increase in fruit mass in relation to N-P-K fertilization.

An increase in shoot dry mass (SDM) was observed with increasing doses of *Brachiaria* extract via edaphic application and a decrease in shoot dry mass for foliar spray (Fig. 2F). When evaluating the application of a biofertilizer via foliar spray in hybrid sweet pepper, Freitas *et al.* (2011) registered a decrease in the values of the characteristics evaluated when the plants were subjected to higher doses.

Edaphic application of increasing doses of the *B. decumbens* aqueous extract provided an increase in total dry mass (TDM) (Fig. 2G). At an extract dose of 100%, the highest total dry mass (74.12 g) was obtained, which represents a 278.74% increase when compared to the dose of 0% (19.57 g). When evaluating the nutritional status and productivity of sweet pepper fertilized with swine biofertilizer, Sediyama *et al.* (2014) registered the best results when higher doses were applied.

The results obtained in the present study may be associated with the presence of nutrients and other compounds in *B. decumbens* leaves. Large amounts of auxins and phenolic compounds have been reported in *Cyperus rotundus* L. extracts, a plant that shows characteristics of aggressiveness similar to *Brachiaria*, once it adapts to various soil and climate conditions and excels over other plants (Rezende *et al.*, 2013). Auxin regulates various growth and developmental processes throughout the life cycle of plants, such as cell division, cell expansion and cell fate (Salehin *et al.*, 2015). From this perspective, the best results found with the application of *B. decumbens* extract may also be related to the high levels of auxin present, which promoted better plant development, which is evidenced by the reduction of days to flowering and higher plant height, leaf number, leaf area, and dry mass.

The application of *Brachiaria* extract can be an accessible alternative in the cultivation of ornamental potted pepper.

This aqueous extract can be used to promote plant growth with great potential to be tested for its efficiency in other crops.

Conclusions

Brachiaria decumbens extract application reduces the number of days to flowering in pepper, making it possible to anticipate its commercialization. In addition, it promotes better development of pepper plants, evidenced by higher plant height, leaf number, leaf area, and dry mass.

The edaphic application of *B. decumbens* extract promotes better results when compared to the foliar sprays. The edaphic application of *B. decumbens* extract at 100% dosage is recommended for promoting the best development of ornamental pepper plants.

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Selection of sowing date and biofertilization as alternatives to improve the yield and profitability of the F68 rice variety

Selección de fecha de siembra y biofertilización como alternativas para mejorar el rendimiento y rentabilidad de la variedad de arroz F68

Yeison Mauricio Quevedo-Amaya[1], José Isidro Beltrán-Medina[1], José Álvaro Hoyos-Cartagena[1], John Edinson Calderón-Carvajal[1], and Eduardo Barragán-Quijano[1]*

ABSTRACT

Multiple factors influence rice yield. Developing management practices that increase crop yield and an efficient use of resources are challenging to modern agriculture. Consequently, the aim of this study was to evaluate biological nitrogen fixation and bacterial phosphorous solubilization (biofertilization) practices with the selection of the sowing date. Three sowing dates (May, July and August) were evaluated when interacting with two mineral nutrition treatments using a randomized complete block design in a split-plot arrangement. Leaf carbon balance, leaf area index, interception and radiation use efficiency, harvest index, dry matter accumulation, nutritional status, and yield were quantified. Results showed that the maximum yield was obtained in the sowing date of August. Additionally, yield increased by 18.92% with the biofertilization treatment, reaching 35.18% of profitability compared to the local production practice. High yields were related to a higher carbon balance during flowering, which was 11.56% and 54.04% higher in August than in July and May, respectively, due to a lower night temperature. In addition, a high efficient use of radiation, which in August was 17.56% and 41.23% higher than in July and May, respectively, contributed to obtain higher yields and this behavior is related to the selection of the sowing date. Likewise, a rapid development of the leaf area index and an optimum foliar nitrogen concentration (>3%) were observed. This allowed for greater efficient use of radiation and is attributed to the activity of nitrogen-fixing and phosphate solubilizing bacteria that also act as plant growth promoters.

Key words: *Azotobacter chroococcum*, plant growth promoters, respiration, biological nitrogen fixation, photosynthetically active radiation, night temperature.

RESUMEN

El rendimiento del arroz está influenciado por múltiples factores. Desarrollar prácticas de manejo que aumenten el rendimiento y sean más eficientes en el uso de los recursos es un reto de la agricultura. En consecuencia, el objetivo de este estudio fue evaluar las prácticas fijación biológica de nitrógeno y solubilización de fosforo por bacterias (biofertilización) con la selección de fecha de siembra. Se evaluaron tres fechas de siembra (mayo, julio y agosto) en interacción con dos tratamientos de nutrición mineral empleando un diseño en bloques completos al azar en arreglo de franjas divididas. Se cuantificó el balance de carbono, índice de área foliar, intercepción y uso eficiente de radiación, índice de cosecha, acumulación de masa seca, estado nutricional y rendimiento. Los resultados mostraron que el máximo rendimiento se obtuvo en la fecha de siembra de agosto; adicionalmente, con la biofertilización el rendimiento se incrementó en 18.92%, alcanzando una rentabilidad del 35.18% en comparación con la práctica local de producción. El alto rendimiento se relacionó con un mayor balance de carbono durante la floración, que fue un 11.56 y 54.04% mayor en agosto, comparado con julio y mayo respectivamente, debido a una menor temperatura nocturna. Asimismo, un alto uso eficiente de la radiación que en agosto fue un 17.56 and 41.23% mayor que julio y mayo respectivamente, contribuyó a mayores rendimientos y este comportamiento se relacionó con la selección de la fecha de siembra. Además, se observó un rápido desarrollo del índice de área foliar y de la concentración optima del nitrógeno foliar (>3%). Esto permitió alcanzar un mayor uso eficiente de la radiación y se atribuye a la actividad de las bacterias fijadoras de nitrógeno y solubilizadoras de fosforo que también son promotoras del crecimiento.

Palabras clave: *Azotobacter chroococcum*, promotores del crecimiento vegetal, respiración, fijación biológica del nitrógeno, radiación fotosintéticamente activa, temperatura nocturna.

Introduction

Rice is an essential commodity for more than 60% of the world population (Patel *et al*., 2010). The crop yield is influenced by climatic factors, physical and chemical soil conditions, water management, sowing date, variety, sowing rate, weed control, and fertilization (Yosef Tabar, 2013). Furthermore, climate change is considered a potential

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[1] Corporación colombiana de investigación agropecuaria - AGROSAVIA, Centro de investigación Nataima, El Espinal, (Colombia).
* Corresponding author: ebarragan@agrosavia.co

limitation for rice producers from the socio-economic point of view (Delerce *et al.,* 2016). For Colombia, an increase of up to 7°C in temperature and a decrease in precipitation of 10% (IPCC, 2014) could generate crop yield instability between 5% and 29% (Iizumi *et al.,* 2014).

Crop management practices maintain or improve yield in function of the environmental offer through the selection of sowing dates based on the varietal requirements. With this strategy, it is possible to escape abiotic stress during critical growth phases or to ensure maximum solar radiation uptake by the crop (Van Ittersum *et al*., 2008; Zhu *et al.,* 2013). Studies have been carried out around the world to identify optimal sowing dates for different rice varieties to maximize their yield (Akbar *et al.,* 2010; Khalifa *et al.,* 2014; Osman *et al.,* 2015; Pal *et al.,* 2017). However, in Colombia, there are only two evaluation reports that include this practice (Garcés and Restrepo, 2015; Quevedo *et al.,* 2019). Nonetheless, these were evaluated with other varieties that are no longer available for producers and in localities with different edaphoclimatic characteristics; moreover, the impact of these practices was not evaluated in economic terms.

Currently, the increase in crop productivity is due to an increase in fertilization rates. However, in the context of climate change and with limited resources, the indiscriminate application of fertilizers will be a limiting factor in production (Han *et al*., 2018). In rice crops, nitrogen fertilization is carried out with high doses generating a low efficiency in the use of nitrogen and further pollution problems (Tilman, 2001; Ju *et al.,* 2015). Regarding phosphorus, this element is not available for the plant because it is easily fixed, i.e., through adsorption or precipitation (Roberts and Johnston, 2015). Considering the above and that the majority of the agricultural soils worldwide are deficient in phosphorus (Velázquez *et al*., 2017), producers usually apply high amounts of this element, generating eutrophication, contamination with heavy metals, and depletion of the resource (Krüger and Adam, 2017).

Based on this problem, several practices have been developed using biological nitrogen fixation processes to increase the availability of the element for the plant (Reinhold and Hurek, 2011). These practices are implemented in addition to the use of phosphorus solubilizing bacteria that transforms the insoluble phosphorus into assimilable forms for the plants (Lavakush *et al.,* 2014). Some of these microorganisms act as plant growth promoters by releasing phytohormones such as auxins, gibberellins, and cytokinins (Castanheira *et al.,* 2014; Shabanamol *et al.,* 2018). This practice is considered eco-friendly and effective to reduce crop fertilization costs (Hallmann *et al*., 1997), and has also been evaluated with promising results because it increases yield and reduces the fertilization with phosphorus up to 40% (Roger and Ladha, 1992; Lavakush *et al*., 2014). The use of *Azospirillum brasilense* and *Pseudomonas fluorescens* in rice crops increase aerial biomass production, harvest index, and grain yield. This behavior was attribuited to their performance as plant growth promoters (García de Salamone *et al*., 2012). In Colombia, some products act as nitrogen fixers and phosphorus solubilizers in rice crops. However, there is only one report in Colombia of the effect of *Azotobacter chroococcum* Beijerinck acting as nitrogen fixating bacteria and plant growth promoter on cotton crops (Romero *et al*., 2017). However, the effect of this practice at the agronomic and physiological level is not known for rice crops in addition to the financial benefits with its application. Therefore, it was necessary to evaluate some of the products used by rice producers in the country such as the biofertilizer Monibac® based on the nitrogen fixating bacteria *A. chroococcum* and a biofertilizer under development based on *Rhizobium pusense*, which operates as a phosphorus solubilizer and plant growth promoter.

Considering the previously mentioned state of the art and that currently there is no knowledge of the effect of the integration of practices, such as the selection of sowing date and biofertilization on rice crops, at the agronomic, physiological and financial levels, our hypothesis is as follows: the selection of the optimal sowing date with the implementation of a biofertilization practice increases the yield and profitability of rice crop. Accordingly, the aim of this study was to evaluate the agronomic, physiological, and financial behavior of variety Fedearroz 68 at three sowing dates with and without biofertilization.

Materials and methods

Study site and plant material

This research was carried out in the municipality of El Espinal, located in the center of the province of Tolima, Colombia, during the year 2017. The experimental site was the Nataima research center of AGROSAVIA georeferenced with coordinates 4°11’28.7” N and 74°57’39.2” W. The soil of the area has a clay-loam texture and is of an alluvial origin. It belongs to the order inceptisol.

The plant material used was the variety Fedearroz 68 that was selected because it is one of the most cultivated varieties in this agro-ecological zone and for its high yield and precocity.

The climatic variables were monitored with a Davis Vantage Pro 2 meteorological station (Davis Instruments, San Francisco, USA).

Experimental design

The study employed a randomized complete block design in a split-plot arrangement. The main plot corresponded to the three sowing dates (SD): May 26, July 7, and August 8, 2017. The subplot corresponded to two mineral nutrition treatments (biofertilization and local production practice). Each treatment was comprised of the SD x nutrition treatment interaction with three replicates, each with an area of 1600 m^2.

Application of treatments

Two mineral nutrition treatments were assessed in this study. The first was a local production practice that included the application of chemically synthetized fertilizers in the following rates usually applied by the local producers: 120 kg ha^{-1} of nitrogen, 80 kg ha^{-1} of P_2O_5, and 120 kg ha^{-1} of K_2O. The second is the biofertilization treatment that included the 100% chemical synthesis fertilization applied by the local producers, plus the inoculation with nitrogen-fixing bacteria (*A. chroococcum* strain AC1 and AC10) at a concentration $1x^9$ colony forming units (CFU) cm^{-3}, whose commercial name is Monibac® with a dose of 1000 cm^3 ha^{-1}. Additionally, phosphorus and zinc solubilizing bacteria (*R. pusense* strain B02) obtained from the germplasm bank of AGROSAVIA microorganisms at a concentration of $1x^{10}$ CFU cm^{-3}. These microorganisms were applied at a dose of 500 g ha^{-1}. The bacteria was sprayed to the soil between 21 and 23 d after plant emergence.

Agronomic management of the crop

Mechanized planting was carried out in furrows with a sowing rate of 120 kg ha^{-1}. Additionally, mineral nutrition was divided into four applications administered in the same quantities for both treatments. Agronomic crop management was used at the scale of a commercial crop. Tillage of primary and secondary soil was performed. A mechanized planting by furrows was carried out. Control of weeds using pre-emergent and post-emergent herbicides selective to the crop was also performed: pendimethalin 2500 cm^3 ha^{-1}, clomazone 1200 cm^3 ha^{-1}, buthaclor 3000 cm^3 ha^{-1}, sodium bispiribac 150 cm^3 ha^{-1}, cyhalofop 1500 cm^3 ha^{-1}. Pest and disease controls were carried out with chemical synthesis products based on periodic monitoring. At phenological stages 30 and 61 the following active ingredients were applied: lambdacihalotrina + thiamethoxam (insecticide) at a dose of 125 cm^3 ha^{-1} and azoxystrobin + diphenoconazole (fungicide) at a dose of 500 cm^3 ha^{-1}.

Estimation of gas exchange variables

Net photosynthesis was estimated at phenological stage 65 (i.e., complete flowering: anthers visible in most panicles) and evaluated in completely expanded young leaves using an open system portable gas exchange meter LI-6400 XT (Li-Cor, Lincoln, USA). The photosynthetic photon flux density was 1600 μmol photons m^{-2} s^{-1}, the concentration of CO_2 inside the chamber was 400 μmol CO_2 mol^{-1}, and the vapor pressure deficit remained between 1.5 and 1.7 kPa. Data were collected after reaching the steady-state equilibrium (~10 min). Further, the leaf area inside the chamber was measured to correct the data (Quevedo *et al.,* 2019). For the evaluation of respiration and leaf carbon balance (LCB), the methodologies described by Da Silva *et al.* (2017) were followed, using an open system portable gas exchange meter LI-6400 XT (Li-Cor, Lincoln, USA). The photosynthetic photon flux density was 0 μmol photons m^{-2} s^{-1}, with a concentration of CO_2 inside the chamber of 400 μmol CO_2 mol^{-1}. The plants were previously adapted to darkness for 30 min, and the temperature was adjusted inside the chamber with the maximum night temperature of the previous night to simulate field conditions. With the net photosynthesis and respiration data, Equation 1 was used to calculate LCB.

$$\text{LCB} = \frac{\textit{Net photosynthesis}}{\textit{Respiration}} \qquad (1)$$

Leaf area index (LAI)

From phenological stages 21 (i.e., start of tillering: first visible tillers) up to 99 (i.e., harvest), the leaf area index (LAI) was estimated every 10 d. To perform such task, a ceptometer ACCUPAR LP-80 (Decagon devices, Hopkins, USA) was used. The evaluation was conducted between 11:00 and 13:00 h. The ceptometer was calibrated according to the manufacturer's instructions, and a leaf distribution value of 0.96 was established. The sensor was located at approximately 45 degrees from the direction of the furrow (Fang *et al.*, 2014).

Interception of photosynthetically active radiation (IPAR), radiation use efficiency (RUE) and harvest index

With the LAI data, third-order logistic regressions were performed, and the daily LAI value was calculated by replacing data in this regression equation. With daily accumulated solar radiation data, daily photosynthetically active radiation (PAR) was estimated, which is equivalent to 50% of the global solar radiation (Garcés and Restrepo, 2015). With this data, Equation 2 was applied to calculate the daily intercepted photosynthetically active radiation (IPAR). The extinction coefficient (k) was assumed to be

0.55, and the calculation of the total IPAR of the crop was carried out by adding the daily IPAR.

$$IPAR = PAR \times exp^{(-k \times LAI)} \quad (2)$$

Further, Equation 3 was used to calculate the radiation use efficiency (RUE) using the sum of the total solar radiation and the total dry weight obtained at phenological stage 99, as follows:

$$RUE = \frac{Dry\ weight}{Accumulated\ solar\ radiation} \quad (3)$$

The harvest index was estimated at phenological stage 99 and the aereal dry matter and the grain weigth were also quantified. Once this information was recorded, the harvest index was calculated (Zhang *et al*., 2013).

Concentration of nutrients in tissue

One hundred g of foliar tissue were collected from the two youngest leaves during phenological stage 65. These samples were analyzed by spectrophotometry in the Soil and Water Laboratory of the Tibaitata research center (AGROSAVIA), where the nitrogen and phosphorus contents were estimated in percentage, in addition to zinc in mg kg^{-1}.

Yield

At phenological stage 99, four samples of 1 m^2 were collected per experimental unit (Garcés and Restrepo, 2015). From these samples, the whole grains were weighed and the green paddy yield was calculated in kg ha^{-1}, and adjusted to a grain moisture content of 22% (Quevedo *et al*., 2019).

Financial analysis

Financial analysis exercises of SD and nutrition practices as well as their interaction were carried out. In this analysis, cost structures were elaborated where the direct and indirect costs were included. The green paddy rice sale price in US dollars was set at US$0.960 kg^{-1}. With this information, the total utility, profitability, monthly profitability, unit production cost, and unit utility were calculated.

Data analysis

Data analysis was performed using general and mixed linear models considering SD and nutrition as a fixed effect, and the block as a random factor. These models were selected according to the Akaike and Bayesian information criteria. The mean comparison test used was Di Rienzo, Guzmán and Casanoves (DGC). The results show that only the factor or interaction had statistical significance ($P \leq 0.05$). Linear and quadratic regression analyses were also performed among the variables LCB, nitrogen content, and yield. The selection of these variables that are highly related to yield was performed using Pearson's correlation coefficient and its *P*-value. The analyzes described were carried out with the software RStudio version 3.5.1 (RStudio Inc, Boston, USA).

Results

Environmental offer

Table 1 shows the diurnal and nocturnal temperature, and accumulted solar radiation conditions to which the rice plants were subjected. It is noteworthy that the maximum night temperature was lower in the August SD (26.18°C) because it was 0.98°C and 1.24°C below the SD conditions of May and July, respectively. The accumulated solar radiation at the fruit development stage (71-79) was higher for the August SD (4,307.08 Cal cm^{-2}/d) than in July (3,877.34 Cal cm^{-2}/d) and May (4,156.56 Cal cm^{-2}/d).

Crop yield

Crop yield values showed significant differences for SD factors and nutrition treatments; however, the interaction of these factors did not show significant differences. The SD of August showed the highest yield (8,744.43 kg ha^{-1} ± 259.24) being 10.22% and 33.43% higher than in July and May, respectively (Fig. 1A). Regarding the nutrition treatments, the biofertilization treatment obtained the highest yield (8,251.88 kg ha^{-1} ± 178.14), which is 18.92% higher compared to the local production treatment (Fig. 1B).

Dry matter accumulation and harvest index

The dry matter accumulation showed significant differences for SD. The effect of nutrition treatments was not significative. The higher dry matter was for the August SD (4051.95 g m^{-2} ± 168.31) and the lower dry matter was for the May SD (2830.59 g m^{-2} ± 113.16) (Fig. 2A). The harvest index showed significative diferences among SD. The August (0.52 ± 0.01) and July (0.53 ± 0.01) SD were equal to a significant level, whereas the May SD showed the lowest harvest index (0.47 ± 0.01) (Fig. 2B).

Radiation use efficiency (RUE)

The RUE showed significant differences for SD factors and nutrition treatments, but not for the interaction of these factors. The SD of August showed the highest RUE (1.31 mg MJ^{-1} ± 0.03), namely, 17.56 and 41.23% higher than the values obtained in July and May, respectively (Fig. 3A). For the nutrition treatments factor, the biofertilization treatment obtained higher RUE values (1.13 mg MJ^{-1} ± 0.02) which are 14.16% higher than those obtained with the local production practices treatment (Fig. 3B).

TABLE 1. Temperature conditions between 70 and 100 days after the emergence of the rice plants established on three different sowing dates.

Sowing date	Phenological state*	Days after emergence	Maximum day temperature (°C)	Minimum night temperature (°C)	Maximum night temperature (°C)	Accumulated solar radiation (Cal cm^{-2}/day
May	51-59	70-80	34.56	23.33	26.79	4,210.01
May	61-69	81-90	35.80	23.88	28.21	4,565.91
May	71-77	91-100	32.97	23.60	26.48	4,156.56
July	51-59	70-80	34.28	24.47	27.43	4,510.17
July	61-69	81-90	34.31	24.24	27.62	3,775.17
July	71-77	91-100	32.90	23.16	27.22	3,877.34
August	51-59	70-80	32.40	23.08	26.49	3,928.64
August	61-69	81-90	33.56	23.17	26.43	4,139.45
August	71-77	91-100	30.97	22.47	25.62	4,307.08

*indicated phenological state according to the BBCH scale (Lancashire *et al.,* 1991). Beginning of panicle emergence: 51; end of panicle emergence: 59; beginning of flowering: 61; end of flowering: 69; watery ripe: 71; late milk: 77.

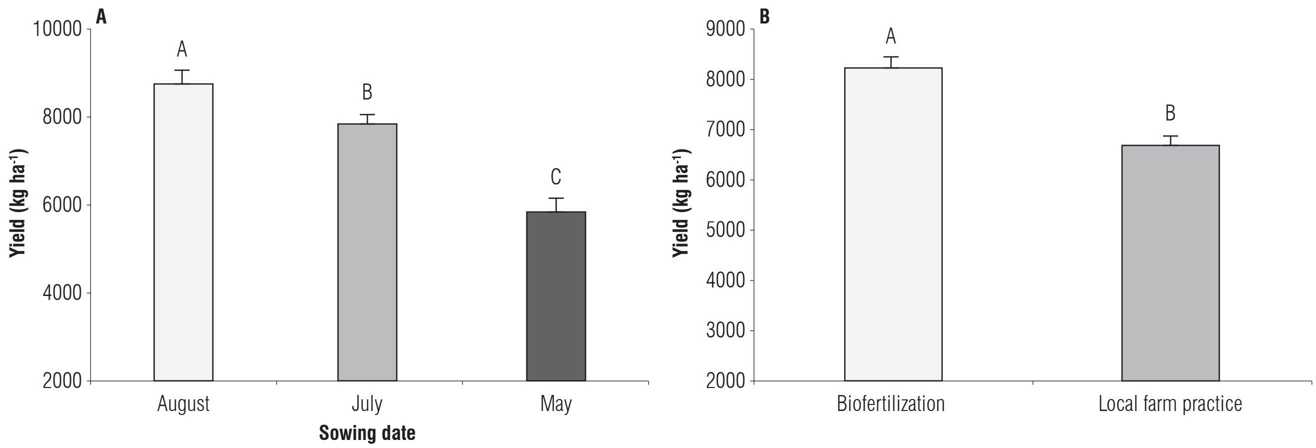

FIGURE 1. Rice yield obtained at three sowing dates with two nutrition treatments. A) rice yield for three planting dates, and B) rice yield for two nutrition treatments. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

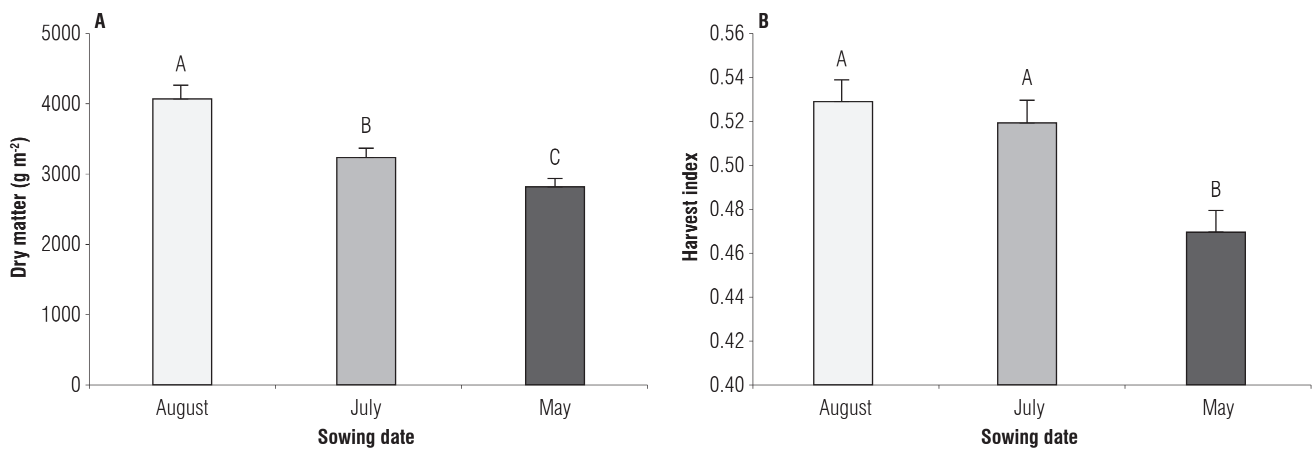

FIGURE 2. Dry matter accumulation and harvest index in rice plants at three sowing dates. A) dry matter, and B) harvest index. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

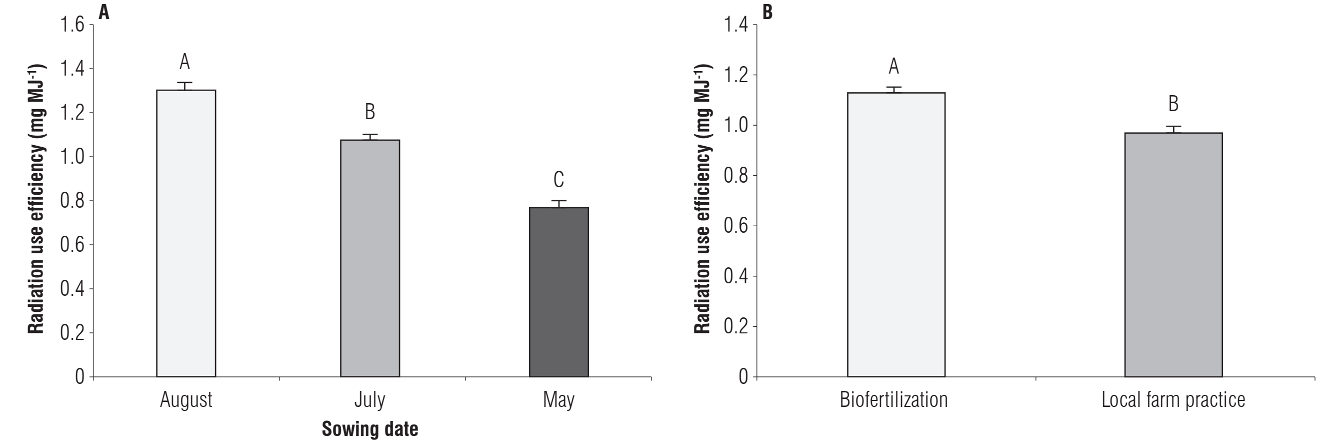

FIGURE 3. Radiation use efficiency in rice plants obtained at three sowing dates with two nutrition treatments. A) radiation use efficiency in three sowing dates, and B) radiation use efficiency for two nutrition treatments. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

Leaf carbon balance (LCB)

The LCB showed statistical differences at a significant level only regarding the effect of SD. The SD of August and July are equal at the statistical level (Fig. 4). However, the LCB in the SD of August is 11.56 and 54.04% higher than that of July and May, respectively.

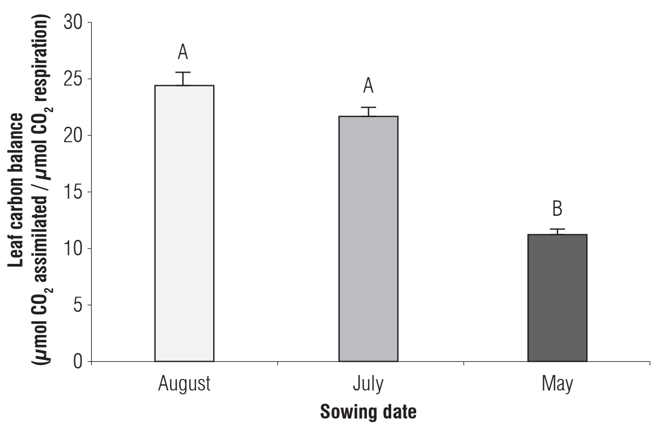

FIGURE 4. Leaf carbon balance in rice plants at phenological phase 65 (i.e., complete flowering: anthers visible in most panicles) at three sowing dates. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

Leaf area index (LAI) and interception of photosynthetically active radiation (IPAR)

The LAI varied significantly only by the SD factor. The SD of August reached a LAI of 10.38, while in May and July it was lower, reaching values of 7.84 and 5.7, respectively; these two SD are statistically equal (Fig. 5). Figure 6 shows the effect of nutrition treatments on the LAI variable. Figures 6A, 6B and 6C show that although the maximum LAI is very similar in each of the SD, with the biofertilization treatment leaf area development is more accelerated compared to the local production practice treatment.

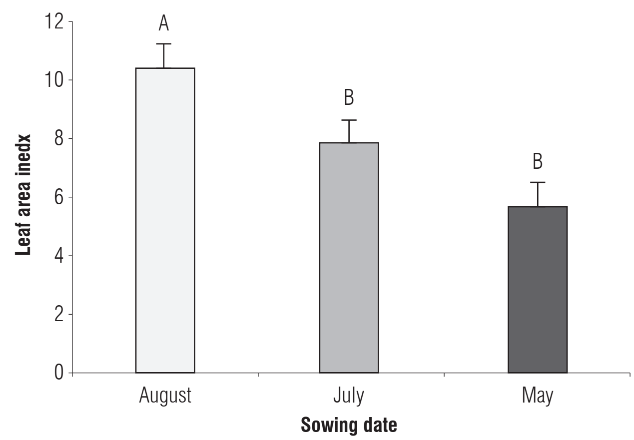

FIGURE 5. Leaf area index of rice plants at phenological stage 65 (i.e., complete flowering: anthers visible in most panicles) at three sowing dates. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

Regarding the IPAR, Table 2 shows that this variable was lower for the SD of August compared to the ones of May and July, which showed similar behaviors. Interestingly, the biofertilization treatment in the SD of August increased the IPAR by 10.05%.

TABLE 2. Interception of photosynthetically active solar radiation (IPAR) on three sowing dates under two mineral nutrition treatments.

Sowing date	Nutrition treatment	IPAR (MJ m^{-2})	Effect of biofertilization (%)
May	Local farm practice	732,740.0	
May	Biofertilization	735,393.0	0.36
July	Local farm practice	742,940.4	
July	Biofertilization	759,110.4	2.18
August	Local farm practice	634,755.5	
August	Biofertilization	698,553.7	10.05

MJ:Megajoule.

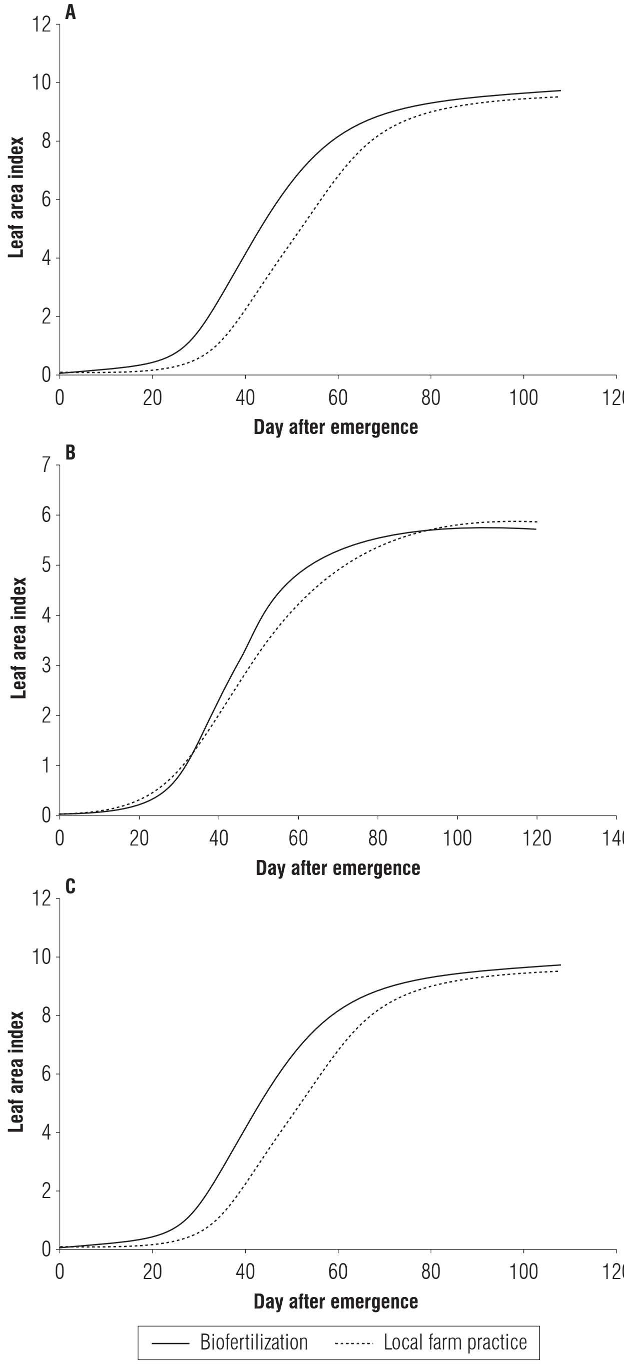

FIGURE 6. Leaf area index for rice plants obtained with two nutrition treatments in three sowing dates (SD). A) May SD, B) July SD, and C) August SD. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

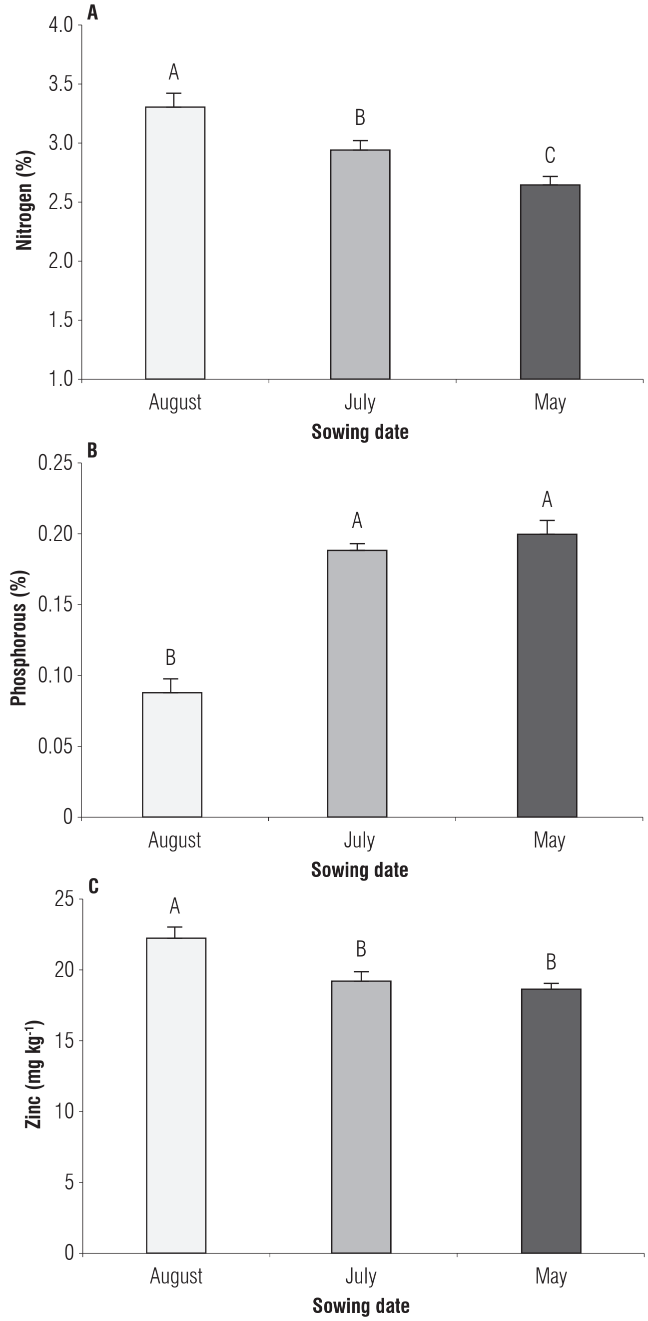

FIGURE 7. Nutritional status of rice plants at phenological stage 65 (i.e., complete flowering: anthers visible in most panicles). A) foliar nitrogen content in rice plants in three sowing dates, B) foliar phosphorus content in rice plants in three sowing dates, and C) foliar zinc content in rice plants in three sowing dates. Error bars correspond to the standard error. Different letters indicate treatments with significant differences ($P \leq 0.05$).

Nutritional status

The nutritional status of the rice plants evaluated by the content of nitrogen, phosphorus, and zinc, only showed significant differences for the SD factor. The effect of nutritional treatments was not significant. The nitrogen content was significantly higher in the SD of August, while the lowest content was observed in May (Fig. 7A). Regarding the phosphorus content, the SD of July and May are equal at a significant level, but higher compared to August (Fig. 7B). As for the zinc content, the SD of May and July showed equal values at a significant level, while in the one of August it was significantly higher compared to the other SD (Fig. 7C).

Relationship between leaf carbon balance (LCB) and nitrogen concentration with yield

Figure 8 shows the two variables that were most related to yield (LCB and nitrogen concentration). In Figure 8A, a positive linear relationship between LCB and yield is observed. Figure 8B shows a quadratic relationship between nitrogen content and yield, with 3.2% nitrogen at the leaf level observed at the inflection point.

Financial analysis of the treatments assessed

The financial analysis showed that the economic benefit was affected by the effect of the agronomic management practices (treatments) evaluated. Table 3 shows the financial analysis for the SD x nutrition treatment interaction. This interaction showed that the SD of August with the biofertilization practice showed the highest profit (US$800.73), in which 35.18% profitability was reached; this contrasts with the local production practice that showed 50% lower profitability for this SD. On the other hand, the May SD was highlighted for not obtaining any financial profit, for this reason, negative profitability was found for each of the nutrition treatments, reaching 1.63% and 19.47% for the biofertilization and local production practices, respectively. This allows observing that the exercise of producing rice in this SD is completely unviable in terms of economic efficiency for the evaluated variety (Fedearroz 68). Proof of this is the high production cost per kg of paddy rice produced, which is higher than US¢9 for the biofertilization practice, and US¢12 for the local production practice compared to the SD of the month of August.

Additionally, the July SD must be highlighted as it showed a negative return of 3.34% with the local production practice. However, this contrasts with what was obtained with the biofertilization practice that yields a positive return of 20.35%, that is, a net profit of US$483.63. This is mainly

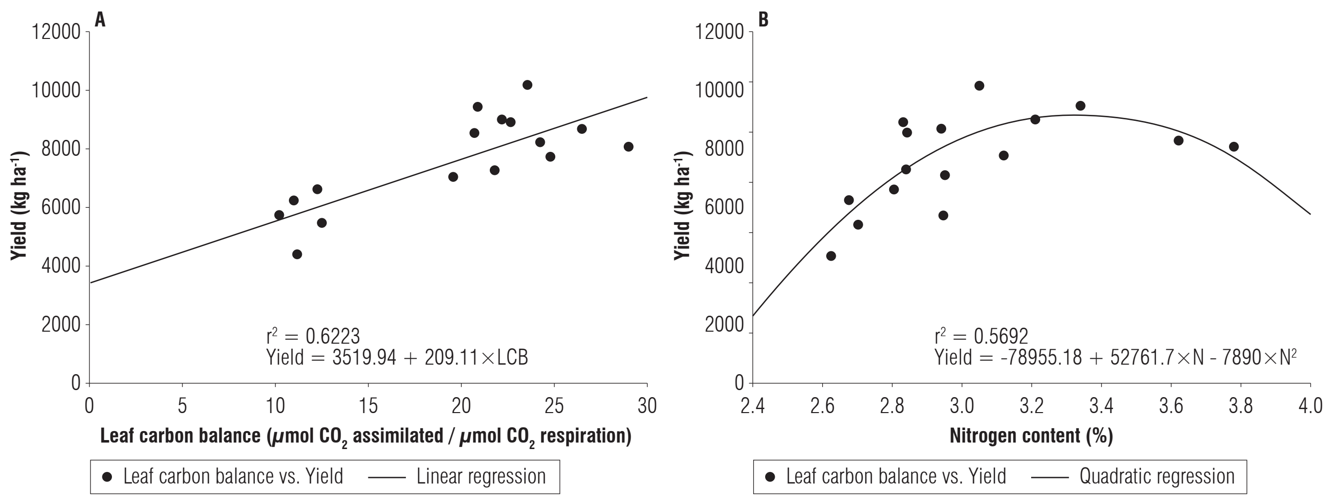

FIGURE 8. Correlation of yield with two variables evaluated at phenological stage 65 (i.e., complete flowering: anthers visible in most panicles). A) correlation of yield with leaf carbon balance, and B) correlation of yield with foliar nitrogen content. Dots correspond to the original data. Solid lines refer to the linear and quadratic regressions.

TABLE 3. Financial analysis of the interaction between sowing date and mineral nutrition treatment.

Sowing date	Nutrition treatment	Total sales ($)*	Total costs ($)*	Accumulated profit ($)*	Profitability (%)	Monthly profitability (%)	Unit cost (kg)	Unit profit (kg)
May	Biofertilization	2104.14	2139.10	-34.96	-1.63	-0.33	0.33	-0.01
May	Local farm practice	1681.14	2087.64	-406.49	-19.47	-3.89	0.40	-0.08
July	Biofertilization	2871.63	2388.00	483.63	20.25	4.05	0.27	0.05
July	Local farm practice	2236.16	2313.36	-77.21	-3.34	-0.67	0.34	-0.01
August	Biofertilization	3076.70	2275.97	800.73	35.18	7.04	0.24	0.08
August	Local farm practice	2612.04	2219.96	392.09	17.66	3.53	0.28	0.05

*Values are given in United States dollars (USD) with an average exchange rate for 2017.

due to a lower unit production cost (US¢27 kg^{-1} of rice) which generated US¢5 of net profit (Tab. 3).

In the analysis of the SD practice as an independent factor (Tab. 4), the SD in May showed a negative level of profitability (-10.55%) compared to the SD in July and August. Notably, in August, a higher profit of US$1,193 and a 26.42% profitability were obtained.

TABLE 4. Results of the accumulated profit and profitability in three sowing dates under two mineral nutrition treatments.

Sowing date	Profit (US$)*	Profitability (%)	Monthly profit (US$)*	Monthly profitability (%)
May	-441.46	-10.55	-88.29	-2.11
July	406.43	8.45	81.28	1.691
August	1192.81	26.42	238.56	5.28

*Values are given in United States dollars (USD) with an average exchange rate of 2017.

Considering the differential result of the yield with the biofertilization practice in the SD evaluated, it was found that it increases a 0.22% in production costs. However, this excess cost allowed achieving 17.93% profitability in the three assessed SD, while the implementation of the local production practice showed a loss of 1.71%. With this exercise, it can be inferred that the implementation of the biofertilization practice allowed achieving US$1,249.4 in profit, while with the local production practice, losses of US$91.62 were obtained in the three SD evaluated, as can be seen in Table 5.

TABLE 5. Accumulated profit and profitability for two mineral nutrition treatments during three sowing dates.

Nutrition treatment	Profit (US$)*	Profitability (%)	Monthly profit (US$)*	Monthly profitability (%)
Biofertilization	1249.4	17.93	249.88	3.58
Local farm practice	-91.62	-1.71	-18.324	-0.34

*Values are given in United States dollars (USD) with an average exchange rate of 2017.

Discussion

Rice yield is influenced significantly by the effect of the SD. The highest yield was obtained in the August SD, so the environmental offer in this SD is optimal for the Fedearroz 68 variety. Nonetheless, high RUE values were observed, and this variable is related to the amount of solar radiation, the capacity of the canopy to capture solar radiation and the efficiency of its conversion into phytomass (Parry *et al.,* 2011; Ceotto *et al.,* 2013). In this case, the accumulated solar radiation at the late milk stage in the August SD (4,307.08 Cal cm^{-2}/day) was higher than other SD. The solar radiation collection capacity estimated by the LAI was higher than 10 during the August SD, which is considered the optimal LAI for rice plants to obtain a maximum yield (Mae *et al*., 2006; Quevedo *et al.,* 2019). However, it is noteworthy that the lowest IPAR was obtained during this SD, which is contradictory to what was found by Garcés and Restrepo (2015) in the municipality of Saldaña (province of Tolima), where they identified that the SD where the highest IPAR value was obtained was the one with the maximum yield (7000 kg ha^{-1}). This may be because the Fedearroz 68 variety in the presence of high radiation suffers a photoinhibition phenomenon.

For the efficiency in the conversion of solar radiation into carbohydrates, the LCB was directly related to the yield. For this reason, in the SD of August, the highest yield and the LCB reached 25 µmol CO_2 / µmol CO_2 respiration; this, however, is explained by the lower nighttime temperature that occurred in this SD. The above is favorable for rice plants since this environmental condition decreases their respiration rate (Alvarado *et al.,* 2017). Furthermore, this condition affects the availability of carbohydrates for grain filling, so the magnitude of respiration can increase or reduce yield (Peraudeau *et al.,* 2015). The high night temperature could generate damage of cell membrane by the generation of reactive species of oxygen (Xue *et al*., 2012). The partitioning to developing sink (grain) was determinated through the harvest index which is affected by the high night temperature (Zhang *et al*., 2013). In this research, it was observed that the harvest index and dry matter accumulation were higher on the August and July SD, when the night temperature was lower than in the May SD. This is related with the high yield on the August SD and the low yield on the May SD.

Regarding the ability to fix CO_2, no clear relationship was found between the net photosynthesis evaluated at the leaf level and crop yield, which is contradictory to what Hidayati *et al.* (2016) found. However, it is hypothesized that in the SD of August the photosynthesis at the canopy level was higher than in the other SD, given that only in this SD a nitrogen concentration higher than 3% was reached, which is considered ideal (Ray, 2013). Nonetheless, this can be related to a high concentration of chlorophyll (Lee *et al*., 2011) and RuBisCO (Imai *et al*., 2008). In addition to this, authors as Mae *et al.* (2006) consider that the optimal LAI has a value of 10.38, so considering the above mentioned, the photosynthesis at the canopy level may be higher compared to the other SD. However, this hypothesis must be verified in future studies.

The nitrogen content was related to yield in a quadratic way; hence, the maximum yield was found with a foliar nitrogen content of 3.3%. However, if the content is higher than this value, the yield shows a decreasing tendency. This can be attributed to the higher availability of the element in the soil solution, which increases vegetative development, and specifically, the development of late tillers that affect the IPAR and the distribution of carbohydrates (Wang *et al*., 2017).

Regarding the effect of the nutrition treatments on yield, biofertilization was found to increase yield by 18.92%; this was similar to what was found in other studies in which this practice increased yield between 19.8% and 29.32% (Roger and Ladha, 1992; Lavakush *et al*., 2014). However, no significant changes were observed in the concentration of nitrogen, phosphorus, and zinc in tissue at phenological stage 65 as a result of this practice. This suggested that the biofertilization did not increase the availability in soil of nitrogen, phosphorus and zinc. Despite this, biofertilization affected the growth level because the plants under this treatment developed their LAI more quickly, which allowed increasing IPAR up to 10.05%. This behavior can be attributed to the fact that the phosphate solubilizing bacterium *R. pusense* strain B02 and *A. chroococcum* act as plant growth promoters as they produce indole acetic acid that is an auxin (Castanheira *et al*., 2014; Romero *et al.,* 2017) controlling cell division and foliar expansion processes (Li *et al.,* 2007). Moreover, they have also been found to affect the degree of foliar inclination, which generates a higher leaf area exposed to light (Zhao *et al.,* 2013). For these reasons, it is believed that leaf development and IPAR were higher with the biofertilization treatment. By increasing the uptake of light, the CO_2 fixation capacity increases (Garcés and Restrepo, 2015), so the plant would have greater availability of photoassimilates to fill a higher number of demanding organs, producing the increase in yield that was observed in this study.

The financial analysis of the evaluated practices allowed establishing the level of productivity of the local production practice in economic terms. In this practice, low percentages of profitability were obtained, which in many cases generates economic losses. The above was evidenced in two of the three SD evaluated; the SD of May showed a negative return of 10.55% with losses of US$441.4, while in the SD of July there was a positive profitability of 8.45% and a profit of US$406.46. The latter differs with the results found by Akbar *et al.* (2010), who evaluated the Super Basmati variety in six SD, estimating a loss of US$40.47 ha^{-1}. Additionally, Bashir *et al.* (2010) also reported that the July SD cultivated with the KS-282 variety recorded a loss of US$184.54. In contrast, Osman *et al.* (2015) stated that this SD shows a higher net profit with US$746.07 ha^{-1} in the semi-arid conditions of Sudan, which reach a yield of 2900 kg ha^{-1} with the Nerica 4 variety. A 17.66% profitability was obtained in the August SD with the local production practice, However, with the implementation of the biofertilization practice, the profitability increased up to 35.18%. This practice was also crucial in the July SD, given that it showed a return of 20.25% yielding a cumulative profit of US$1,284.36 ha^{-1}, while with the local production practice, a negative return of -3.34% was obtained. Considering the above, we can conclude that the use of biofertilizers is a viable alternative from the technical and economic points of view, which is similar to what was stated by Mohammadi and Sohrabi (2012), who also reported that the use of biofertilizers plays a key role in soil productivity and sustainability.

Conclusions

Crop yield and profitability was affected by the management practices evaluated. With the selection of the sowing date, it was possible to maximize the yield of the Fedearroz 68 variety, given by the occurrence of low nocturnal temperatures during flowering and an ideal range of solar radiation. With the biofertilization practice, plant growth was improved allowing a better light interception and, therefore, higher radiation use efficiency.

The selection of the sowing date does not change the production costs but increases the profitability of up to 26.42%. Further, with the biofertilization practice, the production cost increases by 0.22%, while the profitability increases by up to 35.18%. This allowed reducing the production unit cost to US$0.24 kg^{-1}. For this reason, we conclude that these practices are viable from the technical and economic points of view because they increase productivity and economic benefit. However, this should be evaluated in other varieties and agroecological zones, to allow the elaboration of recommendations for a large part of the rice producing areas of Colombia.

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Can *Amplicephalus funzaensis* Linnavuori 1968 (Hemiptera: Cicadellidae) transmit phytoplasmas to strawberry?

¿Puede *Amplicephalus funzaensis* Linnavuori 1968 (Hemiptera: Cicadellidae) transmitir fitoplasmas a fresa?

Daniela Montaño Novoa[1], Helena Luisa Brochero[2], and Liliana Franco-Lara[1]*

ABSTRACT

Phytoplasmas are plant pathogenic bacteria of the class Mollicutes that lack cell walls, are restricted to the phloem of their plant hosts, are difficult to culture, and are transmitted by insect vectors. Phytoplasmas from 16SrI and 16SrVII groups have been associated with diseases in urban trees in the Bogota plateau and with potato and strawberry crops in Cundinamarca, Colombia. The objective of this work was to evaluate if the vector *Amplicephalus funzaensis* (Hemipera: Cicadellidae) could transmit phytoplasmas to *Fragaria x ananassa* under semi-controlled conditions. A transmission assay on *F. x ananassa* var. Monterrey was performed with *A. funzaensis* from a population naturally infected with phytoplasmas, whose host was *Cenchrus clandestinus*. Seven months after herbivory by these insects, the plants did not show symptoms associated to phytoplasmas, even though more than one third of the insects used carried phytoplasmas. In total, 120 *A. funzaensis* individuals were tested for the presence of phytoplasmas by molecular methods; of these, 46 (38%) were positive for phytoplasmas, showing the existence of insect populations with a high number of individuals that are a potential source of inoculum for the pathogen transmission. Additionally, for the molecular identification of *A. funzaensis*, a DNA barcode was generated from the *cyitochrome c oxidase* (*COI*) gene.

Key words: insect vectors, DNA barcoding, cytochrome c oxidase, *COI* gene.

RESUMEN

Los fitoplasmas son bacterias patógenas de plantas de la clase Mollicutes que carecen de pared celular, están restringidas al floema de sus hospederos vegetales, son difícilmente cultivables y son transmitidos por insectos vectores. Fitoplasmas de los grupos 16SrI y 16SrVII se han asociado a enfermedades en árboles urbanos de la Sabana de Bogotá y en cultivos de fresa y papa de Cundinamarca, Colombia. El objetivo de este trabajo fue evaluar si el conocido vector *Amplicephalus funzaensis* (Hemipera: Cicadellidae) podía transmitir fitoplasmas a fresa *Fragaria x ananassa* en condiciones semicontroladas. *A. funzaensis*, naturalmente infectados con fitoplasmas, cuyo hospedero era *Cenchrus clandestinus* se utilizaron para realizar ensayos de transmisión en plantas *F. x ananassa* var. Monterrey. Siete meses posteriores a la herbivoría por estos insectos, las plantas no presentaban síntomas asociados a fitoplasmas, a pesar de que más de un tercio de estos insectos portaban fitoplasmas. En total, en este trabajo se evaluaron 120 individuos de *A. funzaensis* para la presencia de fitoplasmas por métodos moleculares; de estos, 46 (38%) fueron positivos para fitoplasmas, mostrando la existencia de poblaciones de insectos con un número alto de individuos que son fuente potencial de inóculo para la transmisión del patógeno. Además, para la identificación molecular de *A. funzaensis*, se generó un código de barras de ADN partir de una secuencia del gen *citocromo c oxidasa* (*COI*).

Palabras clave: insectos vectores, código de barras de ADN, citocromo c oxidasa, gen *COI*.

Introduction

Phytoplasmas are bacteria of the class Mollicutes that lack cell walls and are sensitive to tetracycline. It has been stated that they inhabit the phloem cells of plants or the intestine, haemolymph, salivary glands and other internal organs of some hemipteran insects of the families Cicadellidae, Fulgoromorpha, and Psyllidae (Lee *et al*., 2000; Weintraub and Beanland, 2006; Hogenhout *et al*., 2008; Bertaccini and Duduk, 2009). Phytoplasmas are difficult to cultivate (Contaldo *et al*., 2016), so their detection, identification and classification are mainly based on the amplification of the 16SrRNA gene (Gundersen *et al*., 1996) and further analysis by restriction fragment length polymorphism (RFLP) with 17 restriction enzymes that discriminate them into ribosomal groups. To date, 34 16Sr groups are recognized for phytoplasmas (Pérez-López *et al*., 2016). The genus '*Candidatus* Phytoplasma' was adopted for incompletely

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[1] Facultad de Ciencias Básicas y Aplicadas, Universidad Militar Nueva Granada, Km 2 vía Cajicá-Zipaquirá, Cundinamarca, Colombia.
[2] Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Bogota, Colombia.
[*] Corresponding author: liliana.franco@unimilitar.edu.co

described, non-cultivable phytoplasmas. A new species within *Candidatus* is created when the sequence of the 16SrRNA gene contains less than 97.5% of nucleotide identity compared to previously described phytoplasma species (IRPCM, 2004).

The presence of phytoplasmas is associated with diseases in crops, ornamentals, fruit and woody plants belonging to different botanical families (Lee *et al*., 2000; Bertaccini *et al*., 2014). In Colombia, phytoplasmas from the 16SrIII group were reported in *Coffea arabica, Cordia alliodora, Physalis peruviana* and *Solanum quitoense* (Galvis *et al*., 2007; Mejía *et al*., 2014); 16SrV and 16SrXII in *Solanum tuberosum* (Mejía *et al*., 2011; Varela and Franco-Lara, 2017); 16SrIII-L in *Manihot esculenta* (Álvarez *et al*., 2009); 16SrIX in *Catharanthus roseus* (Duduk *et al*., 2008a), and 16SrI in *Elaeis guineensis*, *Zea mays* and *Musa acuminata* (Duduk *et al*., 2008b; Álvarez *et al*., 2014; Aliaga *et al*., 2018). In Bogota, phytoplasmas of the groups 16SrI and 16SrVII are widely distributed in urban trees such as *Acacia melanoxylon, Croton* spp., *Eugenia neomyrtifolia, Fraxinus uhdei, Liquidambar styraciflua, Magnolia grandiflora, Pittosporum undulatum, Populus nigra,* and *Quercus humboldtii* (Perilla-Henao *et al*., 2012; Perilla-Henao and Franco-Lara, 2013; Franco-Lara and Perilla-Henao, 2014; Franco-Lara *et al*., 2017). Phytoplasma diseases in strawberry *Fragaria x ananassa* have been reported in Australia (Padovan *et al*., 1998; Padovan *et al*., 2000), the United States (Harrison *et al*., 1997; Jomantiene *et al*., 1999), France (Danet *et al*., 2003), Italy (Bertaccini *et al*., 1997), and Lithuania (Valiunas *et al*., 2006). In Colombia, phytoplasma groups 16SrI and 16SrVII have been reported in strawberry varieties Camino Real and Ventana, with symptoms of flower phyllody, virescence of achenes, double sepals and aborted fruits (Perilla-Henao and Franco-Lara, 2014).

Amplicephalus funzaensis Linnavuori 1968 (Cicadellidae: Deltocephalinae) and *Exitianus atratus* Linnavuori 1959 (Cicadellidae: Deltocephalinae) are known vectors of phytoplasma groups 16SrI and 16SrVII in Bogota (Perilla-Henao *et al*., 2016). The objective of this study was to determine if *A. funzaensis* can transmit phytoplasmas to *Fragaria x ananassa*, and to generate a DNA barcode for this insect species.

Materials and methods

The transmission assays were carried out at the campus of the Universidad Militar Nueva Granada (UMNG), in Cajica, Cundinamarca (4°55'0" N, 74°1'30" W), at an altitude of 2558 m a.s.l., with average annual temperature of 13.9°C and average annual relative humidity of 87.9% (information obtained in 2016 from the meteorological station at Nueva Granada Campus, when the assays were carried out).

Entomological material

Insects were collected from pastures represented mainly by *Cenchrus clandestinus* using an entomological net and a mouth aspirator. Samplings were performed at the campus UMNG or at the Universidad Nacional de Colombia (UN) campus in Bogota, Colombia (4°38'18.88" N, 74°5'2.76" W). The insects were stored in 90% (v/v) ethanol. Taxonomic identification was based on morphological characters (Linnavuori, 1959; Dietrich, 2005; Silva-Castaño *et al*., 2019). Before performing the transmission experiments, a natural population of *A. funzaensis* that lived on the grass *C. clandestinus* at the campus UMNG in Cajica was tested for the presence of phytoplasmas. In July 2015, 20 individuals were analyzed by nested PCR to test for phytoplasmas. The *A. funzaensis* used for the transmission assays were captured in April 2016 at the campus UMNG from the population in which the natural infection by phytoplasmas had been recorded. In February and March 2016, another group of twelve *A. funzaensis* belonging to two different populations, UMNG campus and UN campus were used to generate a DNA barcode for this species.

Transmission assays

One hundred adult *A. funzaensis* were collected and maintained for 5 min at 4°C to reduce their mobility and separate females and males, before placing them on strawberry *F. x ananassa* variety Monterrey of approximately 6 months old, grown in an aquaponic culture. The plants were propagated by division of the crown and the new plants were potted individually in 7 kg pots using soil and rice husks (2:1) as substrate. Before the experiment, the plants were kept for three months in insect free cages and were watered every 3 d by drip irrigation and fertilized once a month with Hoagland and Arnon (1950) nutrient solution. To confirm that all the plants were free of phytoplasmas before the transmission assays, nested PCR tests were performed, as described below. Treatment 1 consisted of two replicas with five plants each, and treatment 2 (control) consisted of one replica with five plants that were not exposed to *A. funzaensis* hervibory. Plants of both treatments were kept in independent insect free cages. For treatment 1, each plant was infested with ten male and female adults of *A. funzaensis* captured at the campus UMNG in Cajica, from the population in which phytoplasmas had been previously detected. To guarantee that the insects were in contact with the leaves, two 10 cm X 10 cm veil bags containing five insects each were tied to different leaves of each plant.

When the insects died, 1 week later, they were retrieved and stored at -20°C for molecular testing. Two months post-herbivory, leaves of all the plants of treatments 1 and 2 were sampled and tested for phytoplasmas. The plants were kept for 7 months in observation inside cages and monthly observations were made to register the appearance of symptoms. During these months, watering and fertilization were performed as described before.

DNA extraction

Insect DNA extractions were performed following the methodology described by Hung *et al*. (2004). One *A. funzaensis* individual was ground with liquid nitrogen and 30 µl of lysis buffer (0.1 M Tris-HCl pH 8.0, 0.05 M EDTA, 0.01% (v/v) N-Lauroylsarcosine). After grinding, 970 µl of the same buffer were added. The tissue was treated with 1% (w/v) sodium dodecyl sulphate and 0.08 mg ml^{-1} proteinase K, and incubated at 55°C overnight. The next day, 500 µl of 1:1 equilibrated pH 8.0 phenol and chloroform were added and the sample was centrifuged at 10,000 *g* for 10 min. The aqueous phase was removed and the nucleic acids precipitated with 1 ml of absolute ethanol for 15 min at -20°C, and then samples were centrifuged at 10,000 *g* for 25 min. The pellet was washed in 1 ml of 70% (v/v) ethanol. The supernatant was eliminated and the ethanol left to evaporate. Finally, the DNA was resuspended in 50 µl TE buffer (10 mM Tris-HCL, 1 mM EDTA pH 8.0) and stored at -20°C. The DNA concentration of the samples was quantified in a Qubit (Invitrogen®) using the Qubit Assay kit.

The plant DNA extractions were performed using a protocol by Dr. A. Bertaccini (personal communication) and adapted in our laboratory. Extractions started from 0.33 g of leaf midribs. The midribs were ground in liquid nitrogen and extraction buffer I (92 mM $K_2HPO_4 \times 3H_2O$, 30 mM KH_2PO_4, 10% sucrose (w/v), 5% (w/v) PVP-10 and L-ascorbic acid (pH 7.6)). The samples were filtered through sterile cloths, centrifuged at 6500 *g* for 10 min and resuspended in 1.3 ml of extraction buffer II (100 mM Tris-HCL, 100 mM EDTA, 250 mM NaCl) with 25 µl of proteinase K (5 mg/ml) and 145 µl of N-Lauroylsarcosine 10% (w/v). Samples were incubated at 55°C for 2 h. The lysate was centrifuged for 10 min at 8,000 *g* and the pellet discarded. The nucleic acids were precipitated with 0.6 volumes of isopropanol and incubated at 4°C overnight. Next day, the extracts were centrifuged at 8,000 *g* for 30 min and the pellet resuspended in 990 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0], 24.75 ml of 20% (w/v) sodium lauryl sulphate and 19.8 µl of proteinase K (5 µmg ml^{-1})), and incubated at 37°C for 1 h. Later, 175 µl of 5 M NaCl and 140 µl (10% (w/v) cetyl trimethyl ammonium bromide/0.7M NaCl) were added and the lysate incubated at 65°C for 10 min. Two consecutive extractions were performed, the first with an equal volume of chloroform / isoamyl alcohol (24:1) and the second with an equal volume of chloroform. In both cases the phases were separated by centrifugation at 8,000 *g* for 10 min and the organic phase discarded. The supernatant was precipitated with 0.6 volumes of isopropanol at 4°C overnight. Finally, the nucleic acids were pelleted by centrifugation at 11,000 *g* for 20 min and the pellet washed with ethanol 70%. The pellet was resuspended in 50 µl of TE buffer and the DNA was stored at -20°C. Later, the DNA was purified with the PowerClean® Pro DNA Clean-Up kit (Mo Bio Laboratories, Inc.) to eliminate other impurities.

Amplification of the *COI* gene of *A. funzaensis* and sequence analysis

The amplification of a region of the *COI* mtDNA gene was performed with the universal insect primers LCO1490/HCO2198 (Folmer *et al*., 1994). The PCR reactions were conducted at a final volume of 25 µl, with 0.05 U of Taq polymerase (Bioline®), 1X reaction buffer, 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 0.3 µM of each primer and 50 ng of DNA diluted in deionized water. The thermic profile consisted on a denaturation for 10 min at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 45 s at 40°C, extension for 45 s at 72°C and, a final extension period for 10 min at 72°C. The amplicons were purified with the UltraClean® PCR Clean-up Kit (Mo Bio Laboratories, Inc.) and were bi-directionally sequenced in Macrogen, Korea. The sequences were edited with the Geneious R9 software (Biomatters Ltd.) and each individual consensus sequences were built by superposition of the forward and reverse sequences. The consensus sequences were compared using BLASTN with the GenBank database. Additionally, nine *A. funzaensis* sequences obtained in this research, and other Cicadellidae downloaded from the BOLD database (http://www.barcodinglife.org) were used to construct a distance tree by the neighbor-joining method, with 1000 bootstrap, based on the Kimura 2 parameter (K2P) model, using the Mega v.6.0 software (Tamura *et al*., 2013). A sequence of *Psylla galeaformis* (Hemiptera: Psyllidae) was used as outgroup. The Barcode Gap analysis of the *COI* gene sequences was performed by comparison of the intraspecific versus the interspecific sequences, obtained from the distance matrix generated in MEGA v.6.0, according to the K2P model.

Detection of phytoplasmas using PCR

The presence of phytoplasmas was tested by PCR in *F. x ananassa* and *A. funzaensis* DNA extracts. To verify that PCR inhibitors were not present in the plant DNA extracts, amplification of rps16 chloroplast intron was performed, before phytoplasma detection (Oxelman *et al*., 1997). The PCR reactions were conducted in a final volume of 15 µl, with 0.05 U of Biolase (Bioline), 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 µM of primers. Dilutions 1:50 and 1:100 were prepared from the plant DNA extracts in deionized water and 2 µl were used as template. Deionized water was used as negative control in all the PCR reactions. The thermic profile was: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 53°C, extension for 2 min at 72°C, and a final extension period for 10 min at 72°C.

The PCR reactions for phytoplasmas were made as explained before. For *F.* x *ananassa,* 1:20, 1:50 and 1:100 dilutions of the DNA extracts were tested. For the insects, 1:20 dilutions of DNA extracts were analyzed. The PCR with universal primers P1A/P7A (Lee *et al*., 2004) produced amplicons of ~1800 bp, which were diluted 1:20 and used in nested PCRs with the internal primers R16mF2/R16mR1 (Gundersen and Lee, 1996) to obtain ~1430 bp amplicons. Frequently, when low intensity amplicon bands were observed in nested PCRs, the resulting amplicons were amplified again using internal primers R16F2n/R16R2 (Gundersen and Lee, 1996) using 1:20 dilutions of the amplicon as template. Bands with enough DNA were further analyzed by sequencing or RFLP analyses. In these reactions, the positive control corresponded to fragments of the region 16S-23S of the phytoplasmas Maize bushy stunt - MBS (16SrI-B group) and ash yellows ASHY (16SrVII-A group), previously cloned and maintained in an *Escherichia coli* strain. In the *F.* x *ananassa* plants and in the *A. funzaensis*, additional nested PCRs tests were performed with different primer combinations such as P1A/P7A (Lee *et al*., 2004) followed by R16F2n/R16R2, and R16mF2/R16mR1 in primary reactions followed by R16F2n/R16R2. The plant DNA extracts were analyzed in 1:50 and 1:100 dilutions and the insect DNA extracts in 1:10 dilutions. The thermic profile was as described before, except for annealing temperatures that depended on the primer pairs used: P1A/P7A, 55°C; R16mF2/R16mR1, 54°C and R16F2n/R16R2, 54°C. The amplicons were separated using standard electrophoresis in 1% agarose gels in TBE buffer 1X (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), stained with ethidium bromide and visualized under UV light.

To determine phytoplasma ribosomal group affiliation, nested PCR amplifications were performed with the R16F2n/R16R2 primers at a final volume of 30 µl. This volume was divided into four, and each aliquot digested with the restriction endonucleases *Rsa*I, *Mse*I, *Hha*I or *Alu*I (New Englad BioLabs). Digestions were conducted at a final volume of 20 µl, with 0.05 U of enzyme, 1X buffer and 6.5 µl of the PCR product obtained by nested amplification. The reactions were incubated at 37°C overnight. The restriction products were separated using agarose gel electrophoresis at 3% in TBE buffer. Additionally, the selected amplicons obtained with the primers R16F2n/R16R2 were purified with the UltraClean® PCR Clean-up Kit (Mo Bio Laboratories, Inc.) and sequenced bidirectionally in Macrogen, Korea. The sequence analysis was performed as explained before.

Results

Entomological material

Only specimens of *A. funzaensis*, confirmed by morphological and molecular methods, were included in this study (Fig. 1).

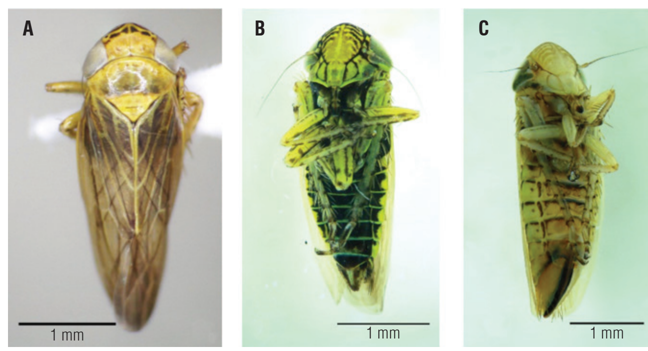

FIGURE 1. *Amplicephalus funzaensis.* (A-B) Adult male, dorsal and ventral views. (C) Female, ventral view.

Transmission assays

At the time of this experiment it was not possible to raise *A. funzaensis* under laboratory conditions, so the transmission experiment was performed with insects from a naturally infected population. In a previous work, a population of *A. funzaensis* infected with 16SrI and 16SrVII phytoplasmas had been found, but before performing the experiment it was tested again for the presence of phytoplasmas. Twelve adults of twenty *A. funzaensis* from that population were found positive for phytoplasmas by nested PCR, but no further RFLP or sequencing analysis were performed because the quantity of the obtained DNA was too small.

For the transmission experiment, a different set of 100 insects were allowed to feed in 10 phytoplasma-free plants, placing 10 insects per plant. DNA extraction is a destructive method, so the detection of the phytoplasmas in these insects was performed after they were allowed to feed on

TABLE 1. Results of transmission assays of phytoplasmas by *Amplicephalus funzaensis* to *Fragaria* x *ananassa* in semi-controlled conditions.

				A. funzaensis (adults)			Plants *Fragaria x ananassa*
		Females	Males	Positive by PCR (b)		Group detected by RFLP/	Detection
	Plants	per plant	per plant (a)	Females	Males	Total individuals tested (c)	by PCR (d)
Plants exposed to herbivory	1	5/10	5/10	4	1	16SrI (2/4)	–
	2	8/10	2/10	5	0	16SrI (4/4)	–
	3	7/10	3/10	2	0	16SrI (2/2)	–
	4	9/10	1/10	6	0	16SrI (5/6)	–
	5	7/10	3/10	4	2	16SrI (4/5)	–
	6	8/10	2/10	3	1	16SrI (4/4)	–
	7	2/10	8/10	0	7	16SrI (6/7) 16SrVII (1/7)*	–
	8	8/10	2/10	7	2	16SrI (4/5)	–
	9	9/10	1/10	1	0	16SrI (1/1)	–
	10	7/10	3/10	2	1	16SrI (1/3)	–
Controls Plants not exposed to herbivory	1	0		–	–	–	–
	2	0		–	–	–	–
	3	0		–	–	–	–
	4	0		–	–	–	–
	5	0		–	–	–	–
Total		70 Females - 30 Males		34	14		

(a) Number of females and males placed on each tested plant, over total number of insects. (b) Number of females and males positive for phytoplasmas by nested PCR.
(c) Number of positive insects for 16SrI or 16SrVII groups, determined by RFLP with restriction enzymes *Rsa*I, *Mse*I, *Hha*I and *Alu*I, over the total of tested insects.
(d) Detection of phytoplasmas in *F.* x *ananassa* by nested PCR and double nested PCR 2 months post-inoculation.
(-) Negative for phytoplasmas.
(*) Samples with mixed infections containing 16SrI and 16SrVII.

the test plants. After two months post-herbivory, all the *F.* × *ananassa* plants were negative for phytoplasmas, regardless of the PCR primer combination used, as were the non-exposed negative control plants. In all the cases, the positive PCR controls generated bands of the expected size and the negatives did not amplify (Figs. 2 A-C). No symptoms associated with phytoplasmas in leaves, flowers, or fruits were observed after 7 months of evaluation (Tab. 1).

The control plants were also negative for the presence of phytoplasmas in tests conducted simultaneously with plants exposed to insects. In all cases, the positive controls generated bands of the expected size and the negatives did not amplify (Figs. 2 A-C).

Of 100 *A. funzaensis* used in the transmission assays, 22 individuals resulted positive for phytoplasmas by nested PCR with primers P1A/P7A followed by R16F2n/R16R2 and 36 individuals were positive with primers R16mF2/R16mR1 and R16F2n/R16R2. Taking into account nested PCR tests with both sets of primers, 48 of 100 individuals that were used in the transmission tests were positive in PCR using primers for phytoplasmas (Fig. 3, Tab.1).

Of the 48 positive insects, 41 were analyzed by RFLP. Of these, 34 were infected with phytoplasmas: 33 were infected with 16SrI group and one simultaneously with 16SrI and 16SrVII groups (Tab. 1). In the others, the RFLP patterns obtained were not consistent with any phytoplasma group profile, suggesting non-specific amplifications (Fig. 4).

DNA barcoded for *A. funzaensis*

Amplicons of the mitochondrial DNA *COI* gene were obtained from 12 individuals, six collected at the UMNG and six at the UN. Consensus sequences of lengths between 667 and 706 bp were obtained from nine individuals (Fig. 5A); the other sequences were illegible. The edited sequences were uploaded in the BOLD database (http://v3.boldsystems.org/) (Ratnasingham and Hebert, 2007). The sequences of all the *A. funzaensis* collected in Bogota at the UN showed a conserved haplotype (Sequences ID: COLCI053-18, COLCI054-18, COLCI055-18, COLCI056-18, and COLCI057-18), but the ones collected at the UMNG in Cajica represented three haplotypes. Haplotype 1 was identical to the Bogota sequence (Sequence ID: COLCI049-18); two sequences called haplotype 2 (Sequences ID: COLCI051-18 and COLCI052-18) presented substitutions T

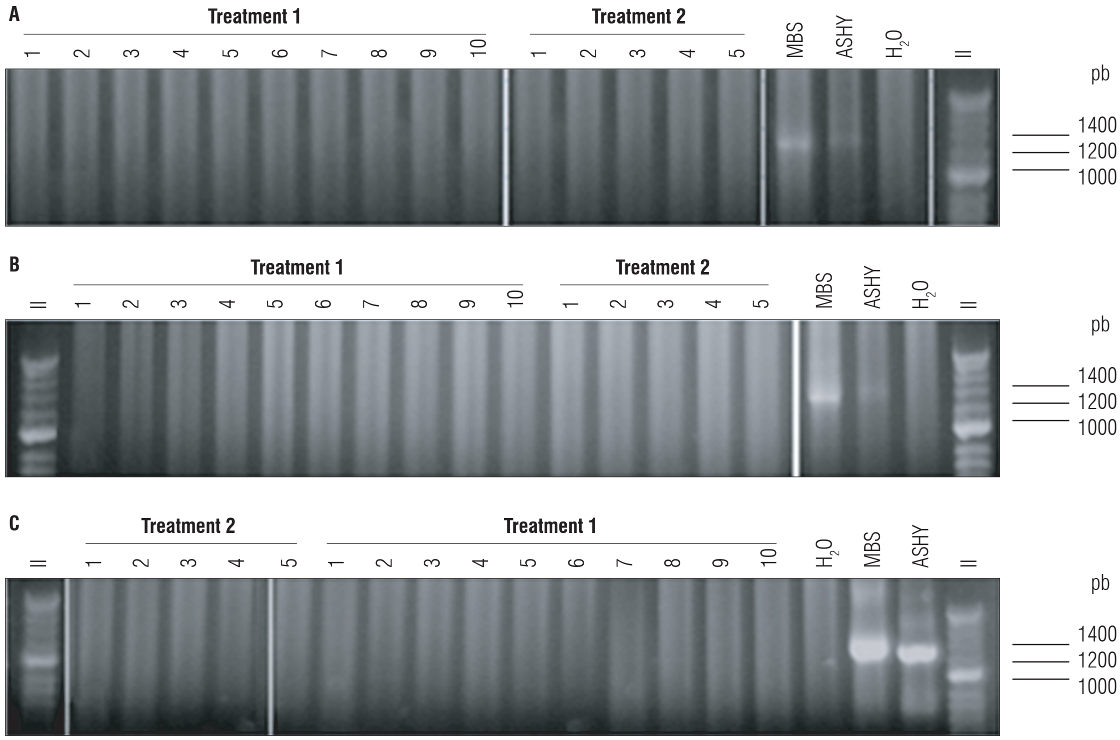

FIGURE 2. Nested and double nested PCR of *Fragaria* x *ananassa* samples exposed to *Amplicephalus funzaensis* (Treatment 1) and control plants (Treatment 2). (A) Double nested PCR with primers P1A/P7A, R16mF2/R16mR1 followed by R16F2n/R16R2. (B) Nested PCR with primers P1A/P7A and R16F2n/R16R2. (C) Nested PCR with primers R16mF2/R16mR1 and R16F2n/R16R2. In all the cases, only the result of the last PCR is shown. MBS: Positive control group 16SrI-B. ASHY: Positive control group 16SrVII-A. Negative control: water. II: Molecular weight marker Hyperladder 50 pb (Bioline®).

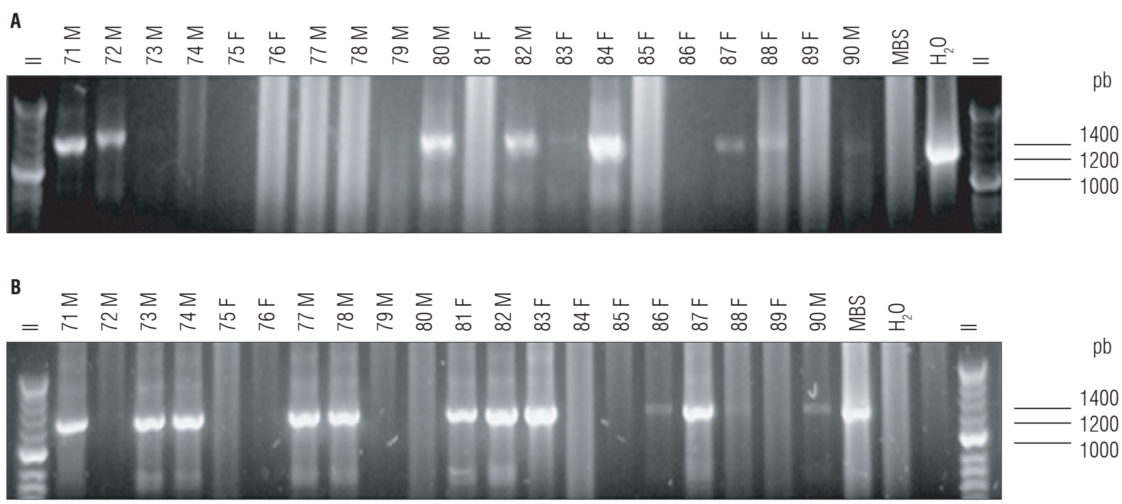

FIGURE 3. Detection of phytoplasmas by nested PCR in the *Amplicephalus funzaensis* used in transmission assays. (A) Nested PCR with primers P1A/P7A followed by R16F2n/R16R2. (B) Nested PCR with R16mF2/R16mR1 followed by R16F2n/R16R2. Only the result of the nested PCR is shown. The number corresponds to the insect code, F: females and M: males. MBS: Positive control group 16SrI-B. Negative control: water. II: Molecular weight marker Hyperladder 50 pb (Bioline®).

by C at positions 183, A by G in 453 and T by C in 517. In haplotype 3, there was a substitution of G by A in position 342 (Sequence ID: COLCI050-18). The sequence similarity of the *COI* sequences obtained for *A. funzaensis* was over 99%. Sequences for *A. funzaensis* were not found in the BOLD or GenBank databases, therefore, Deltocephalinae sequences showing similarities between 86% and 87% were used for comparison.

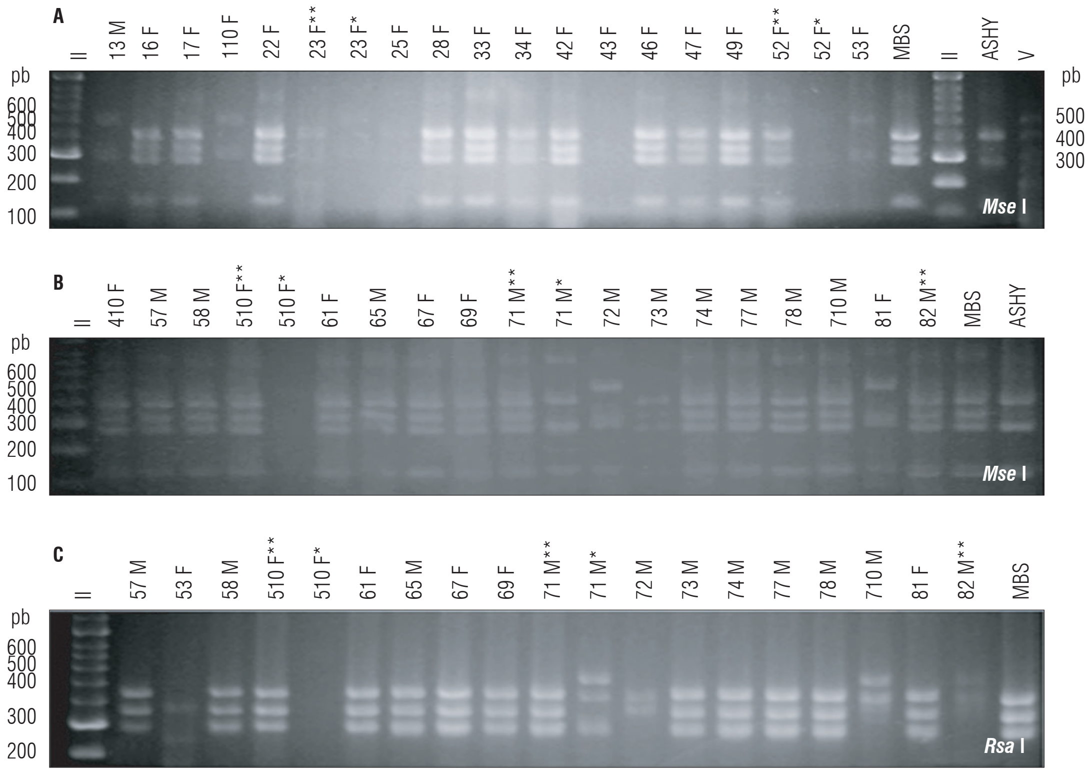

FIGURE 4. Detection of phytoplasmas in *A. funzaensis* used in the transmission assays. (A-B) RFLP with *Mse*I, (C) RFLPs with *Rsa*I, of amplicons obtained by nested PCR with primers P1A/P7A, R16mF2/R16mR1 and R16F2n/R16R2. *Nested PCRs with P1A/P7A followed by R16F2n/R16R2. **Nested PCR with R16mF2/R16mR1 followed by R16F2n/R16R2. The number corresponds to the insect code, F: Females and M: Males. MBS: Positive control group 16SrI-B. ASHY: Positive control group 16SrVII-A. Negative control: Water. II: Molecular weight marker Hyperladder 50 bp (Bioline). V: Molecular weight marker Hyperladder 25 bp (Bioline®).

The nine *A. funzaensis* sequences obtained in this work formed a distinct cluster from other sequences of the *Amplicephalus* genus obtained from BOLD and GenBank. All the sequences clustered by taxa, showing that they had enough differences to separate them as distinct groups (Fig. 5B). The average intraspecific genetic divergence varied between 0.0000 and 0.0245 (mean 0.0048) and the interspecific divergence between 0.1003 and 0.2341 (mean 0.1501). The most divergent species were *P. flavicosta* compared to *A. simplarius* (0.2341), *A. curtisii* compared to *A. littoralis* (0.2233) and *P. flavicosta* compared to *A. littoralis* (0.2233). The less divergent species were *A. littoralis* and *A. simplarius* (0.1003). The *A. funzaensis* sequences were close to *D. balli* (0.1499) and *A. curtisii* (0.1739), and showed the largest divergence with *A. simplarius* (0.2004). The barcode gap analysis showed that for the eight species analyzed, the sequence variability at the intraspecific level was lower than the variability at the interspecific level.

Discussion

A very important aspect of the epidemiology of the diseases associated with phytoplasmas is the identification of their insect vectors. To do this, the insects nearby the symptomatic plants are collected and their ability to transmit phytoplasmas to healthy host plants is tested (Weintraub and Beanland, 2006). The presence of phytoplasmas in an insect does not guarantee its vector ability; therefore, transmission tests are mandatory (Purcell *et al*., 1981; Vega *et al*., 1993). In this work, tests were performed with *A. funzaensis* because it is a known vector of phytoplasma, and it is ubiquitously distributed in the *C. clandestinus* grasslands of the Bogota plateau that frequently surrounds *F.* x *ananassa* crops (Perilla-Henao *et al*., 2016; Silva-Castaño *et al*., 2019).

No evidence was observed of phytoplasma transmission by *A. funzaensis* to *F. x ananassa*. The plants exposed to infected *A. funzaensis* were negative by PCR 2 months post-herbivory and no symptoms were observed for seven months, although the observations performed during the transmission experiment suggested that the insects fed from the test plants and that 36 of the 100 insects used in the assay were infected with phytoplasmas of 16SrI or 16SrVII groups. Several explanations for these results are possible. For instance, that the *F.* x *ananassa* variety Monterrey

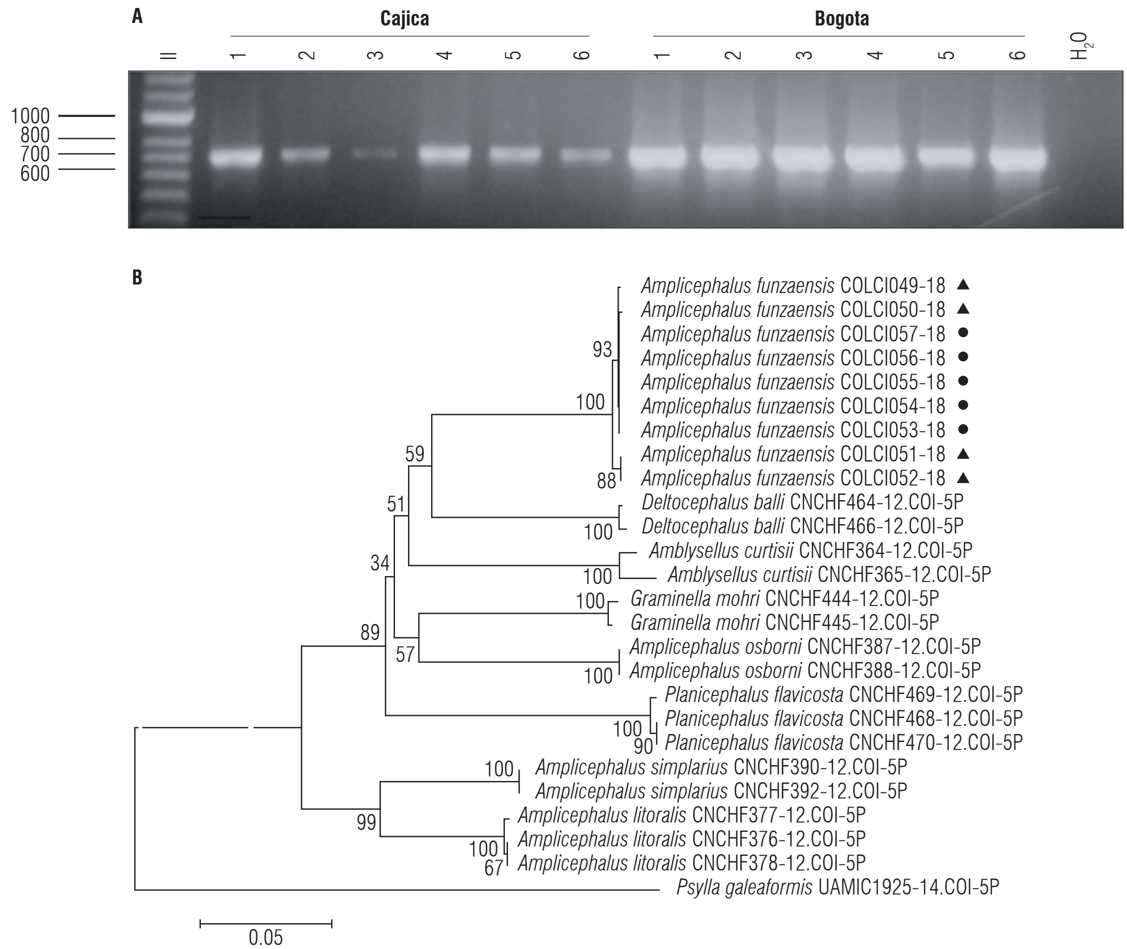

FIGURE 5. (A) Amplification of the *COI* gene of *Amplicephalus funzaensis* by PCR. Negative control: water. II: Molecular weight marker Hyperladder 50 pb (Bioline). (B) Distance tree constructed by neighbor-joining (NJ) based on the Kimura 2 parameter model (K2P) with 1000 bootstrap, using 25 *COI* sequences of the genus Deltocephalini, which includes *Amplicephalus.* The out group was a sequence of *Psylla galeaformis*. Insects collected at UMNG are shown with triangles, and at UN with circles.

plants are not susceptible to phytoplasmas. However, more tests would be necessary to confirm this possibility taking into account that phytoplasma infections in strawberry are widely reported (Padovan *et al*., 1998; Jomantiene *et al*., 1999; Padovan *et al*., 2000; Danet *et al*., 2003; Valiunas *et al*., 2006). Furthermore, phytoplasmas of groups 16SrI and 16SrVII have been detected in strawberries of Camino Real and Ventana in Cundinamarca before (Perilla-Henao and Franco-Lara, 2014). Taking into account that the PCR tests were performed 2 months post-herbivory, it is also possible that the plants at that moment were infected but the phytoplasma title was under the PCR detection level. It would have been desirable to test for the presence of phytoplasmas by PCR in the following period, but the plant tissue collected in the subsequent months was lost due to refrigeration problems. Symptoms were not observed in the next seven months in the plants exposed to insects. Furthermore, it has been known that in different plant hosts, several groups of phytoplasmas can accumulate either in the source tissues, such as in *Euphorbia pulcherrima* and *Cathatantus roseus* with levels of accumulation that can vary one order of magnitude being more concentrated in *C. roseus* than in *E. pulcherrima* (Christensen *et al*., 2004), or in the case of Australian Papaya Dieback Phytoplasma which accumulated in young leaves but not in fruits (Siddique *et al*., 1998), making it difficult to select the sampling tissue in an unknown host.

When this research was carried out, a rearing for *A. funzaensis* was unavailable. In consequence, many variables that influence the transmission efficiency were not controlled. The transmission of phytoplasmas by their insect vectors is of the circulative, propagative type, and occurs in three phases (Weintraub and Beanland, 2006; Perilla-Henao and Casteel, 2016). The acquisition access period (APP) is the feeding time necessary for the insect to acquire phytoplasmas, and the latency period (LP) is the period elapsed between the acquisition and the moment in which the insect is able to transmit phytoplasmas. During LP, the phytoplasmas replicate inside the insect and only when the salivary glands become infected, transmission occurs (Weintraub and Beanland, 2006). The latency period can take as long as 3 weeks (Webb *et al*., 1999). The inoculation access period (IAP) is the time during which the insects are infective, which in this case is all their lives (Alma *et al*., 2019). It is known that insects that test positive for phytoplasmas by nested are not necessary vectors (Purcell *et al*., 1981; Vega *et al*., 1993); to be able to transmit, phytoplasmas must infect the salivary glands of the insect (Weintraub and Beanland, 2006; Alma *et al*., 2019). In this case, the DNA extractions performed on the *A. funzaensis* individuals were performed from the whole body and not only from the head, so it is impossible to know if the salivary glands were infected. The efficiency of transmission is also affected by the insect species. *Euscelidius variegatus* (Hemiptera: Cicadellidae) is considered to be an inefficient insect vector of the Crisantemum Yellows (CY) phytoplasma (group 16SrIB), in comparison to *Macrosteles quadripunctulatus* (Hemiptera: Cicadellidae). It has shown that in *E. variegatus* about 75% of the individuals get their salivary glands infected with CY in experimental conditions in comparison to *M. quadripunctulatus* in which 100% of the individuals get infected salivary glands. The transmission efficiency is attributed to the fact that the intestine and salivary gland membranes of *E. variagatus* act as semi-selective barriers to the pass of phytoplasmas, making them less efficient in transmission (Galetto *et al*., 2009). It was later demonstrated that the CY *Amp* protein (major antigenic membrane) plays a role in the recognition between the phyotplasm and insect membranes in the early stages of the vector infection (Rashidi *et al*., 2015). On the other hand, Palermo *et al*. (2001) showed that in infective individuals of *Macrosteles quadripunctulatus* (Hemiptera: Cicadellidae), a very efficient vector of CY, the infected insects have periods in which they were unable to transmit, but a few days later they recovered this ability (Palermo *et al*., 2001). In this test, the infected *A. funzaensis* were collected from an infected grass patch, but the AAP and LP were not considered. Other variables not considered in this research were the number of insects infected at the beginning of the experiment (Galetto *et al*., 2009), the host plant from which the phytoplasmas were collected (Palermo *et al*., 2001), the plant load (Galetto *et al*., 2014), and the control of temperature which could affect the transmission efficiency (Murral *et al*., 1996; Maggi *et al*., 2014). Recommendations for future experiments include a period of pre-adaptation feeding for the insects before the transmission tests, and longer observation periods and using nymphs instead of adults, as it is generally considered that they are more efficient vectors. Nymphs and adults can be infected with phytoplasmas but their transmission efficiency varies, even if they feed on the same part of the plant if nymphs are exposed earlier in their lives to phytoplasmas (Weintraub, 2007; Alma *et al*., 2019).

The potential transmission ability of insect vectors can vary according to their feeding habits on different plant species, the phytoplasma groups that infect the host plants and the susceptibility of the insect to the colonization of the phytoplasmas, in addition to the intimate molecular relationships that occur between insects and phytoplasmas (Palermo *et al*., 2001; D'Amelio *et al*., 2007; Galetto *et al*., 2009; Bosco and D'Amelio, 2010). There is little information about the interactions between phytoplasmas and different AP and LP which compete for tissues and organs (D'Amelio *et al*., 2007; Bosco and D'Amelio, 2010; Perilla-Henao and Casteel, 2016), as would be the case of the 16SrI and 16SrVII phytoplasmas.

In this experiment, the *A. funzaensis* was collected from a natural population of infected insects growing in grass *C. clandestinus,* a monocot C4 plant, which was placed on strawberry, a dicot C3 plant. It is, therefore, possible that the different diet and nutritional content of the plants, in addition to the stress associated with the experimental conditions found in caged plants inside a greenhouse, may have affected their survival or transmission efficiency.

In total, 120 individuals of *A. funzaensis* were individually tested for the presence of phytoplasmas, including the insects evaluated to test the population used in the transmission experiments, and the insects were allowed to feed on the test plants. Of these individuals, 38% carried phytoplasmas. Of the 100 insects used for the transmission essays, 33 carried phytoplasmas of the 16SrI group and one carried a mix of 16SrI and 16SrVII groups. The existence of *A. funzaensis* populations in which large percentages of insects are infected with phytoplasmas make them a potential risk for dispersion of phytoplasmas in the Bogota plateau.

The groups 16SrI and 16SrVII have been reported as the most common phytoplasma groups in the Bogota plateau infecting trees (Perilla-Henao *et al*., 2012; Perilla-Henao and Franco-Lara, 2013; Franco-Lara and Perilla-Henao, 2014; Franco-Lara *et al*., 2017). Furthermore, 17% of the positive samples by nested PCR produced RFLP patterns that were inconsistent with phytoplasmas, confirming that not specific amplification of DNA can occur with the phytoplasma primers used, as has been reported before (Abeysinghe *et al*., 2016).

This is the first report of a *COI* DNA barcode sequence for *A. funzaensis*. This sequence allows the separation of this species from other members of the same genus such as *A. litoralis, A. osborni* and *A. simplarius*. The two *A. funzaensis* populations studied were separated by a geographical distance of 35 km. The DNA barcode was represented by a major haplotype shared between the two populations and two additional haplotypes that had a few nucleotide substitutions (average genetic distance of 0.0048). This low genetic variability suggests that *A. funzaensis* in the Bogota plateau is a meta-population thriving due to the efficient use of *C. clandestinus* as a host plant, a non-native grass that dominates the landscape. More information is needed to characterize the genetic variability of this species in the zone; in addition, possible differences between its symbionts could enhance its biological efficiency improving its nutritional and reproductive status (Takiya *et al*., 2006). Among the symbionts, phytoplasmas are known to cause differential effects on the fitness of the insect vectors, in some cases improving their general fitness (Beanland *et al*., 2000), or on the contrary negatively affecting their life span, fecundity, and progeny size (Bressan *et al*., 2005).

This work does not provide proof of the ability of *A. funzaensis* to transmit phytoplasmas to *F.* × *ananassa* and more tests are needed. However, the fact that a high number of insects are infected with three groups of phytoplasmas highlights the need for epidemiological surveillance of crops and plants of the urban landscape of the Bogota plateau.

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Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

Desarrollo y validación de escalas de severidad del complejo marchitez del aguacate causado por *Phytophthora cinnamomi*, *Verticillium dahliae* y el desorden hipoxia-anoxia y sus respuestas fisiológicas en plantas de aguacate

Joaquín Guillermo Ramírez-Gil[1]* and Juan Gonzalo Morales-Osorio[2]

ABSTRACT

Avocado wilt complex (AWC) is the most important disease in this crop. AWC may be caused by different causal agents that induce similar symptoms. Accurate scales of disease development (SDD) and physiological changes may be of special importance for the diagnosis and management of AWC. The objective of this work was to design and calibrate a specific SDD for the most common causal agents associated with AWC in Colombia, *Phytophthora cinnamomi* Rands and *Verticillium dahliae* Klebahn, and the hypoxia-anoxia disorder in both seedlings in net house and adult plants under field conditions. Furthermore, physiological responses to infection were determined. The disease was monitored under field and net house conditions. Shoot symptoms were recorded and quantification of inoculum in infected soil and tissue was performed. The visual scale was described based on external symptoms and calibrated with the inocula values by regression analysis. In the net house, net photosynthesis, stomatal conductance, and transpiration were measured during the different stages of disease development. The three causal agents induced a progressive reduction of net photosynthesis, stomatal conductance, and transpiration. The designed scales can be a valuable tool for epidemiological use and support in the diagnosis and management of AWC.

Key words: epidemiological analysis, disease measurement, severity calibration, inoculum, disease management.

RESUMEN

El complejo marchitez del aguacate (CMA) es la enfermedad más importante de este cultivo. El CMA puede ser causado por distintos agentes causales que inducen síntomas similares. Las escalas de desarrollo de la enfermedad (EDE) y los cambios fisiológicos pueden ser de gran importancia para el diagnóstico y manejo del CMA. El objetivo de este trabajo fue diseñar y calibrar EDE específicas para los agentes causales más comunes asociados al CMA en Colombia, *Phytophthora cinnamomi* Rands y *Verticillium dahliae* Klebahn, y el desorden hipoxia-anoxia en plántulas de invernadero y plantas adultas en condiciones de campo. Además, se determinó la respuesta fisiológica durante la infección. La enfermedad se monitoreó en condiciones de campo e invernadero. Los síntomas de la parte aérea de la planta fueron registrados y se cuantificó el inóculo en el suelo y tejido afectado. Se describieron las escalas visuales basadas en síntomas externos las cuales fueron calibradas con la cantidad de inóculo mediante análisis de regresión. En invernadero, se midieron la fotosíntesis neta, la conductancia estomática y la transpiración durante diferentes etapas del desarrollo de la enfermedad. Los tres agentes causales de enfermedad indujeron una reducción progresiva de la fotosíntesis neta, la conductancia estomática y la transpiración. Las escalas diseñadas pueden ser una herramienta valiosa para su uso epidemiológico y de apoyo en el diagnóstico y manejo del AWC.

Palabras clave: análisis epidemiológico, medición de enfermedad, calibración de la severidad, inóculo, manejo de la enfermedad.

Introduction

Avocado wilt complex (AWC) is a disease of high economic importance. It has been called a complex because the expression of the characteristic symptoms in the shoot can be easily confused, since they can be induced by different pathogens and abiotic disorders. Therefore, the causal agents and disorders involved are difficult to identify under traditional diagnostic practices, especially in the first stage of development of the disease. This may lead to erroneous

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[1] Departamento de Agronomía, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Bogota (Colombia).
[2] Departamento de Ciencias Agronómicas, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Medellin (Colombia).
* Corresponding author: jgramireg@unal.edu.co

management practices that worsen the problem and cause large economic losses (Ramírez-Gil *et al*., 2017; Rodríguez *et al*., 2017; Hardham and Blackman, 2018; Ramírez-Gil, 2018; Ramírez-Gil and Morales-Osorio, 2019). AWC can affect various tissues in the avocado, especially the roots, inducing several symptoms. A specific description of symptoms with different causal agents and disorders associated with AWC are presented by Ramírez-Gil (2018), and Ramírez-Gil and Morales-Osorio (2019).

Despite the importance of the disease, not all causal agents or disorders associated with AWC have the same relevance. This may be due to their pathological fitness components or epidemiology within the productive system. The microorganism *Phytophthora cinnamomi* Rands is reported as the most important causal agent of the AWC at a world-wide level (Zentmyer, 1984; Ramírez-Gil *et al*., 2017; Hardham and Blackman, 2018). The fungus *Verticillium* sp. has been less studied, but has been increasingly recognized as important because of problems of root rot associated with the disorder hypoxia-anoxia, especially under tropical conditions in Colombia (Ramírez-Gil *et al*., 2017; Rodríguez *et al*., 2017; Ramírez-Gil, 2018; Ramírez-Gil and Morales-Osorio, 2019). Hypoxia-anoxia is not only considered a disorder that causes the death of avocado plants, but it can also be a stress factor that is highly related to the presence of *P. cinnamomic* (Stolzy *et al*., 1967; Ploetz and Schaffer, 1989; Sanclemente *et al*., 2014; Ramírez-Gil and Morales-Osorio, 2018). This disorder is associated with soil moisture, which reduces the gas content in the porous space of the soil volume (Stolzy *et al*., 1967; Sanclemente *et al*., 2014; Ramírez-Gil and Morales-Osorio, 2019).

To accurately score and evaluate a disease, the availability of an appropriate scale of severity is important. In the case of AWC, the information about scales for measuring disease development is limited mostly to the AWC caused by *P. cinnamomi*, such as the ones reported by Darvas *et al*. (1984) and Coffey (1991) for adult plants. Appropriate scales are not known to evaluate the disease when it is caused by *Verticillium* sp. or the disorder associated with hypoxia-anoxia. Another limitation of these described scales is that they only report the expression of symptoms in the shoot and not the effects caused at the root level. In addition, they are not calibrated for use under different environmental conditions and amount of inoculum. Pathogens and disorders associated with the root and stem, including those of biotic and abiotic origin in the avocado, alter the host plant's metabolism at the morphological and physiological levels. Avocados grown under conditions of hypoxia-anoxia in the soil or with a combination of *P. cinnamomi* infections and hypoxia-anoxia may show symptoms of stunted growth, decreased stomatal conductance, lower net CO_2 assimilation, reduced photosynthetic rates, accelerated senescence, and other physiological responses (Ploetz and Schaffer, 1989; Reeksting *et al*., 2014; Sanclemente *et al*., 2014).

Based on these problems, this research had two objectives: (1) to develop and validate scales of disease development for the most common causal agents and disorder (*P. cinnamomi*, *Vertcillium dahliae*, and hypoxia-anoxia) associated with AWC under tropical conditions in Colombia under field and net house conditions. These include the dynamics through time of the amount of inoculum, the degree of tissue affected in the root and shoot of the plant. (2) To identify the plant physiological responses in seedlings affected by the three causal agents under semi-controlled conditions.

Materials and methods

Location

For the development and calibration of disease scales for the avocado wilt complex (AWC) that is caused by *P. cinnamomi*, *V. dahliae*, as well as for the hypoxia-anoxia disorder, two evaluations were performed. The first was carried out under field conditions. We selected three lots planted with avocado cv. Hass grafted on 6-year-old West Indian rootstock at a planting distance of 7x7 m. The second part of the work was performed in the laboratory of "Fitotecnia Tropical" and the net house at the Universidad Nacional de Colombia, Medellin campus. Details of edaphoclimatic conditions are shown in Supplementary material 1 and 2. The study under field conditions was carried out between 2012 and 2013 and corresponded to four periods of evaluation, performed during the rainy (March-April and September-October) and dry (November-January and June-August) seasons.

Development and validation of specific scales for AWC under field and net house conditions

To develop the scales for AWC under field conditions, diagnostic procedures reported by Ramírez-Gil and Morales-Osorio (2019) were followed, based on plant symptoms and microorganism isolation in semi-selective media. Isolation of *P. cinnamomi* was performed in aseptic vegetable juice (V8-180 ml L^{-1}) with the addition of agar (24 g L^{-1} Difco, USA), ampicillin (200 µg L^{-1}), chloramphenicol (20 µg L^{-1}), and benomyl (100 µg L^{-1}) (V8-AACB). Isolation of *Verticillium* sp. was performed in acidified potato dextrose agar plus lactic acid (PDA-A) (Difco, USA) and vegetable juice agar (V8-AE) (Difco, USA) supplemented with antibiotic (streptomycin 100 μg L^{-1}). Morphological characterization at the genus and species level was performed using the keys of Barnett and Hunter (1972) and Seifert *et al.* (2011) for fungi, and Erwin and Ribeiro (1996) for *Phytophthora* spp. For *P. cinnamomi* and *Verticillium* sp., the identifications were confirmed by sequence analysis of the genomic ITS regions, using the primer pairs ITS5-ITS4 and ITS1-ITS4 and the procedures established by White *et al.* (1990). We selected *V. dahliae* because it was the most commonly identified in the evaluated plots. For hypoxia-anoxia, shoot symptoms, root dissection, and verification of microorganism´s presence or absence were determined (Ramírez-Gil and Morales-Osorio, 2019).

Plants infected with *P. cinnamomi* and *V. dahliae* under natural conditions were selected. The selection of plants affected with hypoxia-anoxia were based on two concepts: (1) plants planted in soil with low slope (less 10%) (Ramírez-Gil, 2018) and (2) plants with high accumulation of water in the soil profile (more than 70% of water saturation) using volumetric humidity values quantified in a V2 moisture sensor (analogy, DF Robot™, reference SKU:SEN0114) with serial communication to an Arduino UNO programming card, and collecting data with a serial communication terminal (Ramírez-Gil *et al.*, 2018). For each pathogen and disorder, a randomized complete block design was used with three blocks (each plot) and each block had three replicates, where the experimental unit consisted of five plants. All the infected plants were left under field conditions with no disease management procedure until they died of these pathologies. Care was taken to avoid the presence of another type of microorganism, based on the isolation of the selected plants and the reduction of the contact with the staff of the plantations.

Infected but asymptomatic plants were selected within the cultivated plots. For each measure five plants were selected with the same degrees of disease or disorder development so as to evaluate the root dynamics. These plants were evaluated once. A detailed description of the symptomatology developed through time in the shoots of the selected plants was registered, such as changes in leaf color, presence of withered leaves, defoliation, and affected tissues. This part was complemented with an evaluation of the root system. The number of affected roots was determined by destructive sampling introducing a stainless-steel cylinder (100 cm^3 volume) at 10 points distributed around a diameter of 3 m equidistant from the base of the stem of the plant at a depth of 60 cm. The material removed was washed with tap water. The avocado roots were selected and a determination of their phytosanitary status as non-viable diseased roots (necrotic) and viable healthy roots (white) was carried out (Ramírez-Gil and Morales-Osorio, 2019). The remaining root samples were kept for inoculum quantification (*P. cinnamomi* and *V. dahliae*) in the laboratory. For the pathology caused by *V. dahliae*, the affected shoot (stem and branches) was also considered and evaluated looking for necrosis or other symptoms.

A definition of the levels of the scale for disease development was carried out by photographic registration and symptom description. A scale from 0 to 5 was assigned, in which the first value (0) indicated an absence of the disease and (5) indicating a dead or irreversibly dying plant. Other values were assigned according to the progress of the symptoms in roots and shoots (Tabs. 1-3). From all of these data the corresponding specific shoot scales were determined (Figs. 1-3).

For the experiments in the net house, a completely randomized experimental design was used with five replicates per treatment. The experimental unit consisted of five seedlings and the evaluation had two repetitions through time. For the net house evaluation, strains of *P. cinnamomi* (Code: PCSOC1) and *Verticillium* sp. (*V. dahliae* Code: VAC3) were obtained from the collection of avocado pathogenic strains kept at the "Fitotecnia Tropical" Laboratory at the Universidad Nacional de Colombia, Medellin campus, which had been morphologically and molecularly characterized. For the reproduction of hypoxia-anoxia conditions, water was applied until 70-90% of water saturation (Ramírez-Gil and Morales-Osorio, 2019) in an Andisol soil (Supplementary material 1). Reproduction of the AWC was previously verified on specimens of avocado cv. Hass for the two causal agents and hypoxia-anoxia disorder.

Avocado seeds of undamaged cv. Hass of similar size were collected. They were incubated for germination in autoclaved quartz (0.1 MPa and 121°C for two cycles of 1 h each) and maintained at 50-70% relative humidity. When seedlings had five fully expanded leaves and the secondary root system showed healthy roots (not necrotic) by visual inspection, cotyledons were removed to induce more root formation (Ramírez-Gil and Morales-Osorio, 2019). Seedlings were then transplanted to plastic pots with a 2 kg capacity on a wet basis. The potting soil was an Andisol from the municipality of El Peñol, Antioquia, Colombia, the description of which is presented in Supplementary material 1. The soil was autoclaved (0.1 MPa and 121°C, for two cycles of 1 h each). Plants were kept under net house conditions, at 40-50% of moisture potential before carrying out the inoculation and reproduction of the hypoxia-anoxia conditions.

Inoculation was performed by adding an aqueous suspension of 200 ml (sterile distilled water) containing inoculum to a final concentration of $1x10^5$ ml^{-1} infective propagules (conidia) of *V. dahliae*, and $1x10^3$ ml^{-1} infective propagules (sporangia) of *P. cinnamomi*. The inocula were added to the root system of each seedling in four equidistant points in each pot (Ramírez-Gil and Morales-Osorio, 2019). Inoculum was grown in potato-dextrose agar medium (PDA-Difco, USA) at 22°C for 10 d, and added to sterile distilled water and dispersed by manual agitation. To achieve a condition of hypoxia-anoxia the soil used for seedling growth was kept at 70-90% of water saturation based on the process described before.

For the definition of the levels of disease development for these two pathologies and hypoxia disorder in net house seedlings, the procedure was similar to the one described above for adult plants in the field, in which symptomatology was monitored in the shoot and at the root level with the modification that the whole seedling was sampled for root analysis. The defined scale consisted of values from 0 to 3, where the value 0 corresponded to healthy plants and 3 to dead or irreversibly damaged to dead plants. With these data, the description of the scale (Tabs. 1-3) and its visual evaluation were defined (Figs. 1-3).

The inoculum and progress of affected tissues present in each of the stages of the disease development defined in Tables 1, 2, and 3 were quantified. For *P. cinnamomi,* 5 g of roots were randomly collected from the sample obtained with the cylinder and these were disinfected (Ramírez-Gil and Morales-Osorio, 2019). The roots were macerated in 200 ml of sterile distilled water. Serial dilutions ($1 \times 10^{-1} \times 10^{-5}$ of sterile distilled water w:v) were prepared. One ml of each dilution was spread in Petri dishes with V8-AACB. For *V. dahliae,* 5 g of a mixture of roots and stem tissues was obtained and processed as described for *P. cinnamomi*. This was then seeded in Petri dishes with PDA-E. For these two microorganisms, the amount of inoculum expressed as colony forming units (CFU) was determined by direct counting from the dilutions performed. For the conditions of hypoxia-anoxia, the level of the disease was quantified as the percentage of dead roots with purple coloration in the inner part (Ramírez-Gil and Morales-Osorio, 2019). The plants with roots affected with a microorganism (i.e. *P. cinnamomi*) were discarded for this analysis.

The calibration for the disease development scales for these two pathogens and the disorder in adult plants under field conditions and seedlings under net house conditions was performed by regression analysis. The analysis was based on the relationship between the scale determined in the shoot and root and the amount of inoculum. The presence of the microorganism in affected tissues for which the scale was the dependent variable, and the amount of inoculum was the independent variable. The principles of normality of residuals (Kolmogorov-Smirnov test), homoscedasticity of residual variances (Levene test) and the uncorrelation of the residual (Durbin-Watson test) were evaluated. The model that best fits the data was chosen using the statistical parameters of correlation coefficient, coefficient of determination, *P* value ($P<0.05$) and the *Akaike information criterion* (AIC). In order to comply with the principles, data associated with the amount of inocula were logarithmically (ln) transformed. As a consequence, in healthy plants in which the inoculum was zero, this value was assumed to be 1 since the function (ln) does not present a real value for 0. In order not to alter the results, 1 was added to each scale. The analysis was run on the free software R (R Development Core Team, 2019).

As a complement to the previous analysis in each of the evaluated seasons (dry-rainy) the average time in days to move from one stage of the disease scale to another, the associated inocula, and the colonization on tissues were determined, according to the scale of disease development proposed. This evaluation was also performed for seedlings in the net house, but without the effect of the dry-rainy season. In the case of the disorder associated with the conditions of hypoxia-anoxia, it was only evaluated during the rainy season since its presence occurs only under conditions of high soil moisture (Ramírez-Gil and Morales-Osorio, 2019).

Simulation of root growth under field and net house conditions and AWC infections

A theoretical model was developed to simulate the effect of root infection by *P. cinnamomi* and *V. dahliae* and the hypoxia-anoxia disorder on root growth under field and net house conditions. This model was developed based on the evaluation of the dynamics of root infections (horizontal and vertical growth) of avocado plants and seedlings according to the measures previously reported. The algorithm implemented in Rootbox (Leitner *et al.*, 2010) was used and run under the MatLab interface (version 15.0).

Physiological response of avocado seedlings to the causal agents and disorder associated with AWC

The physiological responses of Hass avocado seedlings to the infection with *P. cinnamomi*, *V. dahliae* and the hypoxia-anoxia disorder were determined for each of the stages of disease development, according to the evaluation scale proposed for the seedlings (Tabs. 1-3). Net photosynthesis (A), stomatal conductance (gs), and transpiration (E) were directly quantified using a TPS-2 Portable Infrared Analyzer (PhotosynThesis System®, Washington, USA). From the data obtained, water efficiency (WUE) was estimated (WUE=A/E). The quantification of the physiological variables in each of the stages of the scale was performed at the same hour in the morning (9-10 am) and on the same leaf (third fully developed leaf from the apical bud).

A completely randomized design was used with each experimental unit consisting of five plants with three replicates. Quantification was performed for five different points on the same leaf and on the five different plants of each experimental unit. Tests were performed during a 30-day period. Data were quantified each day. Data homoscedasticity and normality were verified using the criteria of Levene and Kolmogorov-Smirnov. Subsequently, data were subjected to an analysis of variance and the means were compared by the Tukey test with a significance of 95% ($P \leq 0.05$). The analysis was run on the free software R (R Development Core Team, 2019).

Results and discussion

Development and calibration of scales for principal pathogens and disorder associated with AWC under field and net house conditions

For the disease development scale of *P. cinnamomi* in the field, the following five stages were determined: Stages 1 and 2 were characterized by leaf yellowing and the absence of active growing buds, leading to stunted growth for stages 3 and 4. The previous symptomatology became more pronounced with concurrent generalized wilt, besides defoliation. For stage 5, plant death or irreversibly damaged leading to death occurred due to defoliation and the presence of dieback. Each of the stages of disease development beginning from stage 1 was characterized by an increase in the number of necrotic secondary and tertiary rootlets as a consequence of *P. cinnamomi* colonization (Tab. 1, Fig. 1).

For *V. dahliae,* the scale of disease development in the field during stages 1 and 2 was characterized by slight yellowing and wilting on one side of the plant. For stages 3 and 4, plant growth stopped, wilt intensified, the leaves became brown and adhered to the plant, and there was the presence of stem necrosis. Root necrosis occurred increasingly in stages 1, 2 and 3 and for the remaining stages it remained constant. In addition, root necrosis was observed in the main, secondary and tertiary roots (Tab. 2, Fig. 2).

In the case of the abiotic disorder originated by hypoxia-anoxia, the first stages of the disease (1 and 2) showed similar symptoms to those caused by *P. cinnamomi* and *V. dahliae* that were characterized by foliar yellowing. For the other stages (3 and 4), there was generalized wilt and plant defoliation. For the root system, a gradual increase of the necrosis as the scale increased was observed in a similar way as for *P. cinnamomi*. However, the difference for hypoxia-anoxia was that this necrosis was generalized in all roots (primary, secondary and tertiary) and the internal color was purple (Tab. 3, Fig. 3).

TABLE 1. Description of the disease developmental scale for *Phytophthora cinnamomi* in avocado cv. Hass grafted on West Indian rootstock and evaluated under field (a) and net house (b) conditions.

Scale value	Appearance of the shoot and disease symptoms	Appearance of roots
0[a]	Healthy plants with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[a]	Visible disease symptoms. Mild leaf yellowing and lack of active growing buds that leads to stunted growth	Diseased rootlets between 10 and 15%
2[a]	Pronounced leaf yellowing and stunted growth	Diseased rootlets between 15.1 and 25%
3[a]	Generalized leaf yellowing, wilting and mild defoliation <35%	Diseased rootlets between 25.1 and 50%
4[a]	Generalized leaf yellowing, wilting and defoliation between 35.1 and 90%	Diseased rootlets between 50.1 and 90%
5[a]	Irreversible damage to death, dieback and severe defoliation >90.1%	Diseased rootlets >90%
0[b]	Healthy seedlings with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[b]	Visible disease symptoms. Generalized leaf yellowing	Diseased rootlets between 10 and 15%
2[b]	Generalized leaf yellowing, stunted growth and mild wilting	Diseased rootlets between 15.1 and 70%
3[b]	Generalized leaf yellowing and wilting, defoliation	Diseased rootlets >70.1%

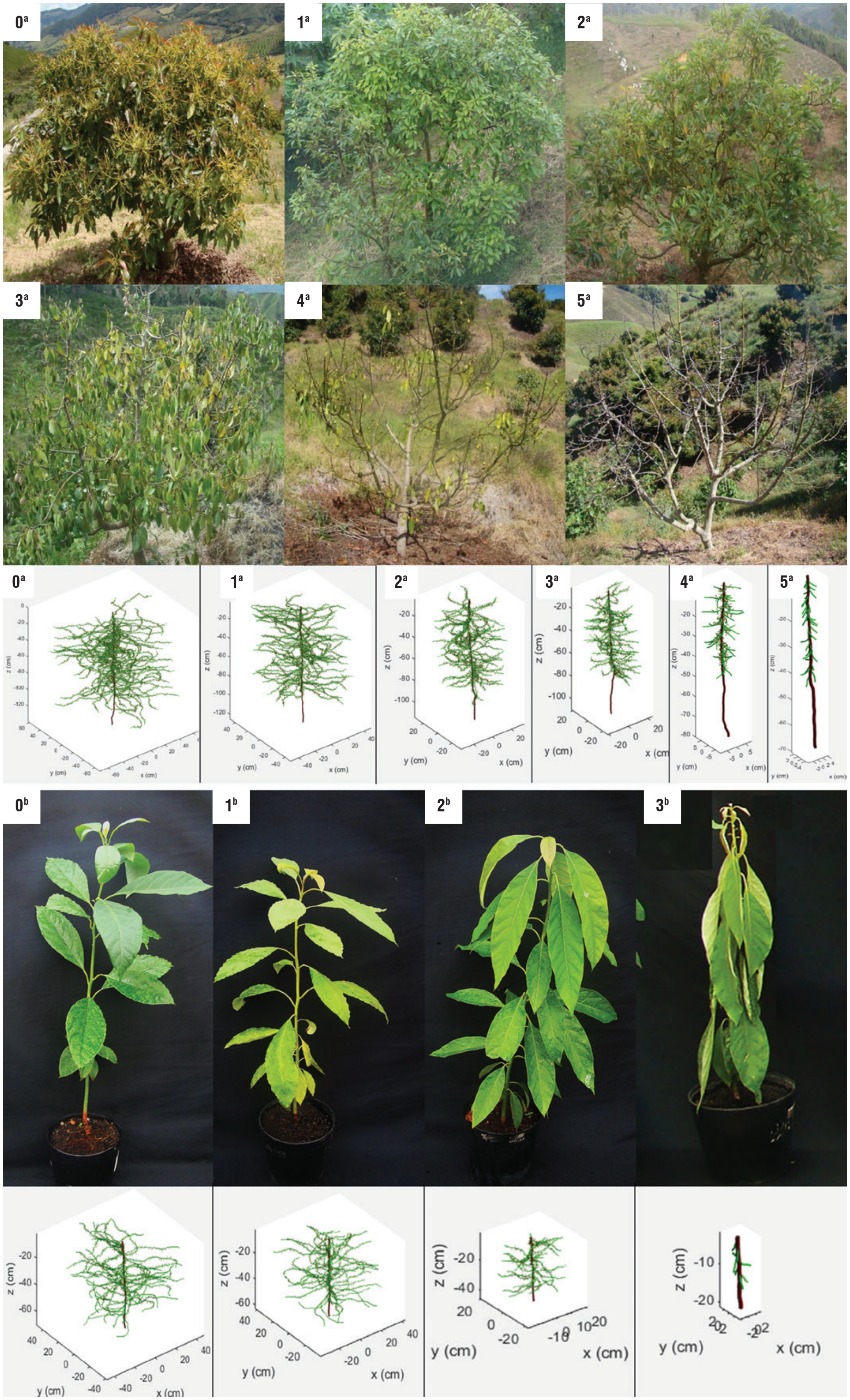

FIGURE 1. Visual and root appearance of disease developmental scale for *Phytophthora cinnamomi* in avocado cv. Hass grafted on West Indian rootstock evaluated under (a) field and (b) net house conditions. Specific description is presented in Table 1.

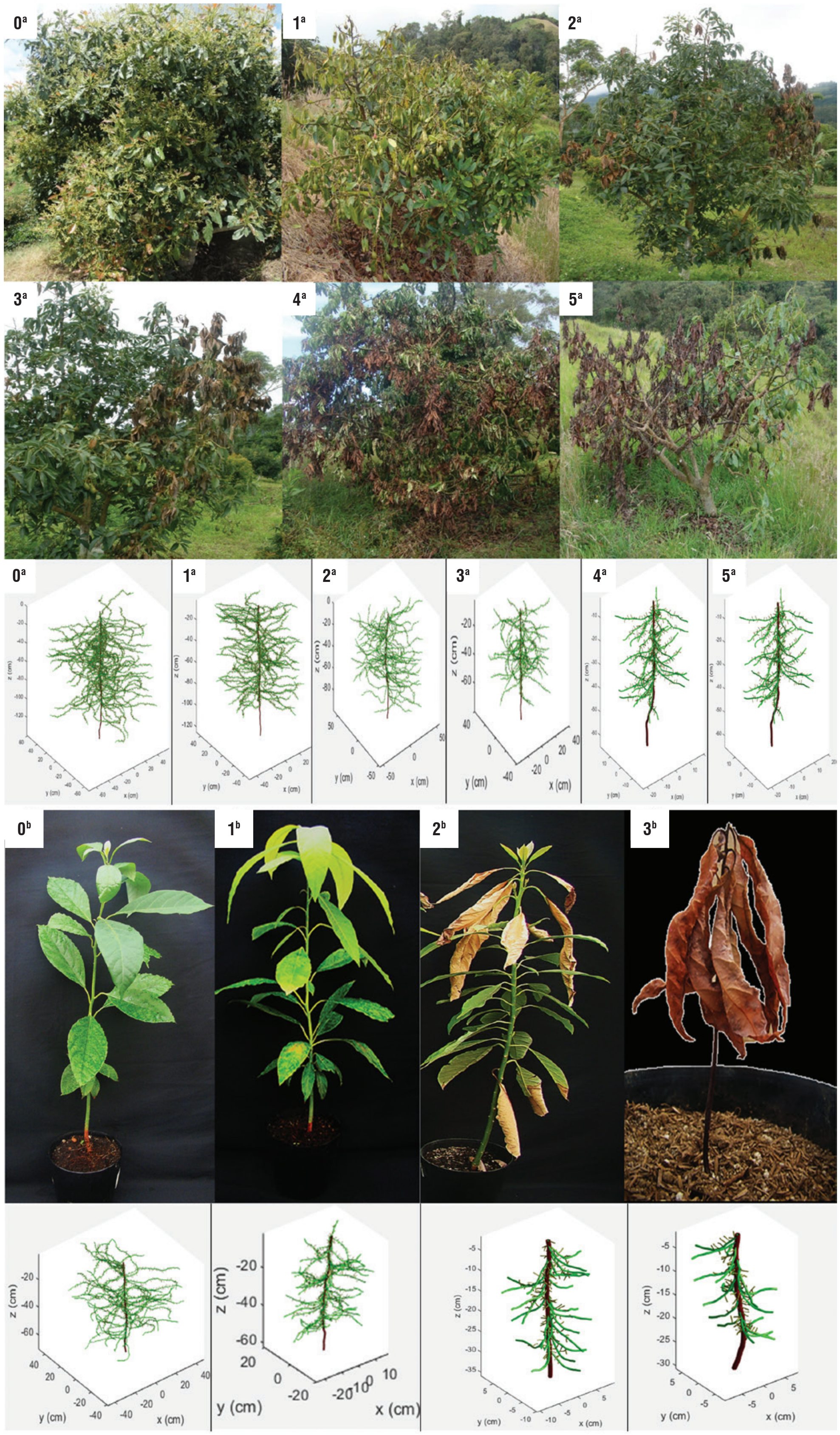

FIGURE 2. Visual and root appearance of disease developmental scale for *Verticillium dahliae* in avocado cv. Hass grafted on West Indian rootstock evaluated under (a) field and (b) net house conditions. Specific descriptions are found in Table 2.

TABLE 2. Description of disease developmental scale for *Verticillium dahliae* in avocado cv. Hass grafted on West Indian rootstock evaluated under field (a) and net house (b) conditions.

Scale value	Appearance of the shoot and disease symptoms	Appearance of roots
0[a]	Healthy plants with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[a]	Visible disease symptoms. Mild leaf yellowing	Necrotic roots between 10.1 and 20%
2[a]	Pronounced unilateral leaf yellowing, mild wilting and stunted growth	Necrotic roots between 20.1 and 25%
3[a]	Pronounced unilateral wilting with leaf death <35%. Leaves remain adhered to the stem	Necrotic roots between 25.1 and 30%
4[a]	Unilateral wilting with leaf death between 35.1 and 70% in the affected portion of the tree Stem necrosis in the affected portion of the tree	Necrotic roots 30.1 and 40%
5[a]	Irreversible damage to death. Stem and leaf death >70.1%	Necrotic roots<40%
0[b]	Healthy seedlings with abundant foliage of dark green color and foliar buds in active growth No disease symptoms	>90% of viable rootlets
1[b]	Visible disease symptoms. Generalized leaf yellowing	Necrotic roots between 10.1 and 20%
2[b]	Leaf death <50%	Necrotic roots 20.1 and 30%
3[b]	Collapse of the seedling, necrotic brown leaves that remain adhered to the stem	Necrotic roots <40%

Under net house conditions, the disease developmental scales showed a similar trend of symptoms in the shoot and in the number of roots affected when compared to what was found for field conditions (Tabs. 1-3, Figs. 1-3). Stages 4 and 5 were not included for seedlings grown under net house conditions. This was because in stage 3, the seedlings infected with *P. cinnamomi* and affected by hypoxia-anoxia already showed a large number of affected roots, and for *V. dahliae* they showed stem necrosis. In stage 3 of the development of the disease, plant death induced by all three causal agents analyzed was already irreversible.

Symptom expression in the shoot of the plants was similar to the three pathogens and the disorder evaluated during the first stage of disease development, mainly characterized by foliar yellowing and wilting. However, clear differentiation of symptoms induced by each causal agent and disorder was observed after stage 2 of the disease's development. *Phytophthora cinnamomi* induced generalized wilting, partial or massive defoliation, dieback and necrosis of secondary feeder roots. In contrast, wilt induced by *V. dahliae* caused generalized ringing and leaf necrosis that remained adhered to the stems. In addition, hypoxia-anoxia caused foliar wilting and defoliation (Figs. 1-3).

AWC symptomatology has been widely reported and explained because the root system is affected, leading to a reduction in nutrient and water uptake from the soil. This induces decompensation resulting from water loss that gives rise to the expression of wilting symptoms in the shoot such as yellowing, scarce foliage, absence of actively growing buds causing stunted growth and wilt (Bingham and Zentmyer, 1954; Zentmyer, 1984; Whiley *et al*., 1987). Similarly, it is reported that conditions of moisture excess in the soil lead to root decay, and as a consequence such conditions decrease the assimilation and translocation of nitrogen (Stolzy *et al*., 1967; Ezin *et al*., 2010). Multiple morphological and physiological changes that make growth stop also accelerate senescence and modulate other physiological processes (Wager, 1942; Stolzy *et al*., 1967;

TABLE 3. Description of disease developmental scale for hypoxia-anoxia in avocado cv. Hass grafted on West Indian rootstock evaluated under field conditions.

Scale value	Appearance of the shoot and disease symptoms	Appearance of roots
0[a]	Healthy plants with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable roots without affected stems
1[a]	Visible disease symptoms. Mild leaf yellowing and lack of active growing buds	Necrotic roots between 10.1 and 20%
2[a]	Generalized and pronounced yellowing	Necrotic roots between 20.1 and 40%
3[a]	Generalized wilting with fast defoliation <30%	Necrotic roots between 40.1 and 70%
4[a]	Severe wilting and defoliation between 30.1 and 70%	Necrotic roots>70.1%
5[a]	Irreversible damage to death, defoliation >70.1%	Necrotic roots>90.1%
0[b]	Healthy seedlings with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[b]	Visible disease symptoms. Mild leaf yellowing and wilting. Lack of active growing buds	Necrotic roots between 10.1 and 20%
2[b]	Generalized yellowing and pronounced wilting	Necrotic roots between 20.1 and 70%
3[b]	Severe defoliation, dead seedling or irreversible damage resulting in death.	Necrotic roots >70%.

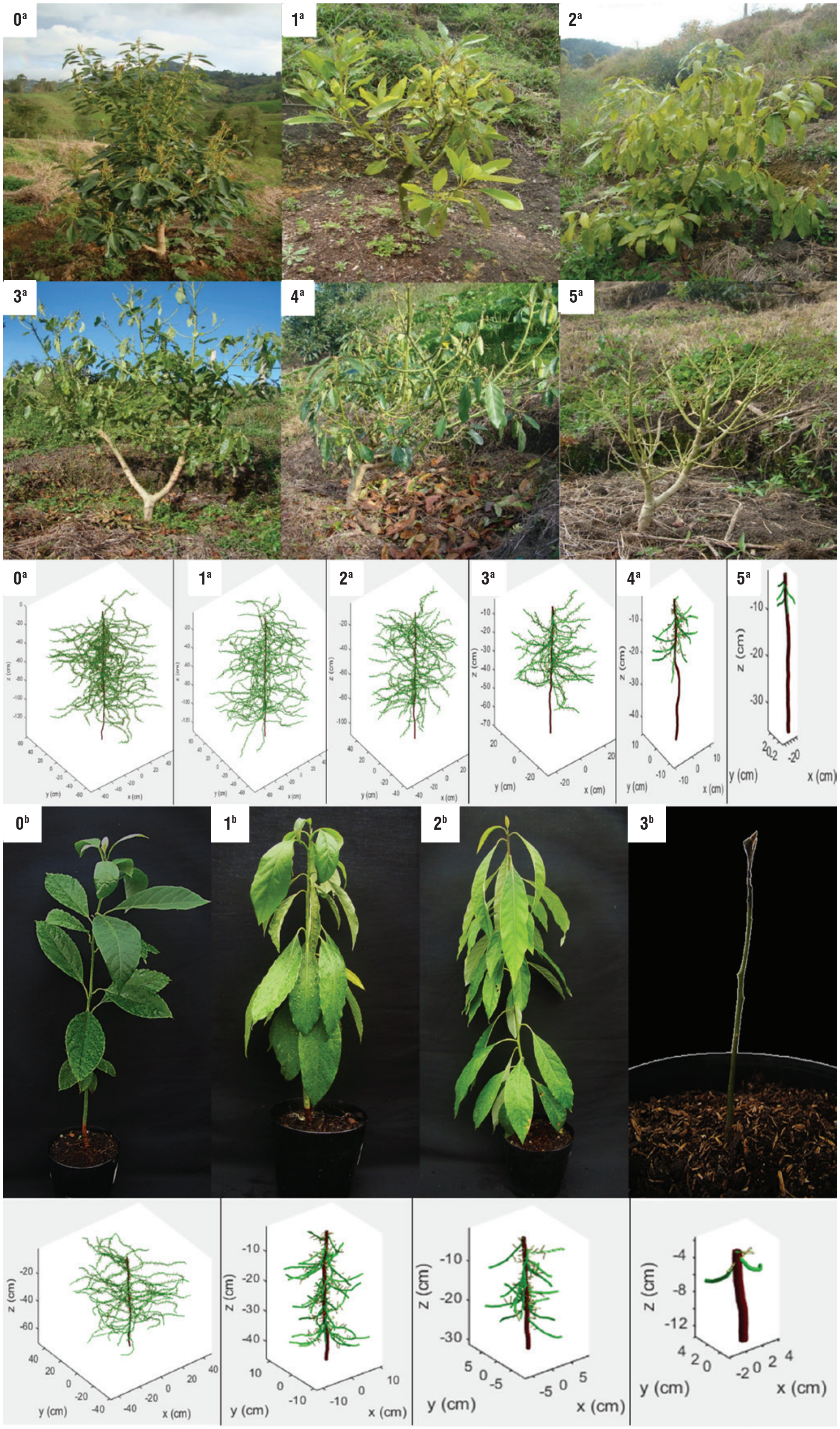

FIGURE 3. Visual and root appearance of disease development scale for hypoxia-anoxia in avocado cv. Hass grafted on West Indian rootstock evaluated under (a) field and (b) net house conditions. Specific description is presented in Table 3.

Sanclemente *et al*., 2014). For the more advanced stages of these evaluated diseases, characteristic expressions of each pathology were observed. *Phytophthora cinnamomi* colonizes and destroys large portions of the root system, almost exclusively limited to secondary and tertiary roots, confirming previous reports (Zentmyer, 1984; Ramírez-Gil and Morales-Osorio, 2019). On the other hand, *V. dahliae* affects the roots and later the mycelium invades the vascular tissues, especially the xylem. From there, it begins its upward movement producing abundant reproductive structures when invading the plant, causing typical unilateral wilting symptoms in the branches (Zentmyer, 1949, 1984). For hypoxia-anoxia, the most marked characteristics are rapid defoliation, reduction of growth, and necrosis of the whole root system (Stolzy *et al*., 1967; Sanclemente *et al*., 2014).

For the *P. cinnamomi* evaluation under field conditions, the regression model that established the relationship between the amount of inoculum and the value of the scale showed a correlation coefficient of 0.95, a coefficient of determination of 91.3%, and a highly significant (P<0.000) and lower value in the AIC. For the other two diseases, the statistical parameters of correlation and determination coefficients were 0.94 and 89.0% for *V. dahliae* and 0.97 and 93.8% for hypoxia-anoxia. They were highly significant (P<0.000) and with lower values in the AIC (Fig. 4). For the relationships between inoculum quantity of *P. cinnamomi*, *V. dahliae* and necrotic roots associated with hypoxia-anoxia, the values of the disease scales developed for seedlings under net house conditions were highly significant (P<0.000), with correlation and determination coefficients of 0.92-85.3, 0.97-95 and 0.84-71.3% (Fig. 4).

The inoculum from soil and plant tissues and necrotic roots and their corresponding values of disease development scale showed a significant (P<0.05) and positive relationships for the three causal agents tested, confirming that these variables are correlated. This result may be useful for estimating theoretical inoculum and progress in values of affected tissues for both field and net house conditions. Precision using the disease scales designed in the present work is likely to be high. This calibration assumes that the descriptive and arbitrary definition of these scales was adequate since good representation of the dynamics of these two diseases and disorder were obtained. This provides a tool for the study of these pathologies, and allows the use of a different scale for each of these causal agents.

Most of the scales developed to date have been designed for *P. cinnamomi* (Darvas *et al*., 1984; Coffey, 1991), but in this research, a contribution has been made by the development of specific scales for the avocado wilt disease complex caused by *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia disorder, both in the roots and shoots of the plants under net house and field conditions during the dry and the rainy seasons. Additionally, the regression analysis showed a high relationship between the scale and the inoculum and necrotic root values.

Evolution through time and the stages of disease development caused by pathogens and disorder associated with AWC

Dynamics through time of the scales of disease development evaluated under field conditions for *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia showed that both pathogens and the disorder caused plant death in a shorter period of time during the rainy season. Hypoxia-anoxia was the fastest developing condition with a mean time of 71.3 d, followed by *P. cinnamomi* (150.5 d), and *V. dahliae* (185.4 d). During the dry season *P. cinnamomi* caused plant death at 205.5 d and *V. dahliae*, took a longer period of time (stage 5) of 215.4 d (Supplementary material 3A and B). As expected, during the dry season no plant deaths by hypoxia-anoxia were recorded. Similarly, for the evaluation under net house conditions, the disease that more rapidly caused seedling death (stage 3) was hypoxia-anoxia (78.4 d) followed by *P. cinnamomi* (115.4 d), and *V. dahliae* (170.5 d) (Supplementary material 3A and B).

The inoculum values and necrotic roots showed a rapid increase (P<0.05) during the first stages (1-3) of the scale of disease development. After stage 3 (stages 4 and 5, plant death) of the disease development scale, the rate of increase of inoculum was slower compared to stages 1, 2 and 3. Variation in the inoculum values showed a similar pattern under field and net house conditions (Supplementary material 3 C-F).

The results associated with the dynamics of the disease in the field confirm that the rainy season accelerates the physiopathological processes associated with the diseases' development for the two causal agents and the particular disorder that we investigated. Soil moisture is considered one of the most important factors in the dynamics of the avocado wilt complex. This depends on the soil's physical and chemical properties, precipitation patterns, water interactions in the sub-surface horizons of the soil and the congruence of drainage networks (Ramírez-Gil, 2018; Ramírez-Gil and Morales-Osorio, 2018). Soil moisture has been widely reported as the most important factor in the

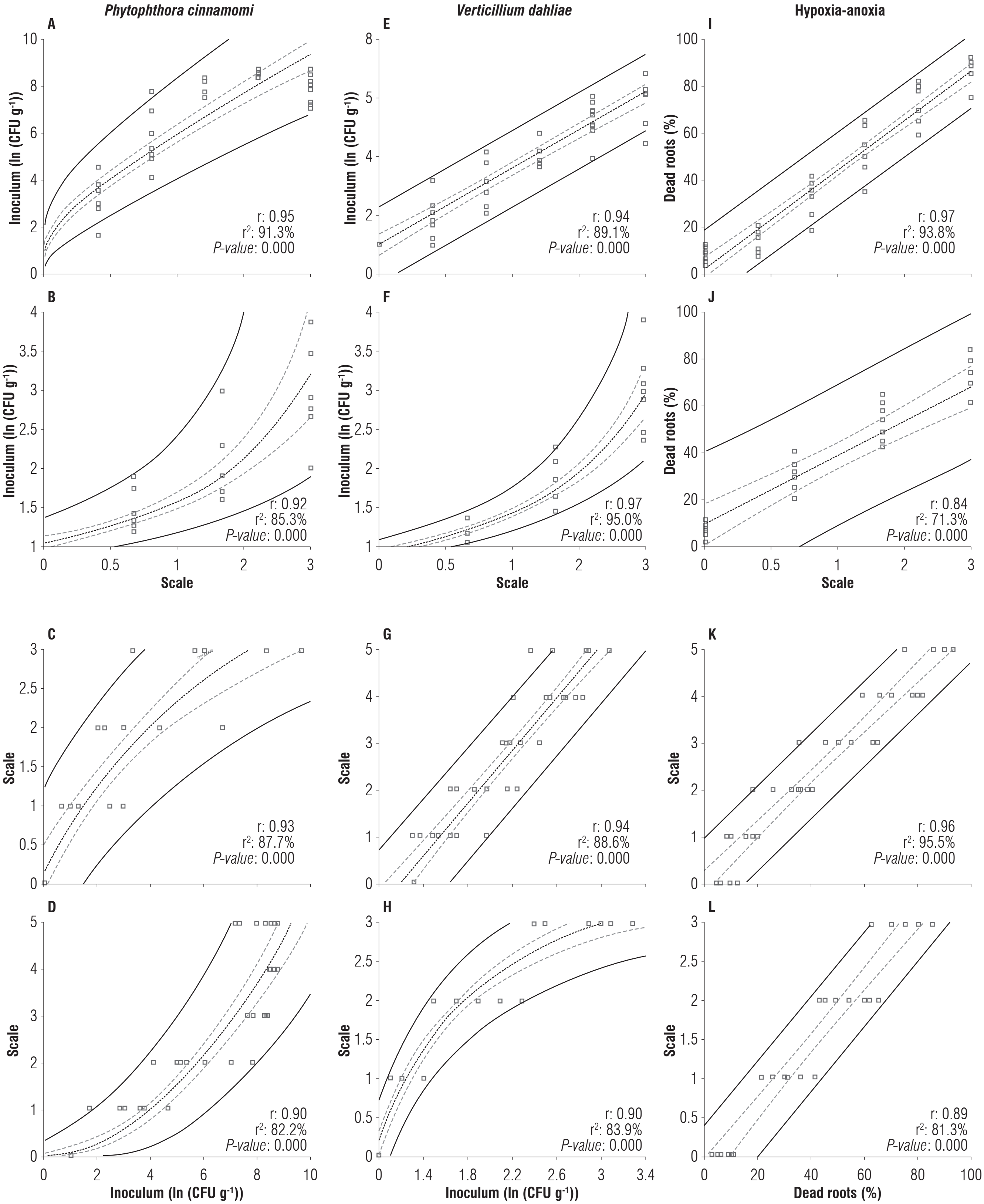

FIGURE 4. Models obtained for relationships between the amount of inoculum and the scale of disease development in *Phytophthora cinnamomi*, *Verticillium dahliae*, and conditions of hypoxia and anoxia under field and net house conditions. A, C, E, G, K, models developed under field conditions data and I, B, D, F, H, J and L models developed under net house conditions. Models were selected based on the correlation and determination coefficient, significance ($P<0.001$). A, B, E, F, I, and J inoculum in function of scale and C, D, G, H, K, and L, scale in function of inoculum.

development of the life cycle of *P. cinnamomi*. Free water is of crucial importance in the dispersion of the inoculum source (Sterne *et al*., 1977; Gisi *et al*., 1980; Coffey, 1991; Ramírez-Gil and Morales-Osorio, 2018). During the dry season we also observed the symptomatology induced by *P. cinnamomi* and *V. dahliae*. Although the amount of inoculum produced was possibly lower under these conditions (Ramírez-Gil and Morales-Osorio, 2018), the soil water deficit and the affected root system made it difficult for the plant to uptake nutrients and water, and this leads to a rapid and marked expression of symptoms.

Physiological responses to the principal pathogens and disorder associated with AWC

The physiological responses of Hass avocado seedlings markedly changed according to the stage of disease development caused by *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia. Net photosynthesis showed a significant decrease ($P<0.05$) for *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia in stage 3 (irreversibly damaged to death) with values of 7.5, 9.1 and 87.2%, respectively, compared to stage 0 (healthy plants) (Fig. 5).

Stomatal conductance and transpiration showed similar behavior to net photosynthesis ($P<0.05$) (Fig. 5). Hypoxia-anoxia induces a maximum reduction of stomatal conductance and transpiration values at stage 2, so no further change was observed in stage 3. Similarly, for *P. cinnamomi* and *V. dahliae,* the values observed for the same variable decreased as the disease scale values increased during all stages of disease development. The indirect variable water use efficiency (Fig. 5) did not show significant differences ($P>0.05$) during the stages 0 and 1 of disease development for all three causal agents of avocado wilt tested. For stages 2 and 3, this parameter significantly increased indicating a more efficient use of water in these stages in the plants affected by *P. cinnamomi*, *V. dahliae,* and hypoxia-anoxia, compared to the plants in stage 1 and healthy plants (stage 0) ($P<0.05$).

The decrease in the values of physiological variables, such as net photosynthesis, stomatal conductance and transpiration, is the plant's response to a source of stress caused by these pathologies, and this becomes more noticeable with the progression of the disease. This condition occurs as a

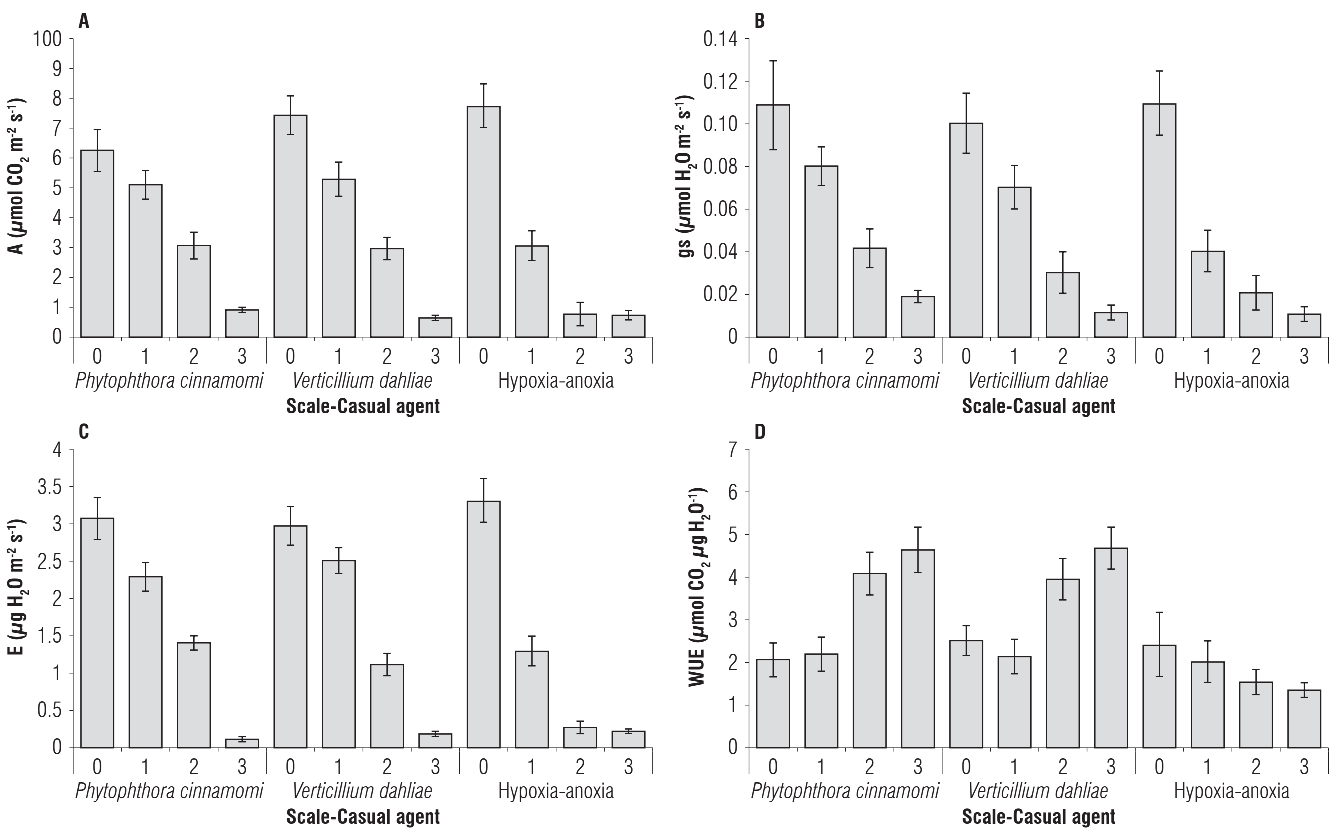

FIGURE 5. Physiological responses of avocado seedlings cv. Hass to *Phytophthora cinnamomi*, *Verticillium dahliae*, and hypoxia and anoxia causing the avocado wilt complex. (A) Net photosynthesis, (B) stomatal conductance, (C) transpiration, (D) water use efficiency. Error bars represent the confidence intervals of the means, validated by the Tukey mean separation test. No overlapping of the error bars indicates significant differences ($P<0.05$).

result of affected plants having many damaged roots and tissues, which reduces the uptake and transport of water in the plant and leads to a water deficit, as occurs for plants infected by *P. cinnamomi* and *V. dahliae* or due to the hypoxia-anoxia disorder.

For these conditions, the physiological responses of avocado seedlings were very similar, and were a little more marked with an excess of humidity, especially during the first stages (Fig. 5). The main mechanism involved that explains the response of the plant is considered to be stomatal closure (Sanclemente *et al*., 2014), since this condition indirectly affects stomatal conductance, net photosynthesis and transpiration (CuiYing *et al*., 2010; Yin *et al*., 2010).

Under a specific stress condition, such as a hypoxia-anoxia or infection by a pathogen, a plant may have adaptive responses, which could be morphological, anatomical or physiological (CuiYing *et al*., 2010). However, if the stressful condition continues and increases, the plant cannot overcome it, leading inevitably to great damage or death as observed in stage 3 of disease development for each one of the causal agents studied in the present research (Ploetz and Schaffer, 1989; Reeksting *et al*., 2014).

In studies of avocado, under conditions of hypoxia-anoxia and the interaction with *P. cinnamomi* there is a decrease in photosynthesis that is accompanied by a reduction in stomatal conductance, transpiration and partial pressure of CO_2 in the intercellular leaf spaces (Ploetz and Schaffer, 1989; Reeksting *et al*., 2014). These results agree with what was found in this research.

Greater water use efficiency occurred in the more advanced stages of the disease. The plant under this condition attempts to maximize the use of each one of the resources that it possesses, especially water, since water deficit in plant tissues becomes elevated when plants have a very reduced and atrophied root system.

Conclusion

Scales developed to evaluate the progress of wilt disease in avocado cv. Hass in field and net house conditions are highly correlated with the amount of inoculum in each of the defined stages. Therefore, they are adequate for evaluating the dynamics through time of each of these pathologies. Avocado seedlings are highly affected by the presence of these pathologies, especially in the late stages close to death, as seen by the physiological responses measured.

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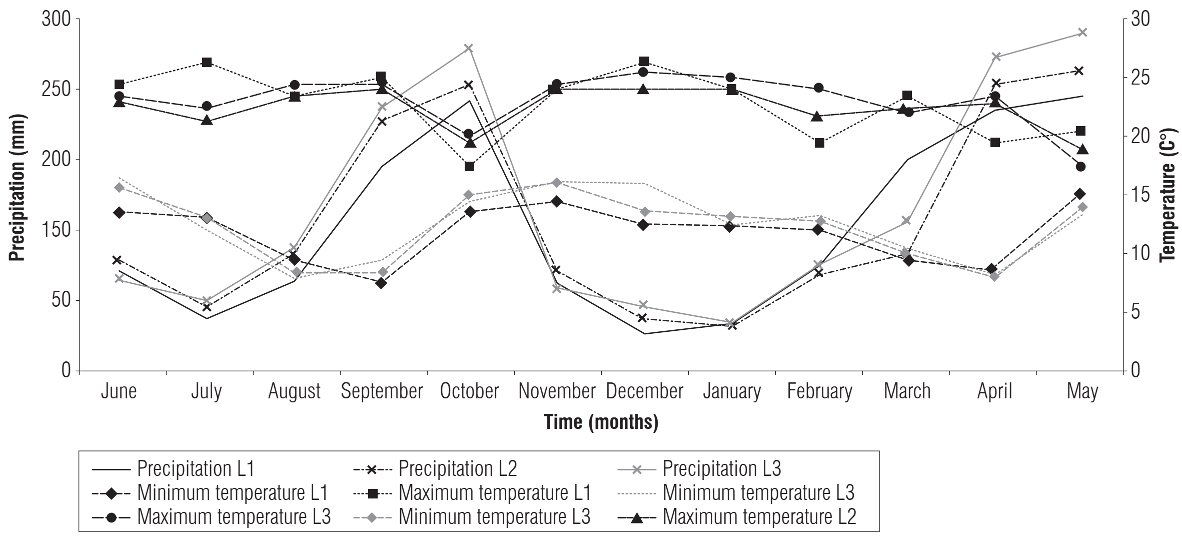
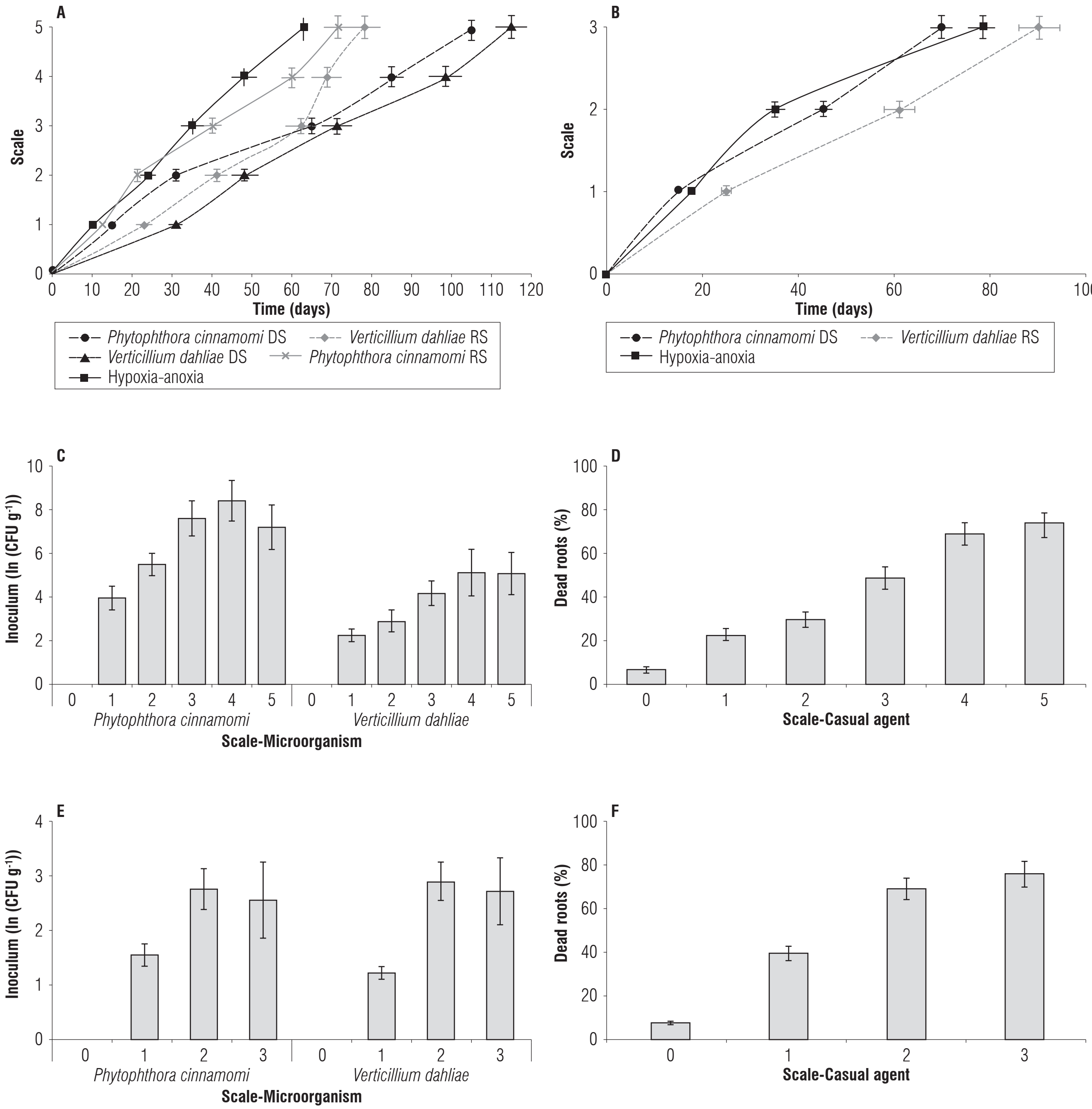
SUPPLEMENTARY MATERIAL 1. Edaphic variables associated with soil in lots and laboratory tests.

Soil	Sn[1]	Si[1]	Cla[1]	pH	OM[2]	Al[3]	Ca[3]	Mg[3]	K[3]	P[4]	S[3]	Cu[5]	Zn[5]	Mg[5]	B[5]
Donmatias[a]	52	28	20	5,2	12.3	0.6	2.55	1.1	0.45	21.3	5.1	0.8	2.3	1.9	0.3
El Retiro[b]	60	22	18	5.3	14.8	0.8	3.5	1.3	0.55	24.4	5.6	0.9	1.9	2.5	0.5
La ceja[c]	58	23	19	5.5	13.1	1.6	1.9	0.9	0.35	15.9	5.9	0.9	2.4	2.1	0.35
Net house[c]	62	15	23	5.1	6.0	0.9	1.1	0.9	0.4	12.3	4.8	0.6	1.7	1.9	0.2

[1]Sand (Sn, %), silt (%), clay (Cla, %) (Bouyoucos). pH (water:soil, 1:2, V:V). [2]Organic matter content (OM, %) (Walkley and Black; aluminium). Aluminium (Al) (1M KCl). Calcium (Ca), magnesium (Mg) and potassium (K) (1M ammonium acetate). Phosphorus (P) (Bray II). Sulfur (S) (0.008 M calcium phosphate solution). Iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn (Olsen-EDTA buffer). Boron (B) (hot water). [3]Interchangeable bases (cmolc kg^{-1}). [4]mg kg^{-1}. [5]Available minor elements (mg kg^{-1}). [a]The plot was located in the Municipality of Donmatias (6.496961 latitude, 75.412118 longitude, 2213 m a.s.l.) in the Northern region of Antioquia (Colombia). [b,c]The plots were located in the municipalities of El Retiro (6.09715 latitude, 75.46478 longitude, 2100 m a.s.l.) and La Ceja (5.95931 latitude, 75.41777 longitude, 2387 m a.s.l.), in the eastern region of Antioquia (Colombia). All plots were in a life zone classified as tropical lower montane wet forest (TLM-wf) *sensu* Holdridge (Holdridge, 1967). [c]Universidad Nacional de Colombia Medellin campus (6°15' N, 75°34' W, 1496 m a.s.l), with an average temperature range of 18-22°C, relative humidity in the range of 75-95% and a photosynthetically active radiation of 650-1920 μmol photons m^{-2} s^{-1}.



SUPPLEMENTARY MATERIAL 2. Climatic conditions associated to the lots evaluated in the two study regions. L1: Lot in Donmatias. L2: Lot in El Retiro. L3: Lot in La Ceja. Climate values represent the average for each month during the years 2011-2012.



SUPPLEMENTARY MATERIAL 3. Variation through time of the disease development scales and inoculum values for *Phytophthora cinnamomi*, *Verticillium dahliae,* and hypoxia and anoxia in avocado cv. Hass evaluated under field and net house conditions. (A) Variation through time of the disease development scale under field conditions. (B) Variation through time of the disease development scale under net house conditions. (C) Variation through time of the inocula values for *Phytophthora cinnamomi* and *Verticillium dahliae* under field conditions. (D) Variation through time of the hypoxia and anoxia scale values under field conditions. (E) Variation through time of the inocula values for *Phytophthora cinnamomi* and *Verticillium dahliae* under net house conditions. (F) Variation through time of hypoxia and anoxia scale values under net house conditions.

Evaluation of Phytoseiidae mites and *Chrysoperla carnea* (Stephens) on the control of *Tetranychus urticae* in *Carica papaya* L.

Evaluación de ácaros Phytoseiidae y *Chrysoperla carnea* (Stephens) sobre el control de *Tetranychus urticae* en *Carica papaya* L.

Yuri Mercedes Mena[1], Nora Cristina Mesa[1]*, Alexander Escobar[2], and Santiago Pérez[3]

ABSTRACT

In order to evaluate the establishment and biological effectiveness of the predators *Phytoseiulus persimilis, Neoseiulus californicus* and *Chrysoperla carnea* on populations of *Tetranychus urticae*, a trial was established at the farm "La Pola" (with a cumulative precipitation of 445.9 mm), located in the municipality of Roldanillo, Valle del Cauca, Colombia. An experiment was performed under field conditions between September 2017 and May 2018 and using the papaya hybrid Tainung-1 between the stages of vegetative growth and fruit filling. Four treatments were established: T1: 12 releases of *P. persimilis* and *N. californicus*; T2: 10 releases of *C. carnea*; T3: treatment with no predator releases (control); and T4: grower's practice (15 applications of pesticides) in a randomized complete block design with three replicates per treatment. Samples were collected every eight days by measuring the following variables: infestation percentage, number of fruits/plant, population of *T. urticae, P. persimilis, N. californicus,* and *C. carnea*/leaf and the diameter of the stem (every 30 d). The largest *T. urticae* populations were found in T3 and the lowest ones in T4. The populations of immature and adult *T. urticae* did not show significant differences between T1 and T2 but there were significant differences between T3 and T4. The variables diameter of the stem, number of leaves per plant, percentage of infestation, and number of fruits per plant showed significant differences between treatments. The T1 and T2 were equal to T3. T4 had the highest number of leaves and fruits per plant. The released predators did not control the populations of the mite *T. urticae* under the conditions of the present experiment.

Key words: settlement, *Neoseiulus californicus*, *Phytoseiulus persimilis*, effectiveness, Tainung-1.

RESUMEN

Con el fin de evaluar el establecimiento y la efectividad biológica de los depredadores *Phytoseiulus persimilis*, *Neoseiulus californicus* y *Chrysoperla carnea* sobre las poblaciones de *Tetranychus urticae*, se estableció un ensayo en la finca La Pola, ubicada en el municipio de Roldanillo, Valle del Cauca, Colombia. El experimento se realizó entre septiembre de 2017 a mayo de 2018 bajo condiciones de campo y con una precipitación acumulada de 445.9 mm utilizando el hibrido de papaya Tainung-1 entre la etapa de crecimiento vegetativo y llenado de frutos. Se establecieron cuatro tratamientos: T1: 12 liberaciones de *P. persimilis* y *N. californicus*; T2: 10 liberaciones de *C. carnea*; T3: testigo absoluto (control), y T4: testigo agricultor (15 aplicaciones de plaguicidas) en un diseño de bloques completos al azar con tres repeticiones por tratamiento. Se hicieron muestreos cada ocho días midiendo las variables porcentaje de infestación, número de frutos/planta, poblaciones de *T. urticae*, *P. persimilis, N. californicus* y *C. carnea*/hoja y diámetro del tallo (cada 30 d). Se encontraron las mayores poblaciones de *T. urticae* en el T3 y las menores para el T4. Las poblaciones de inmaduros y adultos de *T. urticae* no presentaron diferencia significativa entre los T1 y T2, pero si con los T3 y T4. Las variables diámetro del tallo, número de hojas por planta, porcentaje de infestación y número de frutos por planta, presentaron diferencias significativas entre los tratamientos. Los T1 y T2 fueron iguales al T3. El T4 presentó el mayor número de hojas y frutos/planta. Los depredadores liberados no ejercieron un control de las poblaciones del ácaro *T. urticae* bajo las condiciones del presente trabajo experimental.

Palabras clave: establecimiento, *Neoseiulus californicus*, *Phytoseiulus persimilis*, efectividad, Tainung-1.

Introduction

The mite *Tetranychus urticae* Koch is one of the most limiting arthropods for papaya cultivation in various producing regions of the world, including the province of Valle del Cauca in Colombia (Santa-Cecília and Reis, 1986; Vieira *et al*., 2004; Moraes and Flechtmann, 2008; Gómez *et al*., 2014; Migeon and Dorkeld, 2006-2017). *Tetranychus urticae* feeds on a wide range of host plants (1140 plant species) that comprise diverse families (Migeon and Dorkeld,

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[1] Universidad Nacional de Colombia sede Palmira, Valle del Cauca (Colombia).
[2] Bichopolis-BioBee, Tabio, Cundinamarca (Colombia).
[3] Frutas Tropicales S en C, La Union, Valle del Cauca (Colombia).
[*] Corresponding author: ncmesac@unal.edu.co

2006-2017). It frequently begins colonization of host plants on the mature leaves, and as its populations increase, it invades the younger leaves (Marín *et al*., 1995; Seeman and Beard, 2011; Vásquez *et al*., 2012). The mite develops its colonies on the underside of leaves and has a CW type of life (complex and complicated spider web production) according to the classification of Saito (2010). Their web provides shelter and protection for the immature stages against predator attacks, toxic substances, and adverse climatic conditions (Moraes and Flechtmann, 2008; Badii *et al*., 2010). Two-spotted spider mites feed more commonly on older papaya leaves, which initially turn yellow on the upper sides and silver on the lower sides, followed by the appearance of necrotic areas and eventually leaf drop (Collier *et al*., 2007). This damage, predominating in the dry season, directly influences photosynthesis and increases exposure to the sun, with negative consequences for fruit production and marketability. For this reason, pesticide applications in papaya orchards occur year-round, with the well-known risks to the environment and human health (Marín *et al*., 1995).

Biological control of *T. urticae* has proven to be successful in different crops, and the predator mite *Neoseiulus californicus* (McGregor) is one of the major biological control agents of *T. urticae* in rose (*Rosa* spp.) crops in greenhouses of several countries (Marafeli *et al*., 2011). In Colombia, the use of predators of the genera *Phytoseilus* spp. and *Amblyseius* spp. has been reported as an effective control of the mite *T. urticae* in roses (Valcárcel-Calderón, 2013) as well as the effective use of the entomopathogenic fungus *Isaria fumosorosea* Wize (Castro, 2010). For this same species of Tetranychidae, the releases of phytoseids *Phytoseiulus persimilis* Athias-Henriot and *N. californicus* were very effective in nursery conditions for clementine (*Citrus clementina* Hort. ex Tan.) orchards in Spain (Sá, 2012). Alternative products based on the neem *Azadirachta indica* (Juss) have also successfully exercised control of *T. urticae* populations in strawberry plants (Soto *et al*., 2011). However, insecticides and acaricides have always played the central role in controlling this species of mite in different crops such as the papaya in Valle del Cauca (Gómez *et al*., 2014).

Currently, a large number of compounds with different chemical structures and application modes is used (Knowles, 1997; Dekeyser, 2005; Van Leeuwen *et al*., 2009). However, *T. urticae* has a high capacity for rapidly developing resistance to chemical products (Knowles, 1997, Van Leeuwen *et al*., 2008). Its high reproductive potential, inbreeding, the condition of producing haploid males and its short life cycle resulting in many generations per year, facilitate the rapid development of resistance to many acaricides after a limited number of applications (Nauen *et al*., 2000; Van Leeuwen *et al*., 2010). Among all the arthropods, *T. urticae* has the highest prevalence of resistance to pesticides (Van Leeuwen *et al*., 2010; Grbić *et al*., 2011). This very rapid resistance to new and commercially available chemical products affects integrated management programs (Cho *et al*., 1995). Additionally, the excessive use of these chemicals has increased the health risks of farmers and consumers, in addition to the negative impact on the environment (Morera, 2015).

Some of the most effective biological controllers for the control of *T. urticae* are Phytoseiidae mites such as *P. persimilis* and *N. californicus* (Cloyd *et al*., 2006; Rhodes *et al*., 2006). Both species possess important attributes such as a shorter development cycle than that of *T. urticae*, a rapid development of populations, and high search capacity and voracity, among other details (Acosta, 2000; Gómez *et al*., 2014). McMurtry *et al*. (2013) classify *P. persimilis* as a specialized predator of mite species of the genus *Tetranychus* (Type: Ia), and they classify *N. californicus* as a selective predator of mites of the family Tetranychidae (Type: II), which might be used in programs of integrated pest management.

Some studies have been conducted with Phytoseiidae mites in papaya but under controlled conditions. Collier *et al*. (2004) report *Neoseiulus idaeus* Denmark & Muma, 1973 in Brazil as a good candidate for biological control of *T. urticae*, based on laboratory results due to its high occurrence in papaya production systems with intensive use of acaricides, together with its favorable biological characteristics reported in the literature. López (2014) found that the predatory mites *N. californicus* and *Amblyseius swirskii* Athias-Henriot, 1962 are viable for the control of *T. merganser* in papaya cultivation under greenhouse conditions in Brazil.

Chrysoperla carnea (Neuroptera: Chrysopidae) is indicated as an important natural enemy of soft-bodied phytophagous arthropods, such as red spiders, in part because of its high voracity as well as its polyphagous nature (Pappas *et al*., 2011). It is a species with wide geographical distribution, tolerance to insecticides such as abamectins, and ease of breeding in the laboratory, which allows its mass production in different laboratories of Valle del Cauca (Nasreen *et al*., 2005; Cerna *et al*., 2012). Hassanpour *et al*. (2009) recommend including *C. carnea* in red spider management programs due to its good predatory potential.

Predatory mites of the family Phytoseiidae and predatory insects such as *C. carnea* are widely included in biological control programs in various crops, due to their ability to survive, reproduce and tolerate some chemical compounds (Cloyd *et al*., 2006). But a control exercised by these predators has not been evaluated on *T. urticae* in crops of *C. papaya* in Colombia. The hybrid Tainung-1 is the most cultivated material in the province with yield losses caused by *T. urticae*, considered to be the most limiting pest for the cultivation of papaya in this region of the country (Gómez *et al*., 2014). That is the basis for this research, aimed at evaluating the control exercised by *P. persimilis*, *N. californicus* and *C. carnea* on populations of *T. urticae* in papaya Tainung-1 in Valle del Cauca, under field conditions.

Materials and methods

Obtaining *P. persimilis* and *N. californicus*

Individuals of *P. persimilis* and *N. californicus* used for field trials were obtained from the commercial company Bichopolis-BioBee© SAS., located in the municipality of Tabio, Cundinamarca. The mites were packed and sent in 60 ml plastic bottles whose lid had a dispenser. Each bottle contained 1,000 predatory mites mixed with vermiculite. Shipments from Tabio were made by land following the route Tabio-Cali-Roldanillo (to the farm "La Pola") with an average time of 48 to 72 h from packing to release. The bottles contained the two species of Phytoseiidae, *P. persimilis* and *N. californicus,* in a 600:400 ratio, respectively, for each bottle.

Obtaining *C. carnea*

The individuals of *C. carnea* were obtained from the commercial company Perkins Ltda., located in the city of Palmira. Each package contained 100 eggs of *C. carnea* mixed with rice husk and *Sitotroga cerealella* eggs (Olivier) (Lepidoptera: Gelechiidae). These sealed packages were transported in a cooler with ice at the bottom, one day before the hatching of the eggs, from Palmira to the farm "La Pola" (municipality of Roldanillo).

The experiment under field conditions

The study was carried out between September 2017 and May 2018 under field conditions, at the farm "La Pola" (04°40' N and 76°10' W, at an altitude of 910 m a.s.l.). Climatic conditions during the releases were as follows: temperature 23.1°C (maximum: 25°C, minimum 20.3°C), relative humidity 81.3% (maximum: 95%, minimum 73%), and a cumulative rainfall of 445.9 mm with a maximum peak of 72.2 mm at 9 weeks after transplanting (WAT).

A randomized complete block design was used, with four treatments and three replicates per treatment. The experimental unit consisted of 16 plants of hybrid papaya Tainung-1, aged between the sixth and 36th week after transplanting, between the phenological stages of vegetative growth and fruit filling. A total of 192 plants were used for the experiment with a distance between plants of 1.5 m and a distance between rows of 3.5 m. Four treatments were established as described in Table 1.

The predatory mites were released on the leaves of the middle part of the 16 plants in each plot during the morning hours with a mean temperature of 23.1°C and relative humidity of 81.3%. Before opening the bottle, the cap was strongly tapped, since most of the mites were concentrated here, and the bottle was rotated in a horizontal position to allow an adequate mixture of the predatory mites in the vermiculite. The amount and time of release of the Phytoseiidae mites is listed in Table 1, following the recommendations of the producing company and its experience in the release of these Phytoseiidae under controlled conditions. Each bag of *C. carnea* was hung from each of the 16 plants of the three experimental plots. The release dates of the lacewings are listed in Table 1. The appearance of the first focus of *T. urticae* on the crop was used as the criterion for the release of predators.

TABLE 1. Description of the treatments for evaluating the control of predators on the populations of *Tetranychus urticae* in papaya Tainung-1.

Treatment 1	Release of *P. persimilis* and *N. californicus*. Twelve periodic releases were accomplished from 6 WAT*. Initial releases (20 mites/plant) at 6, 7, 9, 10, 11, 12 and 13 WAT. Flooding releases (1,000 mites/plant) at 15, 16 and 17 WAT. Semi-flooding releases (500 mites/plant) at 19 and 20 WAT.
Treatment 2	Realease of *C. carnea*. Ten periodic releases of 100 larvae/plant were accomplished 6, 10, 11, 13, 14, 16, 17, 19, 20 and 22 WAT.
Treatment 3	Untreated treatment (control). Control of *T. urticae* was not carried out throughout the development of the crop.
Treatment 4	Grower's practice, mite management as is conventionally performed in a commercial crop where approximately 15 applications of chemical synthesis acaricides in rotation (abamectin, spiromesifen, cyflumetofen, fenazaquin, hexithyazox, acequinocyl, milbectin, bifenazate) are applied. This treatment was isolated from the other treatments by a green cloth used as an enclosure at 5 m of height.

*WAT: Weeks after transplanting.

The infestation of *T. urticae* in the experimental lot was natural for all treatments. However, this was increased early on by the presence of a nearby melon (*Cucumis melo* L.) crop, which was found infested with the mite, resulting in the experimental lot (mainly block I) being infested. An initial population mean of 7 adults, 39 immatures and

61 eggs per leaf was found for all treatments evaluated by direct counting on papaya leaves. In order to guarantee the highest percentage of hermaphrodite plants in the experimental lot, two plants were planted per site. Once the flowering took place, thinning was performed leaving a single plant, preferably the hermaphrodite plant. The phenological states presented by the culture of papaya are vegetative development (0-11 WAT), beginning of flowering (12 WAT), first fruiting (16 WAT) and reproductive period (more than 30 WAT). Treatments were begun on the sixth week after the transplanting.

Throughout the development of the crop all necessary agronomic work was carried out: irrigation, weed control, disease and insect vectors of diseases, as well as the necessary fertilizations. Plants that had obvious symptoms of virus were removed, but this practice did not affect the experimental design.

For an evaluation of populations of *T. urticae* before and after the release of predators as well as the populations of *P. persimilis, N. californicus* and *C. carnea*, four plants were randomly tagged in each lot with a weekly monitoring of the incidence of mite damage on the plant (number of leaves with damage/number of total leaves per plant). Leaves considered to be damaged were those that showed obvious chlorosis and food marks. Damage was determined in the inspected leaves using a scale of damage proposed by Tomkiewicz *et al.* (1993) (Tab. 2), registering the percentage of the foliar area affected.

TABLE 2. Chart of evaluation of *Tetranychus urticae*'s damage (Tomkiewicz *et al.*, 1993). Reproduced with permission of Experimental and Applied Acarology.

Rate of foliage damage	Definition	% affected foliage area
0	No damage on the leaf	0
1	Feeding marks in the base of the leaf or in the area near the ribbing.	5
2	Base of the leaf and area close to the ribbing affected.	15
3	Base of the leaf and area along the main ribbings with feeding marks.	30
4	Feeding marks that cover all the leaf, although the leaf still looks green.	60
5	Leaves are yellow and are beginning to wither.	80-100

From each lot, a leaf from the middle part of the plant was collected randomly, and then taken to the Acarology Laboratory of Universidad Nacional de Colombia, Palmira. The populations of each species (eggs, immature and adult stages) were counted with the help of a stereoscope Leica EZ4 8X-35X zoom (Leica Microsystems, Switzerland). Eggs were recognized by their yellowish color and spherical shape, finding them along the leaf veins or above or between the cobwebs. The number of fruits and stem diameters were recorded monthly in order to detect the impact of phytophagous mite populations on crop production. All plants were brought to the first month of harvest and the crop yield was evaluated. Meteorological data were obtained from the RMA ZAR station: Zarzal of Cenicaña.

Data analysis was performed using the SAS 9.3 statistical package (SAS Institute, 2014). Data were subjected to an analysis of variance (ANOVA) and mean separation by least significant difference (LSD) at 5% significance.

Results and discussion

Evaluation of populations of *T. urticae* and predators

The populations of *T. urticae* appeared from the fourth week after transplanting (WAT). The presence of a crop of melon (*C. melo*) at harvest stage located next to the experiment was possibly a focus of the mite pest, increasing the population quickly mainly in block one, for all treatments. The largest populations of *T. urticae* occurred from the sixth week and increased at the beginning of flowering, reaching the highest peaks between 9 and 20 WAT in all treatments. Populations in T1 and T2 had maximum peaks of 2,699 and 1,862 eggs/leaf respectively. In T3, the populations were very high and reached peaks of 5,482 eggs/leaf. In the T4, the highest peak population was 407 eggs/leaf. When the 19th WAT was reached, the pest mite populations decreased considerably to the point of registering zero mites/leaf in all treatments. The development of *T. urticae* populations (eggs, immatures, and adults) in each of the treatments is shown in Figure 1.

In contrast, very few individuals of *P. persimilis* were found in T1 and T2, with population peaks of 50 and 40 individuals/leaf at 14 and 18 WAT respectively. Once the releases of the Phytoseiidae were finished (19 WAT), the populations practically disappeared. The presence of *P. persimilis* in T2 can be explained by air currents and the proximity of these treatments in the experiment, especially T1. No individuals of the species *N. californicus* were recovered in any treatment during all evaluations. Something similar occurred with *C. carnea* whose populations were very low, only a few individuals were found in T2 (4 eggs/leaf).

Regarding eggs, immatures and adult stages, the untreated T3 showed the largest populations of *T. urticae* in contrast to the grower's practice T4 that showed the lowest

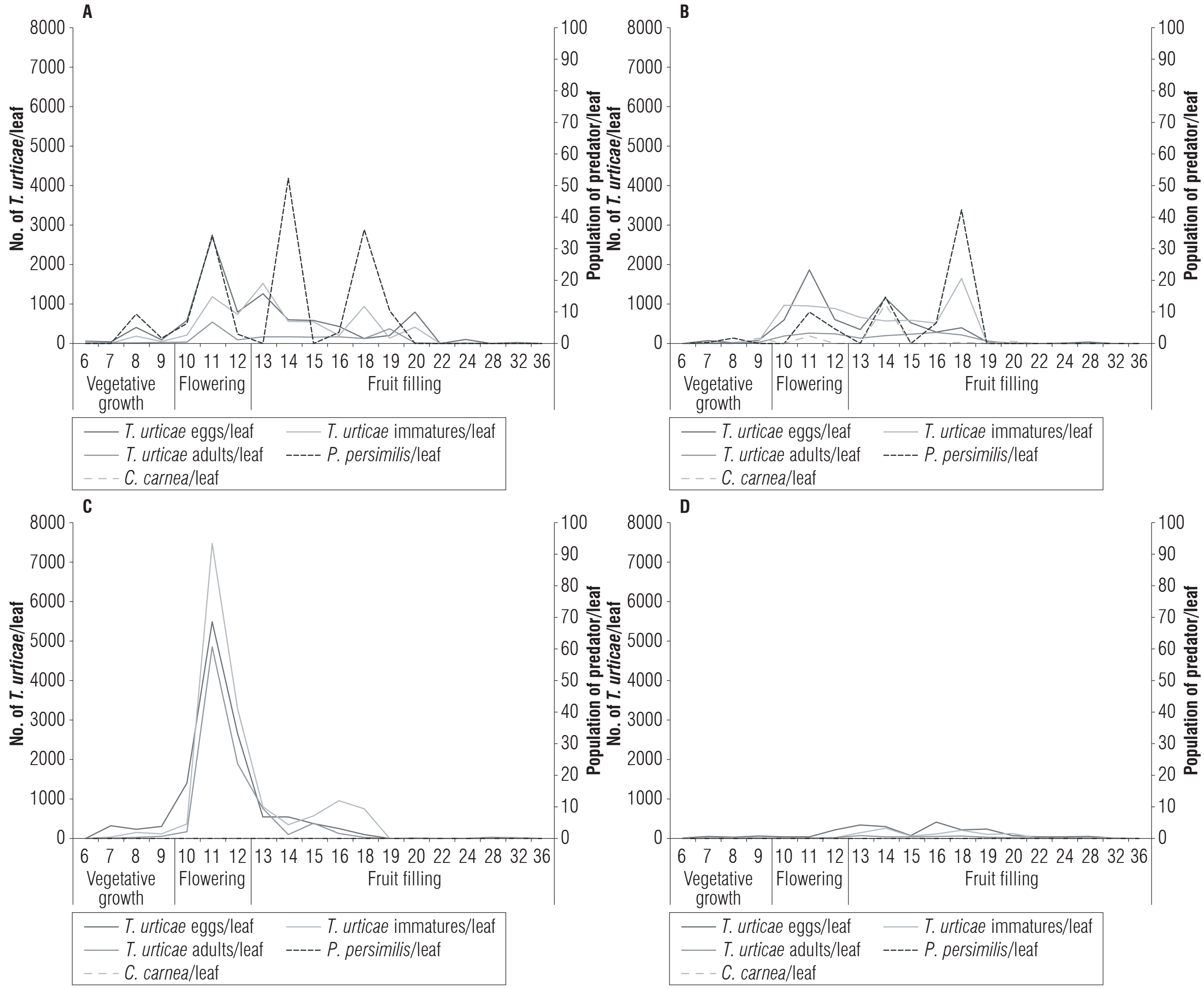

FIGURE 1. Development of the populations of *Tetranychus urticae* and predators in the different treatments A) T1: *Phytoseiulus persimilis* and *Neoseiulus californicus*, B) T2: *Chrysoperla carnea*, C) T3: Untreated (control) and D) T4: Grower's practice.

population levels. Eggs of *T. urticae* per leaf in treatments T1, T2 and T3 showed a significant difference in T4. The immature and adult populations of *T. urticae* showed no significant difference between T1 and T2 but between T3 and T4 (Fig. 2).

For the establishment of Phytoseiidae mites and *C. carnea,* phytoseiids disperse by walking or flying within a crop (Johnson and Croft, 1976; Hoy *et al*., 1985; Jung and Croft, 2000). *Phytoseiulus persimilis* has the longest legs and moves faster than other species, so they move from leaf to leaf, from plant to plant, and can walk a few meters on the ground. These characteristics allow them to ensure a uniform distribution over the plant and adjacent plants, despite the difficulties of flooding releases of phytoseiids on all leaves where the prey is present (Hoy, 2011).

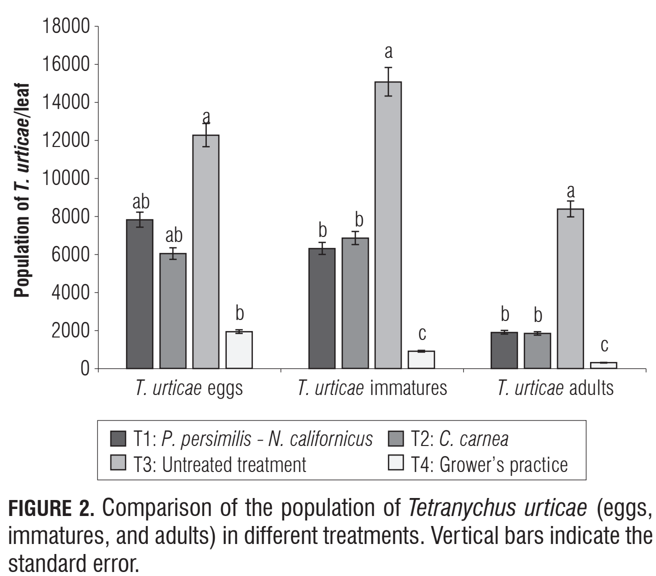

FIGURE 2. Comparison of the population of *Tetranychus urticae* (eggs, immatures, and adults) in different treatments. Vertical bars indicate the standard error.

This was not the situation observed in our experiment. In spite of the flooding releases of *P. persimilis, N. californicus* and *C. carnea*, no establishment of these natural regulators in *C. papaya* was accomplished and, therefore, no control effect on *T. urticae* was observed. Some factors, such as the low initial releases of the predators and the high infestation of *T. urticae* could have influenced these results. The releases were initiated from plants with an average of nine leaves in which the populations of *T. urticae* were on average 48 individuals per leaf (between eggs, immatures, and adults). Releases were initiated at a dose of 20 mites/plant/week approximately at 12 WAT. During this period the maximum number of *T. urticae* populations was 4,418 individuals per leaf, whereas the maximum number of individuals that *P. persimilis* showed at that time was 34 individuals between eggs and mobile stages per leaf.

Hoy (2011) indicates that augmented releases of phytoseiids should be performed early when the pest population is low, since these releases usually involve only adult females, which take time to move to adjacent leaves or plants in search of their pray after the releases. Reyes (2012) recommends the use of this predator with flooding releases in papaya for the control of *Tetranychus merganser* under laboratory conditions, since *P. persimilis* is not settled when showing a low hatching (5.14 eggs per female) when feeding on this Tetranychidae.

Another aspect that can affect the behavior of Phytoseiidae is the long storage period of predatory mites and *C. carnea* between the packaging and the releases. The Phytoseiidae were raised in the municipality of Tabio, Cundinamarca and sent by land courier service to the place of the experiment. The storage period was between 48 to 72 h before being released.

When opening the bottle containing the Phytoseiidae, most were located in the lid of the dispenser. This agrees with Daza *et al*. (2010), who finds that the effectiveness of *P. persimilis* is diminished by the effect of the trip since it affects the quality and survival of the individuals. Mesa and Duque (1994) indicate the convenience of acclimatizing Phytoseiidae before releases by confining them in cages for a time, as pre-adaptation in the field. In our research, releases were performed directly from the jar with vermiculite to the papaya plants after the long transport.

It is important to consider the architecture of the papaya plant, whose leaves have long petioles, the leaves are distant from each other, and often have drops of latex. It is possible that some of these factors may have disturbed the behavior of the predators. According to Konno *et al*. (2004), the latex produced by the leaves of *C. papaya* presents a protease, papain that confers defense against the attack of herbivores, such as polyphagous insects, reducing their rate of growth on that host. *Tetranychus urticae*, has in its genome some detoxifying genes from bacteria, fungi and plants that it uses to combat the defense mechanisms of the plants it feeds on (Grbić *et al*., 2011). These could prevent that this papain had a negative effect on this Tetranychidae, but they could have some effect on *P. persimilis*.

Also, the crop has a leaf blade with a smooth bundle and the underside has protuberant ridges (Arango and Roman 2000), which might prevent the shelter of predators. However, Collier *et al.* (2007) indicates that *T. urticae* reared on papaya or green bean is a suitable prey for the development and reproduction of the predatory mite *Neoseiulus idaeus* under laboratory conditions. This proves that under these conditions the host plant of *T. urticae* does not affect the predator, guaranteeing the success of *N. idaeus* as a biological control agent of *T. urticae* in papaya orchards.

Although there have been very few studies on *N. idaeus* in papaya crops, it may have the potential to regulate populations of two-spot spider mites in this crop, compared to other Phytoseiidae species. *Neoseiulus idaeus* is a type II specialized predator that shows substantial adaptations and

TABLE 3. Variables evaluated to measure the control exerted by predators for the different treatments.

	Treatments	Infestation (%±SEM)	Number of leaves±SEM	Stem diameter (cm±SEM)	Number of fruits*±SEM	Average fruit weight (kg±SEM)*
T1:	*P. persimilis*	78.3±1.3 b[1]	32.9±0.3 ab	21.2±0.7 b	2.7±0.8 b	1.1±0.2 b
T2:	*C. carnea*	80.1±1.2 b	31.7±0.3 b	21.1±0.7 b	2.6±0.4 b	1.1±0.1 b
T3:	Untreated treatment (control)	84.3±1.1 a	30.9±0.3 b	22.3±0.8 b	3.2±0.7 b	0.9±0.2 b
T4:	Grower´s practice	61.5±1.1 c	39.9±0.3 a	24.5±0.1a	6.4±0.8 a	1.5±0.1 a

[1]Means followed by equal letters do not differ from each other according to the Tukey's test ($P<0.05$).

SEM: Standard error of the mean.

*Data collected in the first month of harvest.

preference for spider mites. The presence and abundance of *N. idaeus* throughout the year in commercial orchards with constant acaricide applications indicates that it can develop resistance to pesticides, and this gives more support to its use as a control agent in papaya (Collier *et al*., 2004).

The period in which the experiment took place coincided with the period of strong winds during the year in the municipality of Roldanillo between October and February with average wind speeds of more than 4.6 km/h (Cedar, 2018). Strong wind could carry predators to other plants different from the ones in which the releases were performed. These winds probably contributed to the rapid dispersion of *T. urticae* in the experimental plot since this arthropod has aerial-dispersal behaviors that facilitate its escape from the leaf-surface boundary layer and enable it to become airborne. In addition, as wind speed increases, the dispersion response increases (Smitley and Kennedy, 1985).

In relation to the variables stem diameter, number of leaves per plant, percentage of infestation and number, and the weight of fruits per plant, the analysis of variance showed significant differences between treatments (Tab. 3). Mean values were the same for T1, T2 and T3, showing a reduction in the number of leaves/plant and in the number of fruits/plant and a higher percentage of infestation. In the first month of harvest, the T1 and T2 treatments yielded 58.4% less fruits than the grower's practice, and even though in the T3 a smaller reduction was recorded (50%), the weight of fruits was lower, which is directly related to a lower yield. The grower's practice showed the highest values in the number of leaves per plant and the number and weight per fruit, and the lowest percentage of infestation.

The number of leaves is a clear indicator of productivity of papaya, considering that at least one fruit is formed in the bud of each leaf (Storey 1969; Mahouachi *et al*., 2005). Similarly, the leaves synthesize the carbohydrates that are to be distributed among the different organs of the plant, which allows greater fruit production (Cuéllar and Arrieta, 2010). Thus, a direct relation between the number of leaves and the number of fruits exists, and a decrease in the yield of the crop is observed due to the mites that cause defoliation.

Establishing experiments under real field conditions is important, since the crops grown by the producer entail facing multiple natural factors and problems that cannot be controlled and that do not affect the populations of the pest mite. Therefore, under the conditions of our research the flood releases of predators *P. persimilis* and *N. californicus*, and *C. carnea* were not found to exert control of the *T. urticae* mite populations. It is possible that some abiotic or biotic factors described above did not allow them to settle down in the papaya crop since the populations of *T. urticae* in this crop were so high between weeks 6 and 19 after the transplantation that the number of predators released was not enough or these controllers were not released in a timely enough manner. In addition, perhaps some experimental errors could have been made.

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Closing yield gaps in Colombian direct seeding rice systems: a stochastic frontier analysis

Cierre de brechas de rendimiento en los sistemas colombianos de siembra directa de arroz: un análisis de frontera estocástica

David Arango-Londoño[1,2*], Julián Ramírez-Villegas [2,3,4], Camilo Barrios-Pérez[2,3,6], Osana Bonilla-Findji[2,3], Andy Jarvis[2,3], and Jorge Mario Uribe[5]

ABSTRACT

Rice is one of the most important crops in terms of harvested area and food security both globally and for Colombia. Improvement of technical efficiency levels in rice production in order to close yield gaps in a context in which rice demand increases, natural resources are depleted, and where there are growing expectations about both climate changes and trade agreements is likely the most important challenge that farmers confront. This research assessed the main management factors that limit both rice crop productivity and the likely drivers of non-optimal technical efficiency levels (a proxy for yield gaps). This study focused on both upland and irrigated direct seeding systems across a variety of environments in Colombia. Stochastic frontier models were used to integrate microeconomic theory and empirical regression analysis in conjunction with a large commercial rice production database developed by the Colombian rice growers' federation (Fedearroz). A large variation was found in technical efficiency (from 40 to 95%) levels for both upland and irrigated systems, and major differences were obtained in the limiting factors of the two systems (e.g. seed availability, variety type, market accessibility, fertilizer type, and use rate). This suggests both substantial and varied opportunities for improvements in current technical efficiency levels. Across systems, the correct choice of variety was identified as a common key factor for maximizing yield for a particular environment. For upland systems, optimal choices were F174 and F2000, whereas for irrigated rice F473 was found to produce the highest yield. Additionally, numerical analysis suggests a yield impact of ca. 0.18% for each 1% increase in the nitrogen application rate for upland systems. For irrigated rice, phosphorous rather than nitrogen application rates were found to be more important. Since our analysis is based on farm-scale commercial production data, we argue that once our results are brought to consensus with local extension agents, technicians and agronomists, then management recommendations for closing yield gaps can be used to improve rice productivity.

Key words: empirical models, food security, increasing rice demand, production function, technical efficiency.

RESUMEN

El cultivo de arroz es uno de los más importantes en términos de área cosechada y seguridad alimentaria tanto a nivel global como en Colombia. Mejorar los niveles de eficiencia técnica en la producción de arroz para cerrar las brechas de rendimiento en un contexto en el que la demanda de alimento aumenta, los recursos naturales escasean y las expectativas sobre los impactos potenciales del cambio climático y los tratados de libre comercio crecen es probablemente el desafío más importante que enfrentan los agricultores. Esta investigación evaluó los principales factores de gestión que limitan la productividad del cultivo de arroz, y los posibles impulsores de niveles de eficiencia técnica no óptimos (un proxy de las brechas de rendimiento). El estudio se enfocó en sistemas de siembra directa de tierras altas y de riego en diferentes ambientes en Colombia. Utilizamos modelos de frontera estocástica para integrar la teoría microeconómica y el análisis de regresión empírica junto con una gran base de datos de producción comercial de arroz, desarrollada por la Federación colombiana de productores de arroz (Fedearroz). Se encontró una gran variación en los niveles de eficiencia técnica (del 40 al 95%) para los sistemas de tierras altas y de riego, y se obtuvieron diferencias importantes en los factores limitantes entre los dos sistemas (por ejemplo, disponibilidad de semillas, tipo de variedad, accesibilidad al mercado, tipo de fertilizante, y tasa de uso). Esto sugiere oportunidades sustanciales y variadas para mejorar los niveles actuales de eficiencia técnica. En todos los sistemas, la elección correcta de la variedad se identificó como un factor clave común para maximizar el rendimiento por ambiente. Para los sistemas de tierras altas, las opciones óptimas fueron F174 y F2000, mientras que para el arroz de riego se encontró que F473 era el de mayor rendimiento. Además, el análisis numérico sugiere un impacto en el rendimiento de ca. 0.18% por cada 1% de aumento en la tasa de aplicación de nitrógeno para sistemas de tierras altas. Para el arroz de riego, se encontró que las tasas de aplicación de fósforo en lugar de nitrógeno eran más importantes. Como nuestro análisis se basa en datos de producción comercial a escala de finca, se argumentó que una vez que nuestros resultados llegan a un consenso con los agentes de extensión, técnicos y agrónomos locales, las recomendaciones de gestión para cerrar las brechas de rendimiento se pueden utilizar para mejorar la productividad del arroz.

Palabras clave: modelos empíricos, seguridad alimentaria, aumento de la demanda de arroz, función de producción, eficiencia técnica.

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[1] Department of Natural Sciences and Mathematics, Pontificia Universidad Javeriana, Cali (Colombia).
[2] Research Program on Climate Change, Agriculture and Food Security (CCAFS), Consultative Group for International Agricultural Research (CGIAR), Cali (Colombia).
[3] International Center for Tropical Agriculture (CIAT), Cali (Colombia).
[4] School of Earth and Environment, Institute for Climate and Atmospheric Science (ICAS), University of Leeds, Leeds (UK).
[5] School of Economics, Universidad del Valle, Cali (Colombia).
[6] Laboratory of International Agricultural Development Studies, School of Global Agricultural Science, University of Tokyo, Tokyo (Japan).
[*] Corresponding author: david.arango@javerianacali.edu.co

Introduction

The challenges in terms of food security for human societies for the coming decades are enormous. Recent projections of human population growth indicate that global population will reach ca. 9 billion by 2050 (FAO, 2010; UN, 2010). Global food demand will double and at the same time competition for the use of food crops to produce bioenergy will increase (Wheeler and von Braun, 2013; Tilman and Clark, 2014). Such increasing demand for agricultural products will only raise the pressure for natural resources that are vital for agriculture, and this will be compounded by land and water competition of expanding urban centers (Foley *et al*., 2011; West *et al*., 2014). Sustainable improvement of crop productivity by closing yield gaps is, thus, a top priority for agriculture across the developing world (Licker *et al*., 2010; Lipper *et al*., 2014).

In Colombia, rice is a highly important crop for both food security and farmers' incomes, given its high consumption and acceptance rates. It is, therefore, essential for Colombian daily dietary requirements (Khoury *et al*., 2014). Rice is the second annual crop in terms of area harvested with a total area of ca. 685,138 ha (30% area in annual crops) in 2018 in Colombia after maize, but in terms of production value it is the first in the nation (DANE, 2016). Colombia is the second largest rice producer in Latin America and the Caribbean (MADR, 2012). In spite of its importance in Colombia, for many developing countries the average on-farm irrigated rice yields are about 50% below their potential (FAO, 2004; Lobell *et al*., 2009; Licker *et al*., 2010). In humid tropical conditions, modern rice varieties yield between 10-11 t ha^{-1}, but irrigated rice yields are typically in the range 4-6 t ha^{-1} for Colombia (FAO, 2004). As in many other countries, sub-optimal management in conjunction with climate variability is the main cause for yield gaps.

In this research, the technical efficiency (a proxy for yield gaps) and its drivers were estimated for a representative sample of rice farmers from different rice growing regions in Colombia using stochastic frontier models (SFMs). In Colombia, only SFMs have been used to quantify technical efficiencies of coffee production (Perdomo and Mendieta, 2007; Perdomo and Hueth, 2011). A large number of applications of stochastic frontier models exist for rice in Asian countries (Xua and Jeffrey, 1998; Mythili and Shanmugam, 2000; Tian and Wan, 2000; Chang and Wen, 2011). The vast majority of these studies focus on estimating production frontiers through stochastic frontier models, typically for panel and cross-sectional datasets. All these studies include variables that are economically directly related to production efficiency functions, such as harvested area, fertilizer applications, and labor and machinery use. However, recent studies also include household socio-economic conditions (Villano and Fleming, 2004) and/or environmental variables, such as temperature and precipitation (Chang and Wen, 2011).

The objectives of this study were to quantify technical efficiencies in rice production across different production environments in Colombia, identify management factors affecting efficiency, and propose realistic changes in management that allow increasing technical efficiency. A database from a survey of 771 representative rice farms from the three main rice producing regions of Colombia (north, center and east) during the period 2007-2015 was used to fit an SFM for a range of environments. The SFMs were built separately for upland (eastern and northern regions) and irrigated systems (central and northern regions) as both systems have very different management regimes and yields. The SFMs were then used to quantify technical efficiencies and their driving factors, and finally to use a sensitivity analysis to assess the management changes required to increase efficiency levels. The findings were discussed in light of potential strategies to enhance farmer incomes and welfare by helping them to reach optimal efficiency levels.

Materials and methods

Stochastic frontier modeling

The overall framework and key concepts used throughout this work were introduced by the following terms. The ability of a given production unit to maximize its output, given a particular set of inputs, is what we term 'technical efficiency'. The overall assumption in our analytic framework is that those units that have the highest productivity form the 'production frontier' that can be estimated from data. Our approach follows that of Kumbhakar and Lovell (2000).

Let q_i be the amount of rice (kg per harvest) produced by farm i, defined by a production function f (Eq. 1), which is defined by a vector of j variables measured (as fertilize application, man, and machine labor) at the farm (X_i) with their associated coefficients ($\hat{\beta}_i$), and a stochastic component η_i.

$$q_i = f\left(\hat{\beta} X_{i,j}\right) + \eta_i \quad (1)$$

The component η_i is formed by two independent elements (Eq. 2).

$$\eta_i = v_i - u_i \quad (2)$$

In summary,

$$q_i = f\left(x_{1i}, x_{2i}, x_{3i}, .., \hat{\beta}\right) + \eta_i \text{ where } \eta_i = v_i - u_i \quad (3)$$

The first element in Equation 2 v_i is associated with random variations (error) and is symmetrical and independently distributed with mean zero and constant variance $v_i \sim N(0, \sigma_v)$ and can take positive or negative values $v_i \in (-\infty, +\infty)$. The second element, u_i, is the technical inefficiency observed in rice production (q_i) that is asymmetric $u_i > N(0, \sigma_u)$ and greater than zero and independent from v_i.

Given the characteristics of η_i and the need to have unbiased and consistent model parameters, the estimators ($\hat{\beta}$) of the stochastic frontier function must be computed using a maximum likelihood approach (Aigner *et al*., 1977). The natural logarithm of the likelihood function ($Ln\, f$) is defined by Equation 4.

$$Ln(\sigma_s^2, \hat{\beta}) = -\frac{n}{2}Ln(2\pi) - \frac{n}{2}Ln(\sigma_s^2) + \sum_{i=1}^{n} Ln(1-\varphi(z_i)) - \frac{n}{2\sigma_s^2}\sum_{i=1}^{n}[q_i - f(\hat{\beta}X_{i,j})]^2 \quad (4)$$

where n is the total number of observations (i.e. surveyed farms per system), σ^{s}_{2} is the variance of the model (Eq. 5), and $\varphi(z_i)$ is the cumulative normal standard distribution, in which the parameter γ (Eq. 6) represents the efficiency parameters stemming from both error sources in Eq. 3. When the random effect (v_i) dominates (i.e. $\sigma^{u}_{2} \rightarrow 0$ and $\gamma \rightarrow 0$), high efficiency occurs in a group of farmers (i.e., most farmers adequately use their inputs to maximize production). Conversely, when the asymmetric component (u_i) tends towards infinite (i.e. $\sigma^{u}_{2} \rightarrow \infty$ and $\gamma \geq 1$), the technical inefficiency is the main source of variation in the model (i.e., most farmers use inputs in a non-optimal way and are hence far from the production frontier).

$$\sigma_s^2 = \sigma_u^2 + \sigma_v^2 \quad (5)$$

$$z_i = \frac{q_i - f(\hat{\beta}X_{i,j})}{\sigma_s^2}\sqrt{\frac{\gamma}{1-\gamma}} \quad (6)$$

Finally, the technical efficiency level (TE_i) can be estimated for each rice farm as the ratio between the actual production (q_i) and the maximum achievable production (q^*_i) (Eq. 7).

$$TE_i = \frac{q_i}{q_i^*} = \frac{e^{f(\hat{\beta}X_{i,j}) + v_i - u_i}}{e^{f(\hat{\beta}X_{i,j}) + v_i}} = e^{-u_i} \quad (7)$$

TE_i represents the ratio between the attained and the potential production. Potential production is defined as the production when the rice farmer uses all inputs efficiently. TE_i varies in the range 0-1 with high values indicative of high technical efficiency and vice versa. The TE_i values can be used to identify farms and municipalities where efficiency is low and, hence, where interventions may be urgently required to close yield gaps.

Study region

Our study region is Colombia, and specifically all provinces where rice is grown commercially (Fig. 1). The region comprises 11 different provinces distributed across the country and across a range of climatic conditions. Farmers in the Eastern llanos (provinces of Meta and Casanare), the driest region, grow primarily upland rice with relatively low inputs. The central region (provinces of Huila and Tolima) features the largest production quantities. In this region, rice is grown only under irrigated conditions with relatively high inputs and in a variety of elevations and climatic conditions. The northern Caribbean region is formed by the provinces of Cordoba, Sucre, Bolivar, Magdalena, Guajira, Cesar, and Norte de Santander and features both irrigated and upland systems. This region is the largest in extent and likely the most diverse in inputs and farmer socio-economic conditions.

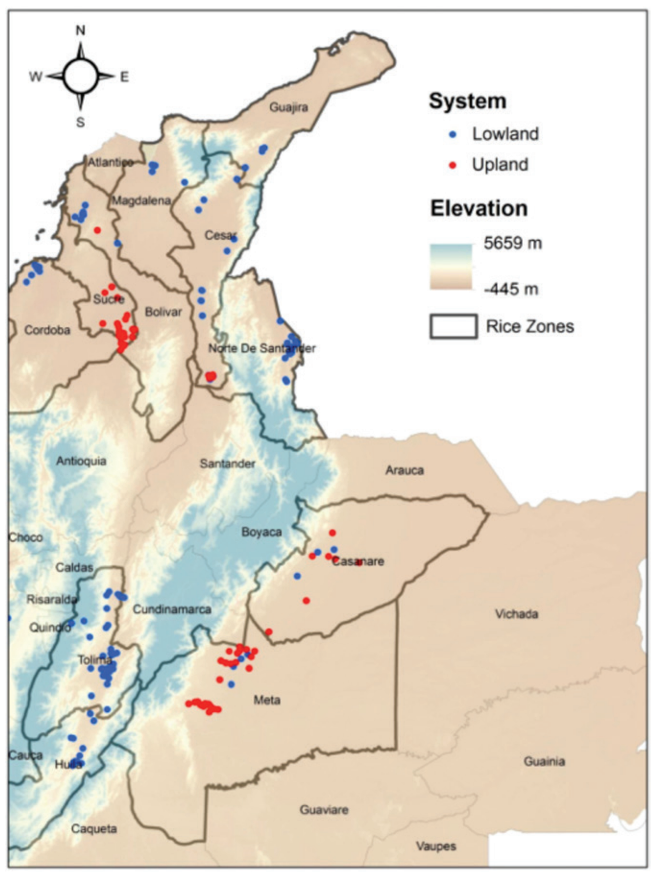

FIGURE 1. Study region and location of rice farms for both irrigated (blue dots) and upland (red dots) systems. Data from Colombia during the period 2007-2015.

Input data

Crop data

Crop management and economic data were gathered from a database of the Colombian National Rice Federation (Fedearroz) through the National Rice Survey (ENA, in Spanish). The survey was carried out in a representative sample of 771 rice farms in the main rice producing areas of Colombia (Fig. 1) for the period 2007-2017, for both irrigated and upland production systems. The survey recorded crop yield, seed quantity, cultivar used, farm size, fertilizer use (quantity and frequency), total use of nitrogen and phosphorous, and man and machine labor hours. As an additional socio-economic factor of potential relevance, we calculated access to farms as the distance from each of the 771 farms to the closest primary, secondary or tertiary road.

Table 1 shows the main characteristics of the two systems. As expected from their contributions to national harvested area and production, there are more irrigated farms (509) than upland farms (262) in the database. There is also a difference between their yield, with irrigated rice yielding ca. 30% more than upland rice (about 1.3 t ha^{-1} more). We note major management differences, with irrigated systems using more fertilizer and more man hours. Due to these differences, we analyzed both systems separately.

TABLE 1. Rice cultivation farm management practices as derived from the National Rice Survey database. Values shown are mean values for each crop system across all observations. Data from Colombia during the period 2007-2015.

Variable / System	Irrigated	Upland	All
Total number of surveyed farms	509	262	771
Yield (kg ha^{-1})	5,992	4,623	5,527
Quantity of seed used (kg ha^{-1})	201.1	203.1	201.8
Average farm size (ha)	54.3	69.1	59.3
Rate of fertilizer per crop cycle (kg ha^{-1})	619.1	401.6	544.9
Total number of fertilizer applications per crop cycle	5.9	3.4	5.1
Rate of total nitrogen use (kg ha^{-1})	154.3	80.9	129.3
Rate of total phosphorous use (kg ha^{-1})	29.9	25.9	28.6
Man labor hours per crop cycle (h)	102.5	77.5	92.5
Machine labor hours per crop cycle (h)	48.8	77.7	60.5

Weather data

We gathered daily weather data for the crop cycle (from sowing to harvest) from the weather station network of the Colombian Institute for Hydrology, Meteorology and Environmental Studies (IDEAM). Total precipitation and minimum and maximum temperature data were available for all weather stations. A quality control process was performed on each of the weather stations using the RClimTool software package (Llanos and Arango, 2015) following Esquivel *et al.* (2018) by detecting and correcting outliers and data gaps. Finally, we assigned the weather information to each farm using geostatistical (autocorrelation) analysis with the geoR library in the R statistical package (R Core Team, 2017). For each farm, we then computed the following variables: total precipitation per crop cycle (P), number of rainy days (i.e. with precipitation above 1 mm, R_D), average minimum temperature for the crop cycle (T_{min}), and average maximum temperature for the crop cycle (T_{max}).

Soil data

Soil data were gathered from the World Soil Information database (ISRIC, 2014). This database consists of globally interpolated chemical and physical soil properties at a resolution of 30 arc-sec (about 1 km around the equator). Using the package 'raster' of the R software version 3.1, we extracted the organic carbon content and the water holding capacity for each of the rice farms.

Environmental classification

As a preliminary step before fitting the SFMs for each crop system (upland, irrigated), climate and soil data were used to derive homogeneous edapho-climatic groups through a cluster analysis using the FactoClass Method (Pardo and Del Campo, 2007). We used a distance index to determine the optimal number of groups. We grouped farms into environments because, regardless of management or socio-economic conditions, the production frontier and optimal achievable production can shift depending upon the environmental conditions prevalent on the farm (Van Wart *et al.*, 2013; Heinemann *et al.*, 2015). The implication is that the biophysical limit of crop yield varies depending on the prevailing climate (Lobell *et al.*, 2009). We thus deemed it necessary to assess these environmental effects through an environmental group classification.

Quantification of technical efficiencies

Once the farms in each production system had been grouped into different environments, we used the 'frontier' package in the R software version 3.1 (Coelli and Henningsen, 2013) to fit the SFMs for each production system. We transformed all continuous variables as well as crop yields into their log forms and used all crop varieties and environmental groups as binary variables in the models. Technical efficiencies for each farm in each crop system were then derived from the models. Finally, we used the model coefficients and the results of an F-test to determine those variables with strong and statistically significant ($P \leq 0.01$, ≤ 0.05, and 0.1) effects on yield.

Assessing the potential for reducing yield gaps

To determine the needed improvements in crop management that would lead to increased technical efficiencies and, hence, to closing yield gaps, we conducted a sensitivity analysis using the production functions derived for each system. For each system, we performed SFM model runs by modifying the most relevant inputs (as identified by the F-test, above), both separately and in combination, making sure input changes did not fall considerably outside their observed ranges. Specifically, for those inputs having a significant positive impact on yield, we increased their observed values by 5%, 10% and 15%, and for those having a negative impact, we decreased their observed values by 5%, 10% and 15%. All sensitivity runs were performed for each of the rice varieties that had a strong effect on yield and for each environment, so that our results would provide sensible environmental-specific management recommendations for improved rice farming.

Results and discussion

Environmental groups

Clustering results indicate that for upland rice, there are four groups of environments, with largely varying edapho-climatic characteristics per environment (Tab. 2). We note large differences in total precipitation and in the number of rainy days. Environmental group (EG) 2 received the most precipitation with a relatively poor distribution (53 rainy days), suggesting the occurrence of extremes. This environment also showed the largest differences between maximum and minimum temperatures and soils with high organic carbon contents. Conversely, EG 3 showed the lowest precipitation (1,012 mm per crop cycle), with a more even distribution as compared to EG 2. EG 1 and 4 showed the best temporal distribution of precipitation that may result in yield advantages, although their organic carbon contents were about half those of EG 2.

For irrigated systems, only three EGs were found (Tab. 2). As with the upland systems we found a large variation in the total seasonal precipitation across irrigated EGs. EG 1 had the largest rainfall amounts (904 mm) followed by EG 2 (710 mm), whereas EG 3 showed the lowest seasonal precipitation (450 mm). Large variations were also found for organic carbon, with EG 2 showing the highest content. We noted only small differences between average seasonal maximum and minimum temperatures, suggesting that temperature might not be a limiting factor for irrigated rice yields. The lower (2-3°C below) maximum temperatures in upland systems compared to irrigated systems highlights the importance of irrigation in allowing evaporative cooling in warm conditions (Peng *et al*., 2004; Julia and Dingkuhn, 2013).

TABLE 2. Environmental groups and their average climatic and soil characteristics for both irrigated and upland rice production. Data from Colombia during the period 2007-2015.

System	EG[1]	P (mm)[1]	R_D (d)[1]	T_{min} (°C)[1]	T_{max} (°C)[1]	ASW (%)[1]	OC (%)[1]
Upland	1	1,425	68	18	35	12	13
	2	2,097	53	12	34	12	22
	3	1,012	41	10	35	12	20
	4	1,507	69	20	35	10	11
Irrigated	1	904	52	17	36	12	13
	2	710	47	17	36	11	29
	3	450	31	16	37	10	10

[1]EG: environmental group, P: total precipitation per crop cycle, R_D: number of rainy days (i.e. with precipitation above 1 mm), T_{min}: average minimum temperature for the crop cycle, T_{max}: average maximum temperature for the crop cycle, ASW: available soil water, OC: soil organic carbon content.

Production frontiers and technical efficiencies

Upland rice

For upland rice, a statistically significant variable was the nitrogen application rate, with a yield impact of ca. 0.18% for each 1% increase in this rate (Tab. 3). This result is expected (Nagai and Makino, 2009; Mueller *et al*., 2012), and is also consistent with previously reported nitrogen yield impacts of 0.08% in a study for Taiwan (Chang and Wen, 2011). The amount of seed was also found to have a significant impact on yield, with a regression coefficient of 0.08 ± 0.04. This result can be explained by the fact that Colombian rice systems are directly seeded and large amounts of seed are often required to ensure appropriate germination rates and sufficiently high yields.

Contrary to what would be expected, we found a negative association (regression coefficient of -0.1 ± 0.04) between man labor hours and yield. While this is somewhat counterintuitive, further analysis of the database indicated that most man labor hours are spent on harvesting activities that would reduce the overall harvesting time but would not increase productivity. It is thus important that increases in man labor time are targeted towards activities that have a greater impact on yield such as fertilizer applications, sowing, and pests, diseases, and weed control.

The survey included seven different varieties, with Fedearroz 733 (F733) being the most common in irrigated systems and overall in the country and F473 being the most used in upland systems, but also to some extent in irrigated systems (Fig. 2). Other varieties such as F2000, F174 and F60 are less often used, although some of them (particularly F2000) have been commercially released only recently. We find that most terms associated with varietal choices were also negatively associated with yields. Our models indicate that F2000 and F473 have no significant difference from the base category (F174), although F473 tends to yield less and F2000 tends to yield slightly more than F174 (Fig. 3B). The other varieties (F733, Fortaleza and IA550) all show statistically significant and negative effects on yields (-0.23, -0.27, and -0.18, respectively, all at $P \leq 0.01$). Since coefficients associated with these varieties were the largest in magnitude across all variables, we conclude that varietal choice is the most important management factor in upland rice systems, with optimal choices being F174 and F2000.

The rest of the variables showed no significant impact, but this does not necessarily mean that they do not have impact under specific conditions or farms. For instance, pesticide doses are not important if the variety used is pest-resistant, but it would be important if the selected variety were sensitive to pest attacks. High noise in the recorded data or low variability across farms could also have precluded the identification of a relevant variable for the yield response. However, for this farm population there are no statistically significant responses for these variables.

Finally, we estimated technical efficiencies for each farmer and found an average technical efficiency level of 77% across all upland rice farms. Despite this relatively high value, we observed some farmers with relatively low

TABLE 3. SFA Coefficient estimates for upland rice systems. Data from Colombia during the period 2007-2015.

Variable	Coefficient	Standard Error	Z Statistic	P Value
(Intercept)	7.39	1.89	3.91	0.00 ***
log(nitrogen)	0.18	0.10	1.88	0.06 *
log(phosphorous)	-0.03	0.04	-0.73	0.46
log(quantity of seed)	0.08	0.04	1.82	0.07 *
log(crop area)	0.01	0.02	0.46	0.64
log(fertilizer use rate)	0.04	0.07	0.60	0.55
log(fertilizer application)	-0.12	0.08	-1.49	0.14
log(pesticide use rate)	-0.06	0.05	-1.13	0.26
log(pesticide application)	-0.01	0.07	-0.15	0.88
log(access)	0.08	0.16	0.51	0.61
log(man hours of labour)	-0.10	0.04	-2.57	0.01 **
log(machine hours of labour)	0.00	0.03	-0.02	0.99
Variety Fedearroz _ 2000	0.01	0.08	0.08	0.94
Variety Fedearroz _ 473	-0.16	0.17	-0.97	0.33
Variety Fedearroz _ 733	-0.23	0.07	-3.33	0.00 ***
Variety Fortaleza	-0.27	0.05	-5.34	0.00 ***
Variety Improarroz _ 1550	-0.18	0.06	-2.81	0.00 ***
Environment 2	-0.10	0.17	-0.58	0.56
Environment 3	-0.13	0.09	-1.49	0.14
Environment 4	0.06	0.07	0.79	0.43
	0.13	0.03	5.25	0.00 ***
	0.95	0.04	21.93	0.00 ***

*** Significant at 1% ** Significant at 5% * Significant at 10%

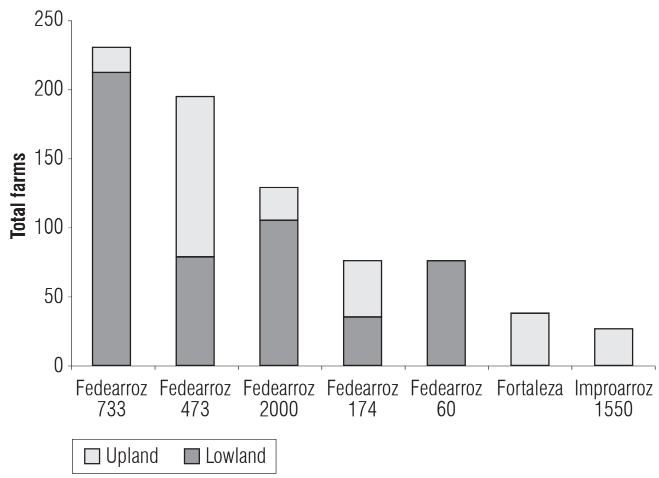

FIGURE 2. Frequency of use of different rice varieties for both rice production systems, as derived from the database used in this study. Data from Colombia during the period 2007-2015.

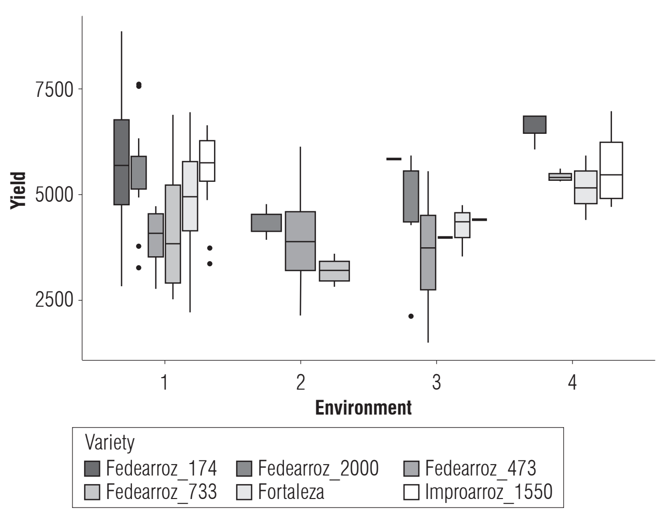

FIGURE 3. Effects of management variables and environmental groups on rice yields for upland rice systems. (A) Model coefficients and (B) yield of all rice varieties for each environment. Horizontal lines show the median, boxes show the interquartile range, whiskers extend to 5-95% of the data, and dots are outliers. Data from Colombia during the period 2007-2015.

efficiencies (below 60%), particularly in the municipalities of Villavicencio, Cumaral, Puerto Lopez and Fuente de Oro, all of which are located in eastern Colombia (Fig. 4).

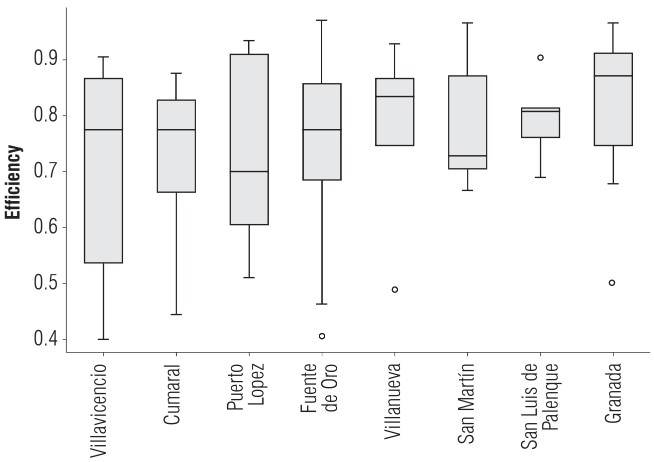

FIGURE 4. Technical efficiencies of upland rice farmers in all municipalities where upland rice is grown. Horizontal lines show the median, boxes show the interquartile range, whiskers extend to 5-95% of the data, and dots are outliers. Data from Colombia during the period 2007-2015.

Irrigated rice

Relevant variables in irrigated systems are different to those in upland systems. This is confirmed by developing system-specific models (Tab. 4). The phosphorous application rate was amongst the most important variables for irrigated rice (coefficient of 0.04). Its positive effect on yield as well as the fact that it had a smaller effect than the nitrogen application rate is in broad agreement with theory (Longstreth and Nobel, 1980; Fujisaka *et al*., 1994). The number of fertilizer applications was also found to be statistically significant, suggesting that a larger number of better temporally distributed yet lower-dosage applications ensures such applications are more efficiently used by the crop in contrast to a reduced number of higher-rate applications.

Access to farms, calculated as the distance from each farm to primary, secondary and tertiary roads was found to affect yields significantly and negatively (coefficient of -0.12). This negative effect indicates that farms that are further away from roads have lower yields. Large distances from farm to roads can limit the timely access of fertilizers and machinery. This could negatively impact farm activity schedules and cause fertilizer, pesticide, or herbicide applications, or machinery-related activities to happen at non-optimal times, thus reducing yields.

Finally, both environments and varieties had a significant impact on yield. In particular, we found that both EG2 and EG3 had a yield advantage as compared to EG1 and that

TABLE 4. Stochastic Frontier Analysis coefficients estimate for irrigated rice system. Data from Colombia during the period 2007-2015.

Variable	Coefficients	Standard Error	Z Statistics	P Value
(Intercept)	9.14	0.55	16.66	0.00 ***
log(nitrogen)	0.05	0.04	1.18	0.24
log(phosphorous)	0.04	0.01	3.18	0.00 ***
log(quantity of seeds)	0.03	0.04	0.63	0.53
log(crop area)	-0.01	0.01	-1.33	0.18
log(fertilizer use rate)	0.07	0.06	1.18	0.24
log(fertilizer aplication)	0.07	0.04	1.66	0.09 *
log(pesticide use rate)	-0.02	0.03	-0.60	0.55
log(pestiside aplication)	-0.04	0.03	-1.23	0.22
log(access)	-0.12	0.03	-3.56	0.00 ***
log(man hours of labour)	0.02	0.04	0.41	0.68
log(machine hours of labour)	0.00	0.02	0.11	0.91
Variety Fedearroz2000	-0.06	0.05	-1.13	0.26
Variety Fedearroz2000473	0.10	0.06	1.63	0.10 *
Variety Fedearroz200060	-0.02	0.06	-0.31	0.76
Variety Fedearroz2000733	-0.06	0.05	-1.09	0.28
Environment 2	0.08	0.04	2.23	0.03 **
Environment 3	0.15	0.03	4.57	0.00 ***
	0.11	0.01	8.23	0.00 ***
	0.97	0.02	50.14	0.00 ***

*** Significant at 1% ** Significant at 5% * Significant at 10%

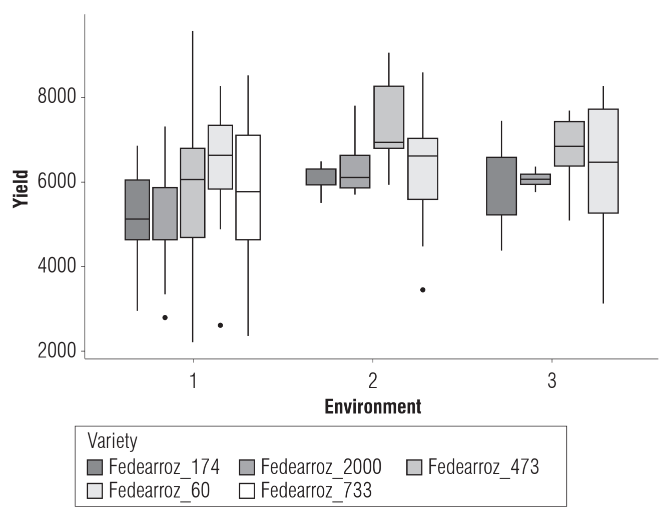

FIGURE 5. Effects of management variables and environmental groups on rice yields for irrigated rice systems. (A) Model coefficients; and (B) yield of all rice varieties for each environment. Horizontal lines show the median, boxes show the interquartile range, whiskers extend to 5-95% of the data, and dots are outliers. Data from Colombia during the period 2007-2015.

the variety F473 had a significantly positive impact (coefficient of 0.1) compared to F174. The other varieties had negative albeit not statistically significant yield impacts (Fig. 5). Our analysis suggests that adequately selecting varieties per environment is critical to increasing yields in irrigated systems (Fig. 5B). The analyses generally highlight the importance of including site-specific variations in yield response models so as to allow the identification of both constraints and opportunities at the scales that are relevant for farmers and extension agents (Jiménez *et al*., 2009, 2011; Delerce *et al*., 2016).

Technical efficiencies for irrigated rice are shown in Figure 6. Notably, the average technical efficiency was 78%, which is very similar to the average efficiency of the upland rice system. However, there are large variations across farmers and environments. Municipalities such as Lorica, La Jagua and Cucuta show low efficiency levels and hence large yield gaps (>40%) for some farmers, whereas the municipalities of Espinal, San Martin, Valledupar and Purificacion show consistently high efficiency levels across farmers. This is consistent with evidence that farmers in northern Colombia are less technologically developed (DANE, 2004).

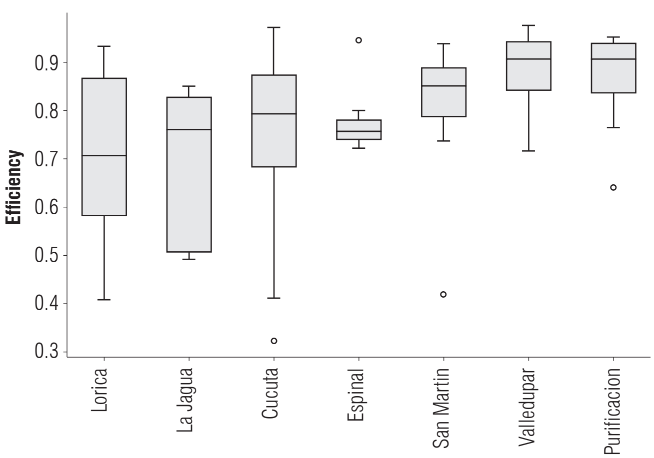

FIGURE 6. Technical efficiencies of irrigated rice farmers in all municipalities where irrigated rice is cultivated. Horizontal lines show the median, boxes show the interquartile range, whiskers extend to 5-95% of the data, and dots are outliers. Data from Colombia during the period 2007-2015.

Conclusions

The stochastic production frontier methodology is a useful alternative to more classical yield gap assessments based on detailed process-based modeling (Bhatia *et al*., 2008; van Bussel *et al*., 2015), and has two clear advantages. First, the method integrates microeconomic theory and empirical analysis, thus allowing the empirical validation of both biophysical and economic hypotheses regarding the cropping systems under study. And secondly, since the method is based on commercial farming information, it has the potential to provide recommendations that are tailored to the farms and farm systems analyzed. The use of georeferenced information at the farm level from a geographically widespread sample of rice farms allowed us to integrate soil and climate data. This not only helped to reduce noise in the responses to environment factors but it also enhanced the capabilities of the methodology beyond only identifying management factors, identifying management x environment interactions, and allowing a more comprehensive assessment of the rice crop systems.

For the upland rice system, our analysis suggests that increasing nitrogen fertilization and seed quantity in upland systems is needed for yield gap closure. Conversely, the amount of man labor hours was found to have a negative effect on productivity, which we attribute to non-optimal use of an excessively large number of man labor hours on harvesting activities. For irrigated systems, we identified the phosphorous application rate as well as the number of fertilizer applications as having a significant and positive effect on yield. For irrigated rice, we also report that accessibility is a limiting factor for a number of farms.

For both rice production systems, we identify two critical aspects that need to be well-managed so as to ensure high levels of production. Most critical is the correct matching of varieties to the prevailing climates of the farms. Our analysis indicates significantly different effects from varieties for the different environments for both production systems. For municipalities with low average efficiency levels, we suggest that improved technical assistance through extension services will be needed to ensure optimal management. For upland systems, we identified Villavicencio, Cumaral, Puerto Lopez and Fuente de Oro, whereas for irrigated systems it would be Lorica, La Jagua and Cucuta. Such technical assistance should be targeted at providing site-specific recommendations and could be based on both our results and the experience of the local extension agents and farmers. Finally, we note that, as this study is based on commercial farming practices and yields across all rice growing areas of Colombia, these recommendations have the potential to directly benefit rice farmers.

Acknowledgments

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Characterization of eggplant producers in the Caribbean region of Colombia: socio-economic aspects and local production technology

Caracterización de los productores de berenjena en la región Caribe de Colombia: aspectos socioeconómicos y tecnología local de producción

Ender Manuel Correa-Álvarez[1], Antonio María Martínez-Reina[2]*, Alfonso Rafael Orozco-Guerrero[1], Gabriel Ernesto Silva-Acosta[1], Lilibet Tordecilla-Zumaqué[2], María del Valle Rodríguez-Pinto[2], Liliana María Grandett-Martínez[2], Carina Cecilia Cordero-Cordero[3], and Jorge Luis Romero-Ferrer[2]

ABSTRACT

Eggplant represents one of the most widely accepted vegetables in the Colombian Caribbean region due to its cultural and socio-economic importance. In this region, 585 ha were cultivated with a production of 4,298 t, in 2018, grouping 900 growers and representing 93% of the national production. However, despite its representativeness, no characterization studies of the species are evidenced. Therefore, the aim of this study was to reveal the socioeconomic, productive, and technological aspects linked to eggplant cultivation in productive areas from the Colombian Caribbean region. In that order, structured surveys were designed and applied. The information was analyzed through multivariate statistical methods, such as Gower's distance and Ward's hierarchical agglomerative clustering method. In general, the results revealed a low-medium technological level associated with eggplant production in this region of Colombia. Of the five groups identified, the highest level of schooling was found in groups G_{IV} and G_V, which also obtained the highest yields with 35 t ha^{-1} and 32 t ha^{-1}, respectively. Sowings predominate in the month of April; this activity is related to the occurrence of precipitation in this period and the general lack of irrigation technologies by growers.

Key words: legumes, agriculture, productivity, food, consumption and returns.

RESUMEN

La berenjena representa una de las hortalizas de mayor aceptación en la región Caribe colombiana dada su importancia cultural y socioeconómica. En esta región se cultivaron 585 ha con una producción de 4.298 t en el año 2018, agrupando 900 cultivadores y representando el 93% de la producción nacional. Sin embargo, a pesar de su representatividad, no se evidencian estudios de caracterización de la especie. Por tanto, el objetivo de esta investigación fue revelar los aspectos socioeconómicos, productivos y tecnológicos asociados al cultivo de berenjena en zonas productoras de la región Caribe colombiana. Para ello, se diseñaron y aplicaron encuestas estructuradas. La información se analizó mediante métodos multivariados, utilizando la distancia de Gower y el agrupamiento jerárquico de Ward. Los resultados revelan, en general, un nivel tecnológico bajo-medio en la producción de berenjena en esta región de Colombia. De los cinco grupos identificados, el mayor grado de escolaridad lo presentaron los grupos G_{IV} y G_V, que a su vez alcanzaron los mayores rendimientos con 35 t ha^{-1} y 32 t ha^{-1}, respectivamente. Las siembras predominan en el mes de abril, aspecto ligado con la ocurrencia de precipitaciones en este periodo y a la carencia general de tecnologías de riego por parte de los agricultores.

Palabras clave: legumbres, agricultura, productividad, alimentación, consumo y retornos.

Introduction

The eggplant (*Solanum melongena* L.) is native to India and was introduced to Colombia by the Spaniards around the 1930s. Nowadays it has high commercial acceptance in the Colombian Caribbean region to the point that it is widely utilized in the regional gastronomy and is prioritized in the productive bets with an export view mainly for the provinces of Cordoba and Sucre (Aramendiz *et al.,* 2008; Tapia *et al.,* 2015). According to official statistics, the Caribbean region of Colombia for the year 2018 obtained a production of around 4.298 t that represents 93% of the national production (Agronet, 2019). Likewise, the imports of eggplant by the United States, Canada, and the Caribbean islands account for approximately 95,000 t, representing a market value close to 105 million dollars/year (FAOSTAT, 2019).

The strategic location of the Colombian tropics whose environmental offer allows the production of eggplant throughout the year, plus the proximity to the main ports

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[1] Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Centro de Investigación Caribia, Sevilla - Guacamayal (Colombia).
[2] Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Centro de Investigación Turipaná, Cerete, Cordoba (Colombia).
[3] Corporación Colombiana de Investigación Agropecuaria (Agrosavia), Centro de Investigación Motilonia, Agustín Codazzi, Cesar (Colombia).
* Corresponding author: amartinezr@agrosavia.co

of the country, generate favorable conditions to obtain competitive and commercial export advantages. These benefits can be exploited by those producers who have or exceed technological aspects to satisfy specialized international and national markets in terms of quality, quantity, continuity, and innocuousness in production. In this sense, typification or characterization studies of agricultural production systems are of great interest because they allow identifying groups of producers within the heterogeneity of socioeconomic and productive conditions. This way, government entities, unions and decision-makers can direct public policies and investment as well as research and technology transfer programs that are tailored to the circumstances, limitations and possibilities of the groups of producers identified (Escobar and Berdegué, 1990; Correa *et al*., 2010; Santos *et al.,* 2014).

The multivariate statistical analysis techniques constitute ideal tools for the characterization and classification of agricultural production systems (Arias and Gálvez, 2010; Correa *et al.,* 2010; Carrillo *et al.,* 2011; Cleves and Jarma, 2014). Among the existing multivariate methods, Gower's coefficient of similarity allows the simultaneous manipulation of quantitative and qualitative variables in a database. With its application, it is possible to find the similarity between individuals to whom a series of common characteristics have been measured. Once the similarity between elements is obtained, the coefficients can be transformed into distances. Subsequently, a grouping of individuals can be carried out in such a way that each group is composed of homogeneous units, and the groups among these will be very heterogeneous (Gower, 1971; Franco and Hidalgo, 2003; Chauza and Villa, 2011).

Despite the importance of the cultivation of eggplant, and especially the Caribbean region that contributes 74% of the national production, there is no history of studies defining the typology of farmers. These studies are necessary at the time of designing technologies in such a way that they respond to the needs of the producer, facilitating their adoption.

The typification of producers in a production system allows us to appreciate the technological level and some socio-cultural variables such as the age of the farmer, the experience in cultivation that is considered important to design models of rural extension and improve production and market conditions.

This research hypothesized that eggplant production in the Caribbean region is carried out with different technological levels, which must be defined to formulate technological recommendations that respond to the needs of different producer groups. In that order of ideas, the ignorance of the elements linked to the eggplant production in the Colombian Caribbean region is a limitation to improve the competitiveness and productivity of this crop in this region.

Accordingly, the aim of the research was to identify the production technologies, agronomic practices and socioeconomic characteristics of eggplant producers in the Colombian Caribbean region, to generate a baseline of the production system as an input to formulate projects on the research agenda of this area. Moreover, this will also be used to measure the impacts of future technologies according to estimations of the social balance of different institutions of the agricultural sector.

Materials and methods

The research was conducted in the second semester of 2018 in eggplant producing areas of the Colombian Caribbean region. For this, aspects related to the socio-economic, technological, and productive characteristics of eggplant producers were studied through the application of surveys with structured questions (Tab. 1). The sample size was established by a simple random sampling design without replacement, considering maximum variance according to Equation 1:

$$n = \frac{N \cdot Z^2_{\propto/2} \cdot p \cdot (1-p)}{(N-1) \cdot \varepsilon^2 + Z^2_{\propto/2} \cdot p \cdot (1-p)} \quad (1)$$

where:

N = population size

$Z^2_{\propto/2}$ = value of the standard normal distribution for a confidence level of 90% (1.645)

p = value of the *a priori* proportion of maximum variance of a proportion variable (0.5)

ε = maximum allowable error of the 12% estimate (0.12)

n = sample size

For this study, the population size was expressed as the number of eggplant producers in the Caribbean region, which was determined by the quotient obtained from the most recent official report of the eggplant harvested area in the region (364 ha for the year 2017) with the eggplant modal planting area (≈ 0.62 ha). The Corporacion Colombiana de Investigacion (Agrosavia) estimated the latter through research (Cadena *et al*., 2011a). Likewise, the values considered for $Z^2_{\propto/2}$, p and ε were 90% (1.645), 0.5 and 12% (0.12), respectively, obtaining a sample size of 46 producers.

One of the advantages of using Ward's method in this research is that it is the only hierarchical grouping method that bases its functionality on a regular sum of squares criterion, which allows obtaining defined groups and minimize the dispersion of each element in each group produced (Murtagh and Legendre, 2014). The measurement of distances through specialized software generates dendrograms that show the similarity/dissimilarity between defined groups.

The qualitative variables were: gender, educational level, state of access roads to the property, land tenure, years of experience as a producer, type of labor, age of family labor, access to credit, origin of income, technical assistance service, production destination, topography, and water availability for irrigation. The quantitative variables were the size of the productive unit, cultivated area, yields, total production, and quantities sold.

The socioeconomic and technological aspects of the production of eggplant (*Solanum melongena* L.) in the Colombian Caribbean region are presented in Table 1, which are the product of fieldwork seasons through surveys applied randomly to farmers in the regions. The socioeconomic

TABLE 1. Socioeconomic and technological aspects inquired to eggplant (*Solanum melongena* L.) farmers in a survey carried out in producing areas of the Colombian Caribbean region.

Variable			
Name			Type
1. Socioeconomic aspects			
1.1. Characteristics of the producer		Gender	Q-ds
		Age (years)	D-q
		Education level	Q-ml
		Cultivation experience (years)	D-q
1. 2. Social environment	**Location**	Province	Q-mnl
		Municipality	Q-mnl
	Public services	Water	Q-ds
		Electricity	Q-ds
		Cell phone	Q-ds
		Household gas	Q-ds
		Propane gas	Q-ds
		Sewerage	Q-ds
		Landline phone	Q-ds
		Internet	Q-ds
		None	Q-ds
	Access roads	Type of road	Q-mnl
		State of the road	Q-ml
1. 3. Productive environment	**Productive unit**	Land tenure	Q-mnl
		Topography of the property	Q-ml
		Planting area (ha)	C-q
		Yield (t ha^{-1})	C-q
		Workforce	Q-ds
		Credit	Q-mnl
	Destination of production	Sale (%)	C-q
		Family consumption (%)	C-q
		Animal consumption (%)	C-q
	Commercialization	Sale site	Q-mnl
		Commercialization unit	Q-mnl

Variable		
Name		Type
2.Technological aspects		
2.1. Soils	Type of preparation	Q-mnl
	Soil analysis	Q-mnl
2.2. Seed	Quality	Q-ds
	Type	Q-mnl
2.3. Sowing	Sowing date	Q-ds
	Planting distance	C-q
	Planting system	Q-ds
	Cultivation cycle (d)	D-q
2.4. Agricultural work	Crop rotation	Q-ds
	Reseeding	Q-ds
	Thinning	Q-ds
	Earthing up	Q-ds
	Pruning	Q-ds
	Stalking	Q-ds
	Removal of crop residues	Q-ds
2.5. Irrigation	Irrigation system	Q-mnl
	Water source	Q-mnl
	Water analysis	Q-ds
2.6. Integrated crop management	Weed control	Q-ds
	Fertilization	Q-ds
	Pest control	Q-ds
	Disease control	Q-ds
2.7. Infrastructure for GAP	Agrochemical preparation area	Q-ds
	Agrochemical storage warehouse	Q-ds
	Showers and baths	Q-ds
	Container storage house	Q-ds
	Crop reception and conditioning area	Q-ds
	Any	Q-ds
2.8. Postharvest work	Classification	Q-ds
	Washing	Q-ds
	Drying	Q-ds
	Packing	Q-ds
	Storage	Q-ds
	Any	Q-ds

Q-ds qualitative double state; Q-mnl: qualitative multistate, non-logical; Q-ml: qualitative multistate, logical; C-q: continuous quantitative; D-q: discontinuous quantitative; GAP: good agricultural practices.

information refers to the age of the producer, experience in cultivation, gender, services, and size of the productive unit. The technological information highlights crop management yields and cultural practices.

For the data analysis, the variables that did not show any variation were discarded. The grouping of producers was carried out through multivariate analysis using Gower's distance (Eq. 2) and Ward's hierarchical agglomerative clustering method, and employing the statistical program InfoStat version 2017 (Di Rienzo *et al.*, 2017).

$$d_{ij}^2 = 1 - S_{ij} \quad (2)$$

where,

$$S_{ij} = \frac{\sum_{h=1}^{p1}\left(1-\frac{|X_{ih}-X_{jh}|}{Rh}\right)+a+\propto}{p_1+(p_2-d)+p_3} \quad (3)$$

S_{ij}: Gower's similarity coefficient
p_1: number of continuous quantitative variables
p_2: number of binary variables
p_3: number of qualitative variables (non-binary)
a: number of matches (1, 1) in the binary variables
d: number of matches (0, 0) in the binary variables
α: number of matches in the qualitative (non-binary) variables
Rh: the range (or path) of the h^{th} quantitative variable

Results

The multivariate analysis of mixed data allowed the formation of five conglomerates (G_I, G_{II}, G_{III}, G_{IV}, and G_V) of farmers, which evidences the existence of divergence in eggplant producers of the Caribbean region (Fig. 1). Groups G_{II} and G_V are comprised of farmers from the province of Cordoba; however, G_{II} is integrated by producers from a single municipality (La Apartada, Cordoba). Furthermore, groups G_{III} and G_{IV} are the most diverse in terms of the place of origin of the farmer, which includes six and seven municipalities, respectively (Tab. 2), showing signs of heterogeneous production processes at the eggplant production area level in the Caribbean region of Colombia.

The G_I group represents 13% of the sample, and its members are characterized by being located in the rural area of the municipalities of La Apartada and Monteria in the province of Cordoba, and Sitionuevo of the province of Magdalena. In this group, about 67% of its members are women with an average age of 55 years, with a low schooling level (no study = 50%, primary = 33.3%), and on average, 18 years of farming experience. The productive units have a flat topography, roads in poor condition, almost no access to public services such as electricity, drinking water, household gas, landline phone, and Internet, and the agricultural land shows varied forms of tenure (own 33.3%, rented 33.3%, on loan 33.3%). The planting areas are on average 1,000 m^2 with average yields of 12.7 t ha^{-1}; production costs are mainly undertaken using their own resources (66.7%) and using family labor. The production is destined for sale (59.2%) and family consumption (40.8%) in a very similar proportion; likewise, the harvested fruits are sold mainly in the production plot (83.3%), either in 50 kg packages or as retail by kilograms (Tab. 2).

In terms of local production technology, farmers that carry out soil preparation manually and do not perform soil analysis characterize group I. They usually use seeds from regional cultivars obtained from their own crops; they plant once per year in April and in monoculture using planting densities of 1.1 m x 1.2 m between plants and rows, respectively, corresponding to 7,572 plants ha^{-1}. These farmers fertilize with chemical sources and achieve productive cycles of 298 d on average (≈ 10 months); a moderate number of its members carry out agricultural work intensively, highlighting reseeding, earthing up, stalking, and removal of crop residues. Likewise, these farmers have not implemented commercial irrigation systems, supplying the water needs of the plants with artisanal manual irrigation and obtaining water from nearby rivers (Magdalena, Sinu, and San Jorge). The infrastructure for the development of good agricultural practices (GAP) is null. However, a significant number of its members carry out the most critical postharvest tasks such as classification, washing, drying, packing, and storage (Tab. 3).

The G_{II} group corresponds to producers settled in the rural area of the municipality of La Apartada (Cordoba). Most of producers are men (66.7%) with an average age of 59 years, a low schooling level (no study = 66.7%, primary = 33.3%) and extensive experience in the cultivation of eggplant (35 years). The productive units have mainly undulated topography, the access road is in good condition, there is good coverage of residential public services (66.6% of water, 100% of electricity and 100% of household gas) and the agricultural land is mostly rented. The average planting area is 3,000 m^2 with average yields of 11.7 t ha^{-1}; production costs are financed by loans from family members and by combining family labor with private wages. The production is destined mainly for sale (90%) and is commercialized by kilograms (66.7%) in the production plot of the farmer (Tab. 2).

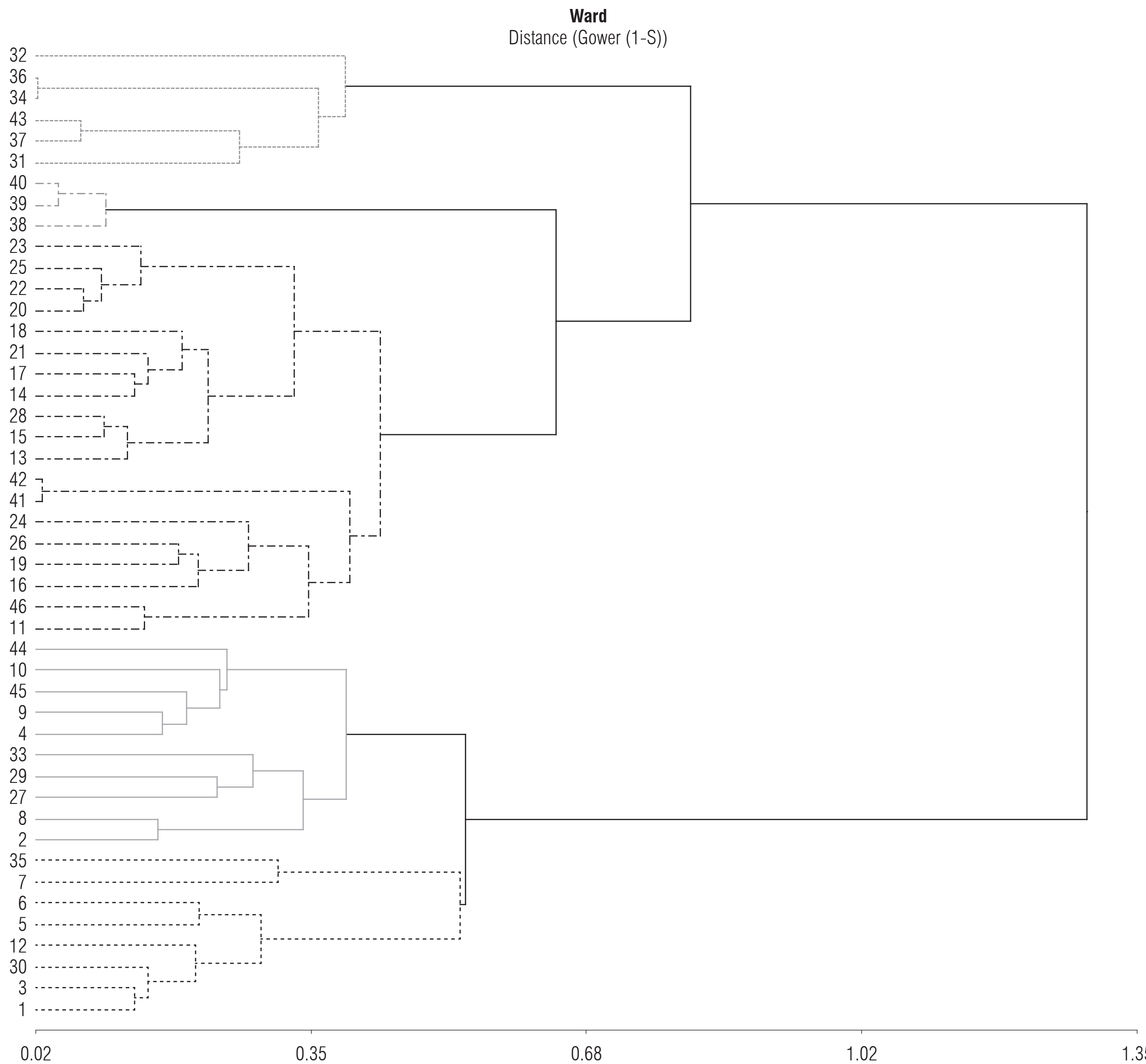

FIGURE 1. The conglomerates of eggplant farmers established using multivariate analyses such as Gower's distance and Ward's hierarchical agglomerative clustering method.

The technological aspects involve land preparation with agricultural equipment and soil analysis, plantings from November to December in monoculture (87%), with planting densities of 0.8 m x 1.5 m between plants and rows, respectively (8,325 plants ha^{-1}), no irrigation is applied, and they use the San Jorge River as a source of water. Further, they use chemical and organic fertilization sources and reach productive cycles of 230 d (≈ 7.7 months), with little implementation of traditional agricultural work. The infrastructure for the development of GAP is null, and the only postharvest work that is carried out is the classification of fruits (Tab. 3).

The G_{III} group covers the largest number of producers, i.e., 41.3% of the sample. Its members are mainly from the province of Sucre (89.5%); most of them are men (94.7%) with an average age of 52 years, a low level of education, and around 19 years of experience in the cultivation of eggplant. The productive units have flat topography, there is poor access to roads, with a coverage of 78.9% and 52.6% of water and electricity services, respectively, and various land tenure types (42.1% belongs to the family, 31.6% is rented, and farmers own the land 26.3%). The average crop area is 6,000 m^2 with average yields around 22 t ha^{-1}; the production costs are assumed mainly with their own

TABLE 2. Socioeconomic aspects of eggplant (*Solanum melongena* L.) farmer groups in production areas of the Colombian Caribbean.

		Stat.	G_I	G_{II}	G_{III}	G_{IV}	G_V
1. 1. Characteristics of the producer							
Gender		%	Female (66.7) Male (33.3)	Male (66.7) Female (33.3)	Male (94.7) Female (5.3)	Male (100)	Male (75) Female (25)
Age (years)		Ẋ	55.2	58.7	51.9	56.0	56.4
Education level		%	None (50) Primary (33.3) Secondary (16.4)	None (66.7) Primary (33.3)	Primary (63.2) None (15.8) Secondary (10.5) Technical (10.5)	Professional (40) Primary (30) Secondary (20) Postgraduate (10)	Secondary (50) Technical (12.5) Professional (37.5)
Cultivation experience (years)		Ẋ	18	35	19.1	12.9	9.6
1. 2. Social environment							
Location	Province	%	Cordoba (83.3) Magdalena (16.7)	Cordoba (100)	Sucre (89.5) La Guajira (10.5)	Cordoba (70) Atlantico (10) Sucre (20)	Cordoba (100)
Public services	Water	%	0.0	66.6	78.9	50.0	62.5
	Electricity		0.0	100.0	52.6	100.0	87.5
	Cell phone		16.6	0.0	15.8	60.0	62.5
	Household gas		0.0	100.0	10.5	0.0	12.5
	Propane gas		0.0	0.0	0.0	20.0	12.5
	Sewerage		0.0	0.0	5.3	0.0	12.5
	Landline phone		0.0	0.0	0.0	10.0	0.0
	Internet		0.0	0.0	0.0	10.0	25
	None		83.4	0.0	15.8	0.0	0.0
Access roads	Type of road	Md	Unpaved road	Bridle path	Unpaved road	Unpaved road	Unpaved road
	State of the road		Bad	Regular	Bad	Good	Regular

continue

TABLE 2 *continuation*. Socioeconomic aspects of eggplant (*Solanum melongena* L.) farmer groups in production areas of the Colombian Caribbean.

1. 3. Productive environment							
		Stat.	**G_I**	**G_{II}**	**G_{III}**	**G_{IV}**	**G_V**
Productive unit	Land tenure	%	Own (33.3) Rented (33.3) On loan (33.3)	Rented (100)	Family (42.1) Rented (31.6) Own (26.3)	Own (40) Family (30) Rented (20) On loan (10)	Family (37.5) Rented (37.5) Own (25)
	Topography of the property	Md	Flat	Undulated	Flat	Flat	Flat
	Planting area (ha)	$\dot{X}$	0.1	0.3	0.6	0.8	1.0
	Yield (t ha^{-1})	$\dot{X}$	12.7	11.7	22.1	35.3	31.8
	Workforce	Md	Family	Family and private wages	Family and private wages	Private wages	Family and private wages
	Credit	%	Does not use credit (66.7) Bank credit (33.3)	Loan from relatives (100)	Does not use credit (78.9) Bank credit (21.1)	Does not use credit (90) Bank credit (10)	Does not use credit (50) Bank credit (25) Governmental productive projects (25)
Destination of production	Sale	%	59.2	90.0	97.8	98.4	94.3
	Family consumption		40.8	10.0	2.2	1.6	5.7
	Animal consumption		0.0	0.0	0.0	0.0	0.0
Commercialization	Sale site	%	Productive plot (83.3) Market place (16.7)	Productive plot (100)	Market place (63.2) Productive plot (26.3) Supermarkets (10.5)	Productive plot (60) Market place (40)	Productive plot (50) Market place (37.5) Other (12.5)
	Commercialization unit	%	Package 50 kg (50) By kg (50)	By kg (66.7) Bulk of 50 kg (33.3)	Package of 50 kg (78.9) By kg (5.3) Other (15.8)	Package of 50 kg (90) By kg (10)	Package of 50 kg (87.5) By Kg (12.5)

Stat.: statistics; $\dot{X}$: average; Md: mode; G_I, G_{II}, G_{III}, G_{IV} and G_V: Groups of farmers established using multivariate analyses (Gower's distance and Ward's hierarchical agglomerative clustering method).

TABLE 3. Technological aspects of eggplant (*Solanum melongena* L.) farmer groups in producing areas of the Colombian Caribbean region.

	Stat.	G_I	G_{II}	G_{III}	G_{IV}	G_V
2.1. Soils						
Type of preparation	Md	Manual	Mechanized	Mechanized	Mechanized	Mechanized
Soil analysis	Md	No	Yes	No	Yes	Yes
2.2. Seed						
Quality	Md	Not certified	Not certified	Not certified	Not certified	Not certified
Type	Md	Regional cultivar	Variety	Variety	Variety	Variety
2.3. Sowing						
Sowing date	Md	April	November and December	April	April	October
Planting density*	$\dot{X}$	1.1 m x 1.2 m	0.8 m x 1.5 m	1.2 m x 1.3 m	1.2 m x 1.2 m	0.9 m x 1.2 m
Planting system	%	Monoculture (100)	Monoculture (100)	Monoculture (94.7) Polyculture (5.3)	Monoculture (90) Polyculture (10)	Monoculture (62.5) Polyculture (37.5)
Cultivation cycle (d)	$\dot{X}$	298	230	252	242	287
2.4. Agricultural work						
Crop rotation		33.3	0.0	0.0	10.0	37.5
Reseeding		100.0	33.3	84.2	100.0	100.0
Thinning		0.0	0.0	21.1	50.0	12.5
Earthing up	%	83.3	0.0	0.0	70.0	25.0
Pruning		16.7	0.0	31.6	90.0	100.0
Stalking		33.3	0.0	0.0	80.0	37.5
Removal of crop residues		33.3	0.0	47.4	70.0	50.0
2.5. Irrigation						
Irrigation system	%	Manual (66.7) No irrigation (33.3)	No irrigation (100)	Manual (10.5) By gravity (10.5) No irrigation (79.9)	Drip (40) By sprinkling (20) By gravity (20) Manual (10)	Drip (50) By sprinkling (12.5) By gravity (12.5) Manual (12.5)
Water source	%	River (100)	River (66.7) Irrigation district (33.3)	River (63.2) Deep well (21.1) Dam (10.5) Aqueduct (5.3)	River (50) Deep well (20) Dam (20) Watering district (10)	River (62.5) Dam (12.5) Watering district (25)
Water analysis	Md	Not performed	Not performed	Not performed	Not performed	Not performed
2.6. Integrated crop management (ICM)						
Weed control	Md	Chemical and manual	Chemical and manual	Chemical and manual	Chemical and manual	Chemical and manual
Fertilization	Md	Chemical	Chemical and organic	Chemical	Chemical	Chemical
Pest control	Md	Chemical	Chemical	Chemical	Chemical	Chemical
Disease control	Md	Chemical	Chemical	Chemical	Chemical	Chemical
2.7. Infrastructure for good agricultural practices						
Agrochemical preparation area		0.0	0.0	0.0	60.0	12.5
Agrochemical storage warehouse		0.0	0.0	5.3	90.0	12.5
Showers and baths		0.0	0.0	0.0	30.0	12.5
Container storage house	%	0.0	0.0	0.0	30.0	0.0
Crop reception and conditioning area		0.0	0.0	0.0	30.0	37.5
Any		0.0	0.0	0.0	10.0	37.5
2.8. Postharvest work						
Classification		83.3	100.0	57.9	80.0	87.5
Washing		50.0	0.0	10.5	40.0	25.0
Drying		33.3	0.0	10.5	20.0	0.0
Packing	%	66.7	0.0	73.7	100.0	0.0
Storage		33.3	0.0	5.3	0.0	0.0
Any		0.0	0.0	10.5	0.0	12.5

Stat.: statistics; $\dot{X}$: average; Md: mode; G_I, G_{II}, G_{III}, G_{IV}, and G_V: groups of farmers established by multivariate analyses (Gower's distance and Ward's hierarchical agglomerative clustering method).
*Distance between plants and rows, respectively.

resources and combining family labor with private wages. The production is mostly destined for sale and is purchased at various points (municipal market, supermarkets, and production plots) in units, by kilograms, or packages of 50 kg according to the nature of the market (Tab. 2).

Regarding technological aspects, farmers use mechanized soil preparation, they do not perform soil analysis, and traditional planting is carried out in April, mainly in monoculture (94.7%) using planting schemes of 1.2 m x 1.3 m to obtain populations of 6,407 plants ha^{-1} in average production cycles of 252 d (8.4 months). The agricultural tasks that are mostly carried out are replanting (84.2%) and removal of crop residues (47.4%). Likewise, the implementation of irrigation in eggplant (21%) is low, but with various water sources for available irrigation (river, deep well, damn and aqueduct). Only 5.3% of its members have an agrochemical storage warehouse and, postharvest work is carried out at a low proportion (Tab. 3).

Group G_{IV} comprises 21.7% of the sample, and its members are scattered in the provinces of Cordoba, Atlantico, and Sucre. All farmers are men with an average age of 56 years and a varied level of schooling. It could be observed that 50% have a professional and postgraduate school degree level with average experience in the crop of approximately 13 years. The productive units have flat topography, the access roads are in good condition, and there is good coverage of public services such as drinking water (50%) and electricity (100%) with agricultural properties mainly owned by the farmer or by the family. The cultivation areas are on average 8,000 m^2 with average yields of 35 t ha^{-1}; the production costs are assumed mainly with their own resources, and the labor used is hired (private wages). The production is destined mainly for sale (98.4%) and commercialized predominantly in packages of 50 kg in the production plot to intermediaries of the region and sold in municipal market places (Tab. 2).

Technological aspects involve mechanized soil preparation with soil analysis, one planting per year in April in monoculture (90%) and using planting densities of 1.2 m x 1.2 m between plants and rows, respectively (≈ 6.944 plants/ha). Likewise, they perform the everyday tasks demanded by the crop (crop rotation, reseeding, thinning, earthing up, pruning, stalking, and removal of crop residues) in high proportion, and at least 80% of the farmers implement irrigation systems to meet the crop's water requirements. In this group, at least 30% of the farmers have GAP infrastructure, with facilities such as an agrochemical storage warehouse (90%), an area for the preparation of agricultural supplies (60%), and carry out postharvest tasks such as classification, washing, drying and packing (Tab. 3).

The G_V group represents 17.4% of the sample, and its members are located in the province of Cordoba and distributed among the municipalities of Cerete, La Apartada, Monteria, and San Pelayo. Seventy-five percent (75%) of the producers are men with an average age of 56 years, they have a variable level of education (50% secondary, 12.5% technical and 37.5% professional), and around 10 years of experience in the cultivation of eggplant. The productive units have flat topography, the access to roads is in a regular state, there is an acceptable coverage of public services (62.5% water, 87.5% electricity, and 25% Internet), and land tenure is diverse (family farms 37.5%, rented 37.5%, and own 25%). The average crop area is 1.0 ha with average yields of around 32 t ha^{-1}; production costs are mainly undertaken using their own resources (50%). However, other funding sources stand out, such as bank loans and government resources through productive projects. The workforce is familiar and complemented with the hiring of family wages, with production destined mainly for sale (94.3%), and commercialized in the production plot and municipal market places in packages of 50 kg and per kilogram (Tab. 2).

The technological aspects of this group involve mechanized soil preparation and soil analysis, one planting per year in October in monocultures (62.5%) and polycultures (37.5%), and using planting distances of 0.9 m x 1.2 m between plants and rows, respectively (≈ 9.259 plants ha^{-1}). Likewise, they perform the everyday tasks demanded by the crop (crop rotation, reseeding, thinning, earthing up, pruning, stalking, and removal of crop residues) in a high proportion; at least 75% implement irrigation systems to meet the water requirements of the crops. In this group, at least 12.5% of the farmers have GAP infrastructure, with facilities such as a harvest reception and conditioning area, an agrochemical storage warehouse (12.5%), an area for the preparation of agricultural supplies (12.5%), showers and baths (12.5%), and postharvest work is carried out, including classification and washing (Tab. 3).

The groups described above share common characteristics such as having unpaved roads, the use of seed selected from their own crops (not certified), they do not perform water analysis for irrigation, they perform weed control by combining herbicides with manual weeding, and use only chemical pesticides for the control of pests and diseases (Tabs. 2 and 3).

Discussion

The results reveal, in general, a low (G_I, $G_{II,}$ and G_{III}) to medium (G_{IV} and G_V) technological level in eggplant production in the Colombian Caribbean region. In this regard, some studies have found that aspects, such as the technological level and the adoption of technologies, have a direct relationship with the schooling level of farmers (Damián and Ramírez, 2008; Aguilar *et al*., 2013; Ayala *et al.,* 2013; Vargas *et al*., 2015; Garrido *et al*., 2017). This is consistent with the results obtained in this study, in which the groups with the highest level of education (G_{IV} and G_V) showed the highest yields (G_{IV} = 35 t ha^{-1}, G_V = 32 t ha^{-1}). Furthermore, this is explained mostly because these groups execute agricultural work implementing irrigation systems, crop nutrition based on soil analysis, and have GAP infrastructure (Tab. 3).

The average age of the eggplant producer groups was similar among the groups, with a range between 52 and 59 years. In related research, Correa *et al*. (2018) found that squash producers in the Caribbean region of Colombia showed a similar average age range (between 42 and 53 years). These results represent the socioeconomic reality of the rural areas, where the generational change of farmers is uncertain due to the precarious conditions of competitiveness of small farmers. This happens mainly because there is a lack of fair marketing scenarios that directly influences the profitability of agricultural products (Villalobos, 1984; Correa *et al.,* 2010; Jaramillo and Riveros, 2013).

Eggplant producers with a lower technological level (G_I, $G_{II,}$ and G_{III}) register cultivation areas between 1,000 m^2 and 6,000 m^2. These results are similar to those obtained by Agrosavia in 2011, in which the areas destined to the crop ranged between 1,000 m^2 and 5,000 m^2 (Cadena *et al.,* 2011a). This shows a null growth in the productive units for this segment of producers. However, the official figures on eggplant planting areas at the regional level show a growth close to 500% between 2011 and 2017 (Agronet, 2018). This growth can be explained by the incursion of producers included in groups G_{IV} and G_V with more extensive productive units (0.8-1.0 ha) and higher capacity for economic and technological investment.

Despite the crucial advances in the generation of knowledge at the local and regional level for the cultivation of eggplant in areas, such as the production of planting material and substrates (Aramendiz *et al.,* 2013), water requirements (Sánchez *et al*., 2004), weed management (Aramendiz *et al*., 2010; Hernández *et al.,* 2015), nutrition (Cantero *et al.,* 2015), phytosanitary management (Tamayo and Jaramillo, 2006, 2013; Gómez *et al.,* 2012), planting densities (Pérez *et al.,* 2006), agronomic crop management (Aramendiz *et al.,* 2008; Tapia *et al.,* 2015), postharvest (Escobar *et al*., 2011) and new eggplant cultivars (Aramendiz *et al.,* 2011; Cadena *et al*., 2011a, 2011b), the adoption of these technologies may be limited by the high level of illiteracy among producers, mainly in the G_I, G_{II} and G_{III} groups, showing a level of illiteracy of around 50%, 67% and 16%, respectively (Tab. 2). In this regard, Salcedo and Guzman (2014) point out that the level of education in Latin America is low with a high level of illiteracy, with Colombia registering an average of 5.6 years of schooling in transitional family agricultural producers (i.e., that have only completed their primary education).

Authors such as Barrientos and Torrico (2014) point out that the socio-economic perspectives of family farming (FF) in countries such as Colombia move mainly to the rhythm of the markets. Furthermore, their transformation from a subsistence condition to a commercial situation and vice versa depends mainly on the availability of sufficient resources for production, mainly regarding land, labor, and financial capital. In this sense, according to the FF typologies, eggplant production in the Colombian Caribbean region can be framed as a transitional FF, whose typology in the country represents around 12% of FF (Salcedo and Guzman, 2014; Sabourin *et al.,* 2015).

The study reveals that the predominant sowing date is in April; this is because the rainy season in the Colombian Caribbean region begins in this period, so the vast majority of farmers establish their crops at this time of the year. This, however, leads to the formation of the phenomenon known as "supply seasonality," which corresponds to the oversupply of production, mainly from June to November (Tab. 3, Supplementary Material 1). In this regard, the dynamics of prices in the primary market places in the region show low seasonality of prices between June and January, with a recovery period during February and May (Fig. 2). These results have great significance by associating the behavior of prices with the productive cycles of the crop, which moves between 7.7 and 10 months (Tab. 3), of which, the first 2.5 months correspond to the vegetative period (unproductive stage) and the remaining period (5 to 8 months) to the reproductive (flowering) and productive (harvest) stages. There is a progressive growth in production during the first six months that descends significantly after the seventh month as a result of crop senescence. This, in turn, explains the recovery of prices due to the substantial decrease in supply. Hence, the preference for a segment of producers that sow between October and December in search of favorable marketing prices is revealed.

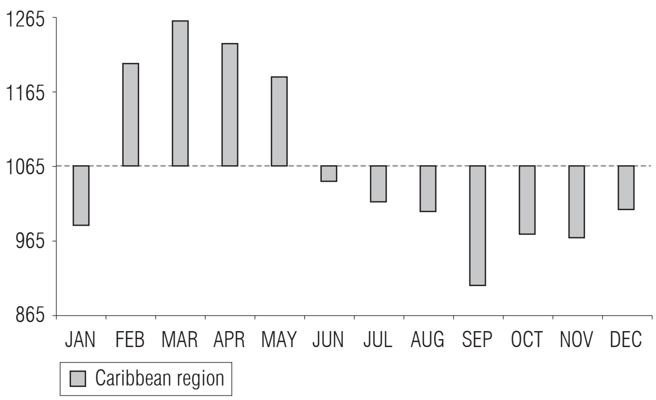

FIGURE 2. Historical behavior of monthly average prices (2013-2018) for eggplant (COP$ kg^{-1}) in the main cities of the Caribbean region of Colombia (Barranquilla, Cartagena, Monteria and Sincelejo) (SIPSA and DANE, 2018).

Conclusions

Eggplant cultivation in the Caribbean Region of Colombia has shown growth in area and production, explained by the increase in consumption and the incorporation of technologies such as irrigation. In addition, this kind of technologies increases yields and decreases seasonality which contributes to its rates of implementation.

Planting dates are associated with the appearance of rains that usually occur in the third week of April, which is a rule for those farmers who do not have nearby water sources and purchasing power to gain access to pressurized irrigation systems, as is the case of farmers located in the flat areas. The other type of farmers, such as those in the Sinu Valley and other productive areas with permanent water sources, take advantage of this asset by growing at different times of the year, thus, breaking the seasonality of production.

The socioeconomic conditions of eggplant producers are heterogeneous, and their technification ranges from low to medium levels. Likewise, as standard features among the groups identified, the crop is cultivated by farmers with average ages between 52 and 59 years who are considered elderly (≥60 years), and with little participation of young people. In the medium term, this can have a significant impact on the reduction of regional production. Therefore, it is imperative to implement a regional rural extension program in the short term, aimed at the adoption of technologies that have been generated, to improve the productive conditions of farmers as well as the sustainability and attractiveness of this economic activity for current and future generations of producers in this region of the country.

The crop is highly dependent on chemical synthesis inputs required for phytosanitary management and crop nutrition, a situation that may be generating a significant environmental impact on the health of producers and consumers, in addition to raising production costs. Therefore, the study reveals that the implementation of research lines aimed at developing integrated pest, disease and nutrition management is a priority to improve the safety and sustainability of the eggplant production systems in the Caribbean region of Colombia.

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SUPPLEMENTARY MATERIAL 1. Monthly precipitation (mm) of some eggplant producing areas in the Caribbean region of Colombia in 2018.

Municipality, province	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Aap
Monteria, Cordoba	18	16	17	97	169	156	137	170	169	155	87	24	1,215
La Apartada, Cordoba	14	29	56	159	243	272	330	312	312	262	123	69	2,181
Cerete, Cordoba	13	20	36	124	215	176	199	209	218	172	110	45	1,536
San Pelayo, Cordoba	7	14	25	104	190	130	162	188	178	144	94	34	1,269
Sincelejo, Sucre	23	27	41	118	186	165	163	185	196	184	128	58	1,475
Corozal, Sucre	18	20	35	92	150	137	139	140	139	135	86	37	1,128
Los Palmitos, Sucre	20	26	37	111	143	148	125	152	156	161	106	33	1,219
Sampues, Sucre	19	35	39	117	167	151	133	170	188	133	107	45	1,304
Dibulla, La Guajira	16	18	27	102	145	79	40	112	202	296	206	41	1,284
Sitionuevo, Magdalena	0	0	5	29	147	111	75	136	153	219	125	23	1,023
Polonuevo, Atlantico	11	4	14	73	154	126	134	146	160	204	128	28	1,181
Ponedera, Atlantico	2	11	15	86	110	109	107	145	185	183	114	36	1,102

Aap: average annual precipitation in mm. Values in bold correspond to rainy months, which were considered based on the accumulated precipitation ≥ 85 mm (IDEAM, 2018).

What does ‘quality’ mean in the context of rural extension and advisory services?

¿Qué significa ‘calidad’ en el contexto de la extensión rural y los servicios de asesoramiento técnico?

Fernando Landini[1]*

ABSTRACT

The quality of rural extension and advisory services is a crucial element in fostering innovation and rural development. This article aims to clarify the concept of quality of rural extension and to develop a preliminary theoretical framework. An ample literature review was conducted in search of articles on service quality and quality of rural extension and advisory services. The first part presents the main results of the literature search on quality of extension services. The definition of quality is not universal. Quality cannot be conceptualized only as farmers' satisfaction or as extension results. It has different dimensions or components and stakeholders have different points of view about it. The second part of this article discusses the definition of service quality and the concept of Total Quality Management and underlines that the concept of quality varies according to industry types or contexts and is the result of complex negotiation among different stakeholders. Finally, a comprehensive theoretical framework for addressing quality of rural extension and advisory services is presented that differentiates among enablers that limit or facilitate the delivery of quality rural extension and advisory services, the production and delivery processes, and results obtained. Here, the key role played by quality self-assessment and organizational learning is highlighted.

Key words: agricultural extension, total quality management, rural development, institutional learning.

RESUMEN

La calidad de los servicios de extensión rural y asesoramiento técnico es fundamental para impulsar procesos de innovación y desarrollo. Este artículo busca clarificar el concepto de calidad de la extensión rural y desarrollar un marco teórico preliminar. Se realizó una amplia revisión bibliográfica sobre la calidad de los servicios y de la extensión rural. La primera parte presenta los principales resultados de la revisión sobre calidad de la extensión: su definición no es universal, no puede ser conceptualizada únicamente ni como satisfacción de los productores ni como resultados de extensión, posee diferentes dimensiones o componentes, y los actores tienen diferentes visiones sobre ella. La segunda parte discute la definición de calidad de servicios y el concepto de Gestión Total de la Calidad y destaca que el concepto de calidad varía según el contexto y el sector productivo, siendo el resultado de complejos procesos de negociación entre diferentes actores. Finalmente se propone un marco teórico completo para abordar la calidad de los servicios de extensión, el cual diferencia entre facilitadores que hacen posible o que limitan la prestación de un servicio de calidad, el proceso mismo de producción y prestación del servicio, y los resultados obtenidos. Se destaca el rol clave que juegan la autoevaluación de la calidad y el aprendizaje organizacional.

Palabras clave: extensión agrícola, gestión total de la calidad, desarrollo rural, aprendizaje institucional.

Introduction

During the last 20 years, different scholars and rural development institutions have progressively paid more attention to the importance of the quality of advisory services provided by rural extension institutions (Israel, 2010; Issa and Issa, 2013; Garst and Franz, 2014; Herman and Grant, 2015; Castaño-Reyes *et al.*, 2017). There are several, intertwined reasons that may help to explain this process. First, in the context of the pressures towards the privatization of rural extension and advisory services (RE&AS) that began in the 80s, concepts such as ‘quality’ and ‘clientele’ that have been traditionally linked to the world of private companies (Fredendall and Lippert, 1995) became notions that could be applied to rural advisory services, now framed in market terms (Turkson, 2009; Anaza *et al.*, 2012). Second, pressures towards privatization, coupled with a questioning of the lack of evidence for the impact of RE&AS, made clear the need for evaluating the results and quality of the services provided to present them to funding agencies or institutions in order to keep up a steady flow of resources (Diehl *et al.*, 2012; Lamm *et al.*, 2013; Franz *et al.*, 2014).

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[1] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) y Universidad de la Cuenca del Plata, Posadas, Misiones (Argentina).

* Corresponding author: landini_fer@hotmail.com

Finally, more recently the rise of demand-driven extension approaches (Qamar, 2011; Masangano *et al.*, 2017) as well as acknowledgment of the value of accountability to clients in the context of RE&AS (Galindo and Israel, 2010; Sseguya *et al.*, 2012) also supported the 'market framing' of the extension practice (Christoplos, 2008). This encouraged the evaluation of the clients' (i.e., famers') satisfaction with RE&AS, since it was considered to be a synonym for service quality (e.g. Fredendall and Lippert, 1995; Terry and Israel, 2004).

In the context of the great importance widely assigned to the quality of RE&AS in the scholarly literature as well as in institutional practices, the concept of quality of RE&AS as well as the strategies to evaluate that quality would be expected to be a fundamental topic of debate in extension science. However, most academic bibliography tends to use the concept superficially, implicitly assuming common-sense definitions when referring to the importance of or the need for improving RE&AS quality (e.g. Danielsen *et al.*, 2013; Garst and Franz, 2014; Myeni *et al.*, 2019). Furthermore, even when the concept of quality of RE&AS is explicitly addressed, the term is usually vaguely defined; or the paper is lacking a proper discussion of what quality is in the context of RE&AS (e.g. Benin *et al.*, 2007; Feng *et al.*, 2007; Dunne *et al.*, 2019). Most importantly, works aimed towards increases in RE&AS quality cannot be based on unclear, vague or decontextualized definitions and conceptualizations of the term; and it is apparent that increasing the quality of RE&AS implies increasing its potential to improve farmer productivity and promote rural development.

Thus, it is clear that the concept of quality has gained greater relevance in the context of RE&AS. However, the term "quality" has been neither properly discussed nor properly defined. The objectives of this review article are: (1) to explore and clarify the concept of quality used in RE&AS academic and institutional literature, pointing out the main limitations; (2) to present the key elements of the current debate on service quality and quality management in order to address such problems; and (3) to develop a preliminary theoretical framework to define quality of RE&AS and to guide the implementation of actions aimed at improving it.

Literature review

In order to meet the objectives of this article, I conducted a two-step literature review. Firstly, I used the descriptors 'quality' + '[rural/agricultural] extension' and 'quality' + 'advisory services' (and their equivalents in Portuguese and Spanish) to search for relevant literature in EBSCO, SCIELO and DOAJ databases, and in websites of relevant institutions such as the Food and Agriculture Organization of the United Nations (FAO), the Global Forum for Rural Advisory Services, and the Inter-American Institute for Cooperation on Agriculture. I read the selected texts and identified the main topics of debate.

Secondly, I used the same databases to search for literature on topics acknowledged as relevant during the previous review, including 'service quality', 'quality management', 'quality standards', 'EFQM [European Foundation for Quality Management] Excellence Model' and 'ISO 9000'. In this case, articles were analyzed and relevant topics for addressing quality of RE&AS were selected.

The following titles summarize the main topics identified during the literature review.

Quality in RE&AS current literature

On the definition of rural extension and advisory services

In this paper, I follow the standard definition used by Christoplos (2010) in the context of the Food and Agriculture Organization of the United Nations (FAO). He defines 'extension' as an admittedly amorphous umbrella term for all the different activities that provide the information and advisory services that are needed and demanded by farmers and other actors in agrifood systems and rural development. Thus, the author defines rural extension as an 'umbrella term' and gives advisory services a central role. In English, the concepts of 'rural/agricultural extension' and 'advisory services' are frequently used as synonyms, with the frequency of the use of one or the other depending on the context and the country. Following this common use, both concepts are used as synonyms in this paper.

Quality with regards to RE&AS: Quality of what?

When addressing RE&AS quality it is essential to be clear about the quality of which aspects, practices, or services we are referring to; because RE&AS involve different activities and, thus, an inaccurate use of the term may lead to confusion. For instance, Issa and Issa (2013) seem to refer indistinctly to the quality of the extension personnel and the quality of extension services. In general, when addressing RE&AS quality, most authors refer to the quality of rural extension/advisory services in general (e.g. Lamm *et al.*, 2013; Anik and Salam, 2015; Jona and Terblanché, 2015; Elahi *et al.*, 2018). Nonetheless, there are also other two, frequently mentioned, aspects of quality with regards to RE&AS. Firstly, there are several authors who focus on the quality of extension programs (Garst and Franz, 2014; Singletary *et al.*, 2016), and not on RE&AS as a whole. On the other hand, other scholars pay attention to the quality of extension agents (Sarker and Itohara, 2009), that is to the quality of the human resources that provide RE&AS.

When analyzed in depth, it is clear that service quality, program quality and advisors' quality (among other alternatives) are different aspects, dimensions, or components to consider when addressing quality in the context of RE&AS. What's more, as they comprise different aspects of RE&AS quality, the indicators that ought to be used to assess them should also be different. Thus, the need to clarify the quality of what we are talking about when addressing quality in the area of RE&AS becomes apparent. Finally, in general terms, the most common reference to quality in this context (and arguably the one with the most practical potential) seems to be service quality, which takes into consideration RE&AS practices as a whole.

Quality as clients' satisfaction or as results?

Having analyzed what aspects of the quality of RE&AS can be addressed, it is now time to discuss what quality means in this context. In order to do so, from now on, the focus will be on RE&AS quality (this is, service quality). In RE&AS academic literature, there are different ways of defining service quality. However, two of the most common definitions are as clients' satisfaction or as extension results.

Many scholars consider (explicitly or implicitly) quality to be farmers' satisfaction with the extension service (e.g. Benin *et al.*, 2007; Issa and Issa, 2013; Singletary *et al.*, 2016). In this way, farmers and their expectations and wishes define what quality is. However, despite the importance of valuing farmers' perspectives, considering their satisfaction as the core aspect of service quality has limitations.

Firstly, Fredendall and Lippert (1995) argue that such an approach is characteristic of private businesses focused on making profit and building customer loyalty. Nonetheless, it is clear that the goals of RE&AS go beyond those that frame market logic (Sulaiman and Davis, 2012; Vicher, 2012), such as reaching public goods (Rivera and Alex, 2004; Franz *et al.*, 2014; Baig *et al.*, 2019), like for instance environmental conservation. Thus, it is clear that, although farmers' (or other clients') satisfaction may (and should) be considered *as part* of service quality, it cannot be its only or foremost dimension.

Secondly, several authors have argued that low-income communities that have no access to certain services tend to be highly satisfied with them even when they are considered of low quality by other types of clients or from a technical point of view. For instance, Comes and Stokiner (2004) have shown that poor women are highly satisfied with having access to health care even if they have to wait several hours to obtain it. In this line, López and Pérez (2014) differentiate between perceived and real quality, pointing out that the former is framed by expectations and these can be unrealistic (very low or very high) when people have no knowledge or experience of different alternatives as occurs with underprivileged social sectors. According to Landini (2016a), farmers should be informed of the different extension service alternatives to allow them to identify what they really want. Thus, using farmers' satisfaction to properly evaluate service quality would require a prior knowledge (or even joint development) of different extension service alternatives, even those that are not available, which is almost never considered when assessing farmers' satisfaction.

The third argument addresses the fact that farmers' expectations and satisfaction may not coincide with (and may even be contrary to) other extension goals or social values that are considered superior or at least equally valuable, particularly in the case of publicly funded RE&AS. What if clients are satisfied with RE&AS but these are not racially equitable, do not support gender equity, or go against key social values or institutional objectives? Or if they are unsatisfied but extension services are in line with institutional priorities and goals? In consequence, farmers' satisfaction should not be considered as the key aspect of service quality, but simply as one element that, combined with others, shapes what quality extension service is (e.g. Danielsen and Kelly, 2010; Rodríguez-Espinosa *et al.*, 2017).

Fourthly, in the previous argument the focus was on farmers' satisfaction. However, why not consider the satisfaction of other relevant actors? Within RE&AS literature, Fredendall and Lippert (1995) highlight that extension institutions have external (farmers) and internal customers, the latter generally comprises extensionists, given that they are clients of internal processes. Why should not extensionists' satisfaction be considered as part of the quality of extension services? According to different authors (Archer *et al.*, 2007a, 2007b; Castaño-Reyes *et al.*, 2017; Rodríguez-Espinosa *et al.*, 2017) excellence criteria in extension should be meaningful for a variety of stakeholders, and not only for farmers. In this line, it is debatable that, even when extension service quality is understood in terms of satisfaction of expectations, only farmers' satisfaction is taken into consideration, and not that of a wider range of actors.

Fifth, in the case of RE&AS, where scientific knowledge is at stake, addressing quality only in terms of farmers' satisfaction would seem to be a limited approach. Danielsen and Kelly (2010), besides valuing clients' satisfaction, propose that technical quality should also be included when developing quality criteria. It could be argued that technically incorrect advice would not lead to clients' satisfaction, but when advice implementation results are unclear due to the entanglement of multiple factors, addressing technical quality directly seems to be preferable.

The second most common frame for understanding extension service quality is assimilating it for reaching desired results (Mueller, 1991; Birner *et al.*, 2009; Faure *et al.*, 2012). In this context, some authors tend to highlight the importance of extension impact (e.g. Herman and Grant, 2015). However, others are more cautious, arguing that impacts such as adoption of technologies yield increase and, even more, poverty reduction are the result of multiple factors, with RE&AS only being one among others (Benin *et al.*, 2007; Birner *et al.*, 2009). Thus, they tend to assess quality in terms of extension performance as an indicator of results (Rivera and Alex, 2004; Danielsen *et al.*, 2013).

With regards to quality as results, there are issues that deserve discussion. Firstly, assimilating extension quality into results leads to the question of deciding which results are to be considered as RE&AS quality. Depending on the extension approach, expected results are different, ranging from technology transfer to fostering innovative processes (Leeuwis and Aarts, 2011; Landini, 2016b). What's more, different stakeholders expect different results. For instance, Christoplos *et al.* (2012) have argued that farmers and the general public (the society as a whole) may have different goals with regards to environmental issues. Likewise, Sayeed *et al.* (2015) point out that governmental authorities may be more interested in reaching objectives such as food security, compared to the ones preferred by farmers (for instance increasing monetary income). Thus, it is clear that different stakeholders may and will have different extension objectives. Acknowledging this, scholars have suggested the construction of quality indicators in a participatory way, taking into account the perspectives of extensionists, policymakers, farmers, and other relevant stakeholders (Archer *et al.*, 2007a, 2007b; Birner *et al.*, 2009; Landini and Bianqui, 2018). Nagel (1997) states that extension approaches are presented in terms of their most important organizational forms and their respective goals. The goal system reflects the power positions of various groups of actors. Thus, if extension quality is going to be understood in terms of its capacity to reach desired results, then the power dynamics underlying the equilibrium or the compromise between different stakeholders' objectives should be acknowledged.

The second main issue when addressing RE&AS quality as results has to do with the relationship between the means and the ends, that is, quality procedures versus quality results. Clearly, the capacity to reach desired results is a sign of RE&AS quality. When analyzing high impact extension programs, Mueller (1991) pays particular attention to the 'roots [...] linked to desired outcomes', which refers to the processes that allow for those results to be reached. As argued previously, the technical quality of extensionists' advice cannot be considered in and by itself a goal but instead as a way of reaching good results (Danielsen and Kelly, 2010). Likewise, there are also means such as extension strategies or approaches linked to specific values that seem to be part of extension quality but cannot be expressed in terms of results. Some examples are the implementation of culturally appropriated and socially inclusive interventions and the use of participatory processes or gender sensitivity (Trigo *et al.*, 2013; Krishna *et al.*, 2019). Thus, the quality of RE&AS cannot be reduced only to reaching desired goals but should also include socially acceptable interventions and technically pertinent recommendations.

Quality criteria and best practices in RE&AS

The RE&AS scholarly literature not only describes RE&AS quality in terms of clients' satisfaction and of reaching desired results. Authors mention a multiplicity of dimensions or quality components that are useful for widening our conception of service quality and that may also be used for assessing it. In this level, these dimensions are divided into two different categories: those referring to the quality of the advice and those that address extension service in general. Additionally, best/good practices in RE&AS are mentioned, given that they can also be helpful towards identifying quality processes in RE&AS.

Characteristics of quality advice: Characteristics that shape what quality advice is

1. The information provided by the adviser is technically accurate and up-to-date in scientific terms (Terry and Israel, 2004; Sarker and Itohara, 2009; Israel, 2010), and it is effective at accomplishing its objectives (increasing productivity, reducing diseases, etc.) (Turkson, 2009). Danielsen *et al.* (2013) describe this in terms of technical quality.
2. The advice is useful, relevant and effective in practical terms, for solving problems or reaching its objectives (Birner *et al.*, 2009; Faure *et al.*, 2012; Jona

and Terblanché, 2015; Ragasa and Mazunda, 2018; Dunne *et al*., 2019). It is also feasible (Mueller, 1991; Lamontagne-Godwin *et al*., 2017). The advice cannot be described as high-quality if farmers have no access to the required resources or do not have the necessary knowledge to put it into practice (Danielsen *et al*., 2013).

3. The advice is easy to understand and use (Fredendall and Lippert, 1995; Israel 2010). It is practical and not too technical (Jona and Terblanché, 2015).
4. The advice is timely and is provided without unnecessary or excessive delays when needed (Benin *et al*., 2007; Birner *et al*., 2009; Danielsen *et al*., 2013; Elahi *et al*., 2018).

Broader characteristics of a quality extension service

Rural extension does not only involve providing advice to farmers. In the following list, the dimensions expressing quality of RE&AS that go beyond the characteristics of advice are presented.

1. Quality extension workers require establishing good interpersonal relationships with farmers and other stakeholders (Turkson, 2009). This involves treating people with respect (Sseguya *et al*., 2012) and building trust with farmers (Landini, 2016c).
2. Extensionists' clientele have to participate in the planning, implementation and evaluation of extension programs (Archer *et al*., 2007a). In this line, quality RE&AS require the incorporation of farmers' and other stakeholders' inputs in order to use them to design extension strategies and to keep beneficiaries informed about the implementation process as well as the results (Mueller, 1991; Christoplos *et al*., 2012).
3. Quality extension service has to be culturally pertinent. That is, it must be respectful of local ways of life, acceptable in terms of people's customs, and even re-organized and based on the beneficiaries' cultural rationale (Singletary *et al*., 2016).
4. Quality RE&AS do not only have to reach desired results but also be efficient (Birner *et al*., 2009; Danielsen *et al*., 2013). In general terms, this would imply that the benefits of the RE&AS are below its cost (Zwane and Groenewald, 2014).

Best practices and quality

Best practices in RE&AS refer to those extension practices or guidelines that have proven from experience to contribute to reaching better extension results. Although best practices have not generally been considered as being ways of referring to RE&AS quality, the fact that they express the means to obtain desired results allows us to think of them in terms of process quality. Although many best extension practices have been proposed, only the most frequently used and the most useful for this context are presented.

1. Implementation of participatory, demand-driven extension approaches. There is considerable agreement in RE&AS that good extension services have to be participatory and structured by demand and not by supply (Trigo *et al*., 2013; Akumu *et al*., 2019).
2. Interdisciplinary approach. Traditionally, RE&AS have been considered a practice focused on technical expertise. Nonetheless, over the last decades, the complexity of rural extension has increased enormously (Leeuwis, 2004; Sulaiman and Davis, 2012). Thus, it is clear that obtaining good extension results requires involving practitioners with different social and technical backgrounds (Landini and Bianqui, 2014).
3. Gender-sensitive approach. Even nowadays it is common that RE&AS address mainly male farmers and do not acknowledge how gender influences farmers' practices. It is clear that women have a key role in agriculture and that extension practices have to be gender-sensitive and aimed towards gender equity (Quaye *et al*., 2019).
4. Dynamic, bi-directional articulation between research and RE&AS in the context of agricultural innovation systems. Within the traditional extension approach, researchers develop technologies and extensionists transfer them to farmers. In contrast, the current understanding of innovation highlights the role of agricultural research and rural extension as part of agricultural innovation systems, in which different stakeholders reflect critically, learn together, and develop new strategies to face existing challenges (Moschitz *et al*., 2015).
5. Flexibility and acknowledgment of diversity. The lack of flexibility of extension programs leads to multiple problems and poor results. Thus, extension programs have to both acknowledge diversity and take into consideration the specificities of particular contexts (Aguirre, 2012).

Evaluation of quality and RE&AS enhancement

One of the key topics when addressing RE&AS quality is quality improvement (Sseguya *et al*., 2012; Sayeed *et al*., 2015; Castaño-Reyes *et al*., 2017). Taking into account the fundamental role of quality evaluation in this process, an overview of the topic is going to be presented. Most evaluation processes mentioned in the academic literature linked to providing quality RE&AS are in the areas of customer satisfaction (Fredendall and Lippert, 1995; Galindo and Israel, 2010) and extension results, impact, or effectiveness (Lindner and Nieto, 1998; Christoplos, 2008; Birner *et al.*, 2009). Interestingly, both approaches should not be thought of as contradictory but as complementary (Terry and Israel, 2004).

Despite their importance, several authors have expressed concerns about the limitations of frequently used procedures for quality evaluation (Faure *et al.*, 2012). Some scholars have highlighted that performance assessment is often irregular and sparse (Danielsen *et al.*, 2013; Castaño-Reyes *et al.*, 2017) and that it usually pays more attention to the private value of programs than to public good (Franz *et al.*, 2014). Likewise, some scholars have drawn attention to the difficulty involved in evaluating RE&AS quality (e.g. Lamm *et al.*, 2013; Herman and Grant, 2015; Lamontagne-Godwin *et al.*, 2017). Different methodologies have been used to assess RE&AS quality. The most common one is the use of questionnaires and surveys specifically designed for impact and customer satisfaction evaluation in RE&AS (Lindner and Nieto, 1998; Galindo and Israel, 2010; Israel, 2013). Nonetheless, there are also reports of the use of general service quality measurement instruments, such as SERVQUAL (Feng *et al.*, 2007) or SERVPERF (Grīnberga-Zālīte and Liepa, 2012). Additionally, other authors have mentioned participatory impact or quality assessments (Castaño-Reyes *et al.*, 2017) and the application of observational tools (Herman and Grant, 2015). Lamm *et al.* (2013) that highlight that quality evaluations tend not to assess behavioral changes.

Different authors underline the importance of RE&AS evaluation for quality improvement and institutional learning (Archer *et al.*, 2007b; Diehl *et al.*, 2012; Lamm *et al.*, 2013). However, most scholars do not address the process of how quality assessment results can turn into service quality improvements. In order to do this, Danielsen and Kelly (2010) highlight the significance of raising extensionists' awareness regarding quality improvement and stimulating critical reflection, while Herman and Grant (2015) suggest developing plans for improvement based on identified strengths and weaknesses. Nonetheless, the most common reference in this context is the use of a Total Quality Management (TQM) approach. TQM will be addressed more in depth later, when analyzing the quality management literature. However, it is worth mentioning that TQM focuses on customer satisfaction and quality improvement and is commonly used in the context of private business; but TQM can also be adapted for improving RE&AS quality (Fredendall and Lippert, 1995; Lindner and Nieto, 1998).

Synthesis and key conclusions

Several interesting conclusions were reached after analyzing quality in RE&AS literature. These are summarized as follows:

1. The concept of quality within RE&AS has gained relevance but has not been properly discussed or clarified.
2. Equating RE&AS quality with farmers' satisfaction or with results has important limitations.
3. Different stakeholders' points of view (and not only farmers') have to be considered when assessing satisfaction with RE&AS and identifying which extension results are valuable.
4. Different stakeholders' expectations and goals regarding RE&AS may differ and even be contradictory. This implies that their assessment of service quality may be different, and that prioritizing the perspective of one stakeholder over another entails power struggles.
5. RE&AS definition of quality is neither general nor universal. What is considered quality within RE&AS will depend on the extension approach and the expected results.
6. Constructing RE&AS quality indicators requires participatory processes that take into account the perspectives of extensionists, policymakers, farmers, and other relevant stakeholders.
7. RE&AS quality entails both quality processes and quality results. Quality processes refer to aspects not necessarily considered within customers' satisfaction and extension results, such as equity of access or transparency in the use of resources.
8. Quality advice has to be technically accurate, useful, easy to understand and use, and timely.
9. RE&AS quality entails extension staff having positive attitudes towards people, involvement of beneficiaries, cultural pertinence, and efficiency.
10. From the perspective of the best extension practices, RE&AS have to adopt a participatory, interdisciplinary, gender-sensitive, horizontal and flexible approach.
11. Quality assessment is essential. Total Quality Management seems to be a useful strategy for quality improvement.

Quality and quality management in the current debate

Nowadays, the concept of quality is a central area of research and debate in the contexts of marketing, business, and many other disciplines. In this heading, key elements of academic literature on quality will be presented and discussed in order to generate useful guidelines for the analysis and enhancement of RE&AS quality.

On the definition of service quality

Multiple and contrasting definitions and conceptualizations of quality can be found in the academic literature. It is apparent that there is no clear, scholarly agreement on what quality is or what it means (Radomir *et al.*, 2012; Torres, 2014; Javed *et al.*, 2019). Several authors have argued that quality is a complex and multidimensional concept (Fatima *et al.*, 2019; Marimon *et al.*, 2019), which makes it difficult to define. Arguably, the existence of different definitions and conceptualizations of quality helps to grasp the concept's complexity and multidimensionality, thus, making them complementary instead of contradictory (Kiauta, 2012). According to Garvin (1984), to rely on a single definition of quality is a frequent source of problems.

Different scholars have highlighted that the interest in quality emerged in the context of the manufacturing industry (Cordero *et al.*, 2013; Torres, 2014; Alzaydi *et al.*, 2018), that is, in terms of product quality. However, there is a consistent agreement regarding the relevant differences between goods and services when addressing quality (Radomir *et al.*, 2012; Prakash and Mohanty, 2013; Polyakova and Mirza, 2015). Torres (2014) presents a definition of service quality that seems to be particularly useful for thinking about RE&AS, given the fact that it simultaneously considers customers' expectations, as well as the points of view of experts and other stakeholders: a service of quality is one whose superior standards create a sense of value that matches or exceeds the customer's ideal expectations. A quality service has enduring characteristics that would fulfill the standards of various stakeholders including consumers and experts.

In the context of service quality, the services' specificities and the market orientation of most academic literature have led most authors to consider service quality as customers' satisfaction or as perceived quality (López and Pérez, 2014; Polyakova and Mirza, 2015). Interestingly, this shows the change from a definition of quality centered on the inherent properties of goods or services to an approach focused on their capacity to fulfill consumers' needs or expectations (Vicher, 2012).

This theoretical presentation allows for some useful reflections on addressing the concept of RE&AS quality. Firstly, it was argued that there is no scholarly agreement on a single definition or conceptualization of quality. In consequence, further debate and discussion of what RE&AS quality means is a must. Secondly, potential disagreements in this debate should not be considered a problem or a limitation but, instead, a contribution to understanding the multiple dimensions of RE&AS quality. Finally, it is important to frame the debate over RE&AS quality in the context of service quality. Nonetheless, this framing should acknowledge that the market-oriented perspective of service quality focused on customer satisfaction is not the best fit for RE&AS, given that it needs to consider other dimensions of quality, such as technical quality as well as the social impact of extension services.

Key debates and discussions for a RE&AS quality framework

Some debates on quality are particularly useful for building a RE&AS quality framework. Firstly, there is an intuitive tendency to understand quality from a realistic perspective, in the sense that quality and its dimensions are usually assumed to exist before any definition of them. If this perspective is accepted, defining quality would imply formulating a good definition of what quality is. However, different authors have argued, perhaps not explicitly, that any definition of quality is the result of a social, constructive process. That means that quality is not pre-existent to its definition. In this sense, it has been highlighted that organizations as well as researchers have to formulate or select the quality definition that best fits their situation and interest (Hernández *et al.*, 2013; Urban, 2013). Interestingly, ISO 9000 Quality Standards do not provide a specific definition of quality for every industry or service area, but just a general one, leaving to each organization or institution the explicit responsibility of identifying their customers and other interested parties' needs and their own contextual quality objectives (ISO, 2015).

Additionally, many scholars have also highlighted that definitions of quality vary among different industry and service areas (Cordero *et al.*, 2013). Thus, it is clear that definitions and relevant dimensions of quality are industry, context and culture-dependant (Prakash and Mohanty, 2013; Terziovski and Guerrero, 2014; Polyakova and Mirza, 2015; Marimon *et al.*, 2019; Subiyakto and Kot, 2020).

A second interesting area of debate is the role of different stakeholders or interested parties in the contextual definitions of quality. Within the market-oriented approach, consumers tend to be seen as the main source that defines what quality is in a specific industry area, company or organization. However, authors have claimed that, although quality must be customer-driven, the concept of quality used by a particular company can be enriched by also using the perspectives of experts and internal stakeholders (company's staff) (López and Pérez, 2014; Torres, 2014; Rodríguez-Espinosa *et al.*, 2017). Additionally, Golder *et al*. (2012) argue that quality attributes have also to be evaluated from an expert point of view, given the fact that customers may not have a clear or accurate perception of them. Interestingly, several authors suggest that different stakeholders can have different perceptions of service quality (Dedeoğlu and Demirer, 2015).

Different authors argue that organizations that offer high-quality products and/or services have to satisfy the needs and expectations of different stakeholders and not solely those of customers (Prakash and Mohanty, 2013). Moreover, Majstorovic (2009) suggests considering different stakeholders, such as owners and employees, as different types of customers, placing them on the same level as traditional customers. Interestingly, the ISO (International Organization for Standardization) 9000 Quality Standards highlight that quality organizations not only have to satisfy customers' needs but also those of other interested parties (Vicher, 2012; ISO, 2015). The ISO 9004/2009 Standard defines interested parties as individuals and other entities that add value to the organization or are otherwise interested in or affected by the activities of the organization. Despite the fact that specific industries or sectors of the economy may have to consider different stakeholders, in general terms, shareholders/owners, employees, suppliers/partners, and even the society as a whole are acknowledged as interested parties (ISO, 2005, 2009; Majstorovic, 2009). Likewise, the Excellence Model of the European Foundation for Quality Management (EFQM) also considers that excellent organizations have to meet the needs and expectations of different stakeholders (Michalska, 2008; Ciravegna, 2015; Castaño-Reyes *et al.*, 2017), even including within their model those of employees and the society as a whole, besides customers and owners (Suárez *et al.*, 2014). In acknowledgement of the fact that different stakeholders may have different and even contradictory needs and expectations, the ISO 9000 Standards highlight that the needs of the interested parties have to be met in a balanced way over the long term.

Finally, the last interesting topic of discussion refers to the components of a comprehensive, theoretical model for service quality. In this context, four key elements are identified: enablers, production processes, products, and results. Enablers are what makes quality processes, products, and results possible. The production process refers to how or in which way a product is built or a service shaped and delivered (Golder *et al.*, 2012; Alzaydi *et al.*, 2018). Products are the goods prepared for customer use or the service delivered to them. Lastly, results are what are obtained through the consumption of goods or services, both in the short and long term. These four elements are addressed in different ways by different approaches to quality.

In the context of the manufacturing industry, process and product are usually the core elements of quality. In this line, product quality is assumed to be the result of quality processes that assure conformance to specifications (Prakash and Mohanty, 2013). In the area of health services, processes and results seem to be the focus (Robledo *et al.*, 2012). Here, processes that follow scientific knowledge are expected to lead to health improvements (Cordero *et al.*, 2013; López and Pérez, 2014). From this perspective, patients' satisfaction with the practitioners and the health system is not neglected (García, 2001), but it is not considered to be the principal component of quality health services. In the context of a market-oriented approach to quality, customer satisfaction is paramount (Golder *et al.*, 2012; Polyakova and Mirza, 2015). Within this debate, customer satisfaction (customer understood either in a limited or broader sense) expresses a specific type of result: that is, customers are satisfied with the service or the product they have received.

The EFQM Excellence Model is composed of two types of elements: enablers and results. Enablers are defined in terms of what an organization does and how it does it, and results are what the organization achieves regarding all interested parties (Michalska, 2008; Castaño-Reyes *et al.*, 2017). The fundamental idea of the model is that merely addressing results does not allow companies to understand how product quality is generated (Robledillo and Velázquez, 2013; Saiz and Olalla, 2013). In this sense, enablers are expected to lead to quality results (Ciravegna 2015; Gómez *et al.*, 2015). Using a different terminology, Vicher (2012) describes organizational or process quality as 'internal quality', and Prakash and Mohanty (2013) highlight the importance of increasing the attention we place on the 'how' aspects of service quality (processes) instead of only focusing on the 'what' aspects (the service or product provided). The EFQM model includes five enablers and four types of results. Enablers include leadership, policy and strategy, people, processes, and partnerships and resources, while results are divided in terms of customers, people (employees), society, and organizational results (García, 2001; Suárez *et al.*, 2014). Interestingly, this idea of multiple result areas clearly resembles the existence of multiple parties or stakeholders with different needs, expectations or interests.

Another topic of the literature on quality refers to specificities of services in contrast to manufactured products. As stated before, several authors emphasize that services are generally produced while they are being consumed. Moreover, it has also been highlighted that, in many cases, services are co-produced in the interaction between providers and consumers (Golder *et al.*, 2012; Prakash and Mohanty, 2013; Alzaydi *et al.*, 2018), which may lead suppliers to lose some control over the service they are providing (Polyakova and Mirza, 2015). Interestingly, analyzed from this perspective, the difference between service production processes and service delivery seems to partially lose weight and relevance, making the limit between them somewhat blurry.

Several ideas for developing a RE&AS quality framework can be drawn from this third area of theoretical discussion. Firstly, the concept of enablers emerges as highly useful, given the fact that acknowledging them helps us to understand how quality is generated and thus develop strategies to improve it. Secondly, the idea of understanding service production and service delivery as part of the same process also emerges as a promising tool for making sense of RE&AS quality dynamics, because services provided by extension workers and advisors imply simultaneously producing and delivering them. Thirdly, different authors understand RE&AS quality in terms of farmers' satisfaction or perceived quality. Nonetheless, following the EFQM model, it seems wise to recognize that in RE&AS there are various interested parties (stakeholders) that expect different results. Thus, a RE&AS quality framework should consider farmers or customers' satisfaction as one among other expected results that encompass quality.

Total quality management

Another area of interest for RE&AS is Total Quality Management (TQM). TQM is a holistic management philosophy aimed at obtaining excellent results through continuous organizational improvement (Suárez *et al.*, 2014; Ciravegna, 2015). TQM requires changes in the organizational culture, involvement of all staff, and clear commitment of top management (Santos and Álvarez, 2007). It goes beyond simply assessing quality or organizational results and its focus is the analysis of the whole institutional dynamic and its relationships with the environment in order to address quality in a much broader sense (García, 2001; Saiz and Olalla, 2013). According to Robledillo and Velázquez (2013), TQM studies all the aspects and dynamics of an organization aimed at reaching quality results in a never-ending, continuous process (Prakash and Mohanty, 2013).

Total Quality Management requires identifying and determining the needs and interests of customers and other interested parties and defining the quality policy and the quality objectives of the organization (ISO, 2005). These guidelines allow for periodical self-assessments that compare the organization with a model of excellence, thus leading to the development and implementation of improvement action plans (Majstorovic, 2009; Saiz and Olalla, 2013).

Despite their differences, the ISO 9000 International Standards and the EFQM Excellence Model are two of the most well-known approaches for increasing business and organizational performance (Guix, 2005; Ciravegna, 2015). The EFQM Excellence Model is a non-prescriptive framework based on the identification of key enablers and results for achieving sustainable excellence (Robledillo and Velázquez, 2013; Suárez *et al.*, 2014). In contrast, although ISO 9000 Quality Standards incorporate different principles of excellence business models (Ciravegna, 2015), they are mostly aimed at standard quality management systems (Santos and Álvarez, 2007).

Beyond the interest of the EFQM and ISO 9000 Standards, there is agreement that they are not a panacea and have a number of limitations when attempting to guide organizations along their path to excellence (Kiauta, 2012; Terziovski and Guerrero, 2014; Marimon *et al.*, 2019). Guix (2005) states that the EFQM Model (but also the ISO 9000 Standards) has difficulties addressing issues of technical expertise, such as the case of public health and, of course, RE&AS. Thus, it is clear that companies and organizations can adopt quality models and strategies such as the EFQM or the ISO 9000 Standards, but they should take into consideration that they will have to find their own way of increasing performance and quality (García, 2001; Hernández *et al.*, 2013; Gómez *et al.*, 2015).

Towards a theoretical framework for RE&AS quality

Up to this point, the current literature on RE&AS quality has been analyzed, as well as that addressing quality in general. In this heading, a proposal for a theoretical framework for addressing RE&AS quality is presented. Figure 1 expresses the proposal graphically.

In order to address RE&AS quality, three different, though articulated, elements are considered: the enablers, the process of producing and delivering the RE&AS, and the results obtained from such advice. Some examples are presented in Figure 1. Nonetheless, each extension institution should identify the most relevant components for their situation and context.

The enablers are the factors or processes that limit or facilitate the delivery of a quality RE&AS. Enablers are specifically highlighted in the EFQM excellence model. When reflecting on the quality of RE&AS, we usually tend to focus on the production and delivery process (mainly the relationship and interaction between advisors and farmers, and the content of the advice, etc.) and on the results obtained. Thus, what makes quality RE&AS possible (i.e. enablers) tends to be neglected. In this context, including enablers in the model helps us to acknowledge their relevance as 'roots' of extension service quality. At the same time, it allows us to better identify the reasons for the low quality of RE&AS and the factor(s) that need to be addressed to improve them. Importantly, enablers seem to be multiple, diverse and highly context-dependent. However, identifying them is essential for developing strategies for quality improvement.

The second and third elements of the model are each composed of a set of quality standards. The idea of differentiating between them is to acknowledge the existence of dimensions of quality RE&AS that refer to quality processes (the production of the extension service and its delivery), while other dimensions refer to quality results that are expected to be obtained through the service. Despite finding support within academic literature on the subject, the standards presented in Figure 1 also have to be considered as examples and identified and jointly constructed for each particular institution in its context.

As mentioned above, the second element of the model entails quality standards referring to the process of production and delivering the extension service. Let us remember that the extension service (as many other services) is co-constructed with the customers (mostly farmers but also

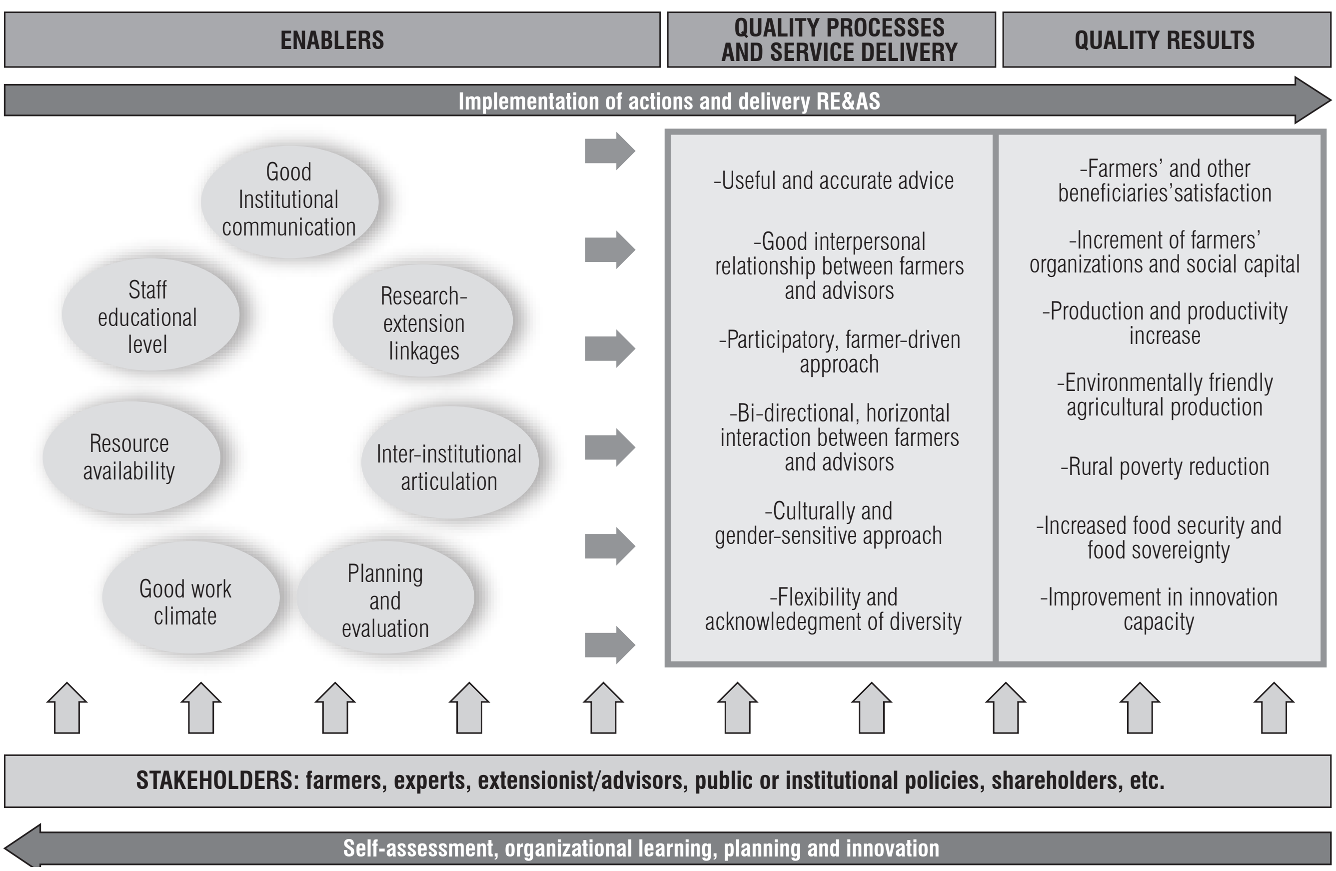

FIGURE 1. Theoretical framework for RE&AS quality.

other stakeholders), and implies that the production and delivery processes cannot be analyzed separately. Briefly, this second component of the model includes aspects related to both the production of the service, such as good interpersonal relationships between extension workers, and to the service itself, for instance usefulness and accuracy of the advice.

Finally, the third element encompasses the expected results of the extension services. Reaching these results also expresses quality. Regarding these quality standards, it is fair to discuss the degree of influence of RE&AS in reaching them. For instance, while being a traditional objective of RE&AS, increasing farmers' productivity is also influenced by the weather and the quality of inputs, among other variables. Importantly, different stakeholders will have to identify and define, among the results under the influence of RE&AS, which ones have to be considered as quality results.

A second aspect of the model refers to the stakeholders or interested parties that should be part of the definition of what quality is when referring to rural extension quality processes, services and results. As stated previously, farmers are not the only ones that can contribute to defining RE&AS quality, nor are extension experts. RE&AS involve different stakeholders whose perspectives have to be considered. In Figure 1, farmers, extension experts and extension staff are included, among others. The stakeholders to be considered as well as the procedures and the degree of their involvement (and power of influence) will vary and will have to be defined in each case, depending on the specific institutional and social context. For instance, relevant stakeholders will differ according to the institutional type (public or private) and the main objective of RE&AS (i.e. increasing farmers' productivity or addressing serious food insecurity situations). Thus, when we have to replace the examples indicated in Figure 1 with enablers and quality standards suitable for a specific context, we will need to identify contextually relevant stakeholders.

Finally, the model also includes two arrows. The first one goes from left to right, expressing the process of extension service delivery, focused on the provision of quality services. The second goes from right to left, expressing the process of quality self-assessment and organizational learning. This means that RE&AS organizations are expected to develop quality improvement strategies that lead to innovations in their service delivery. Analyzing both arrows together, they show a feedback process wherein extension service delivery is evaluated in terms of the presence of the enablers and the fulfilment of quality standards, which leads to a learning, planning and innovational process that will be put into practice in RE&AS delivery in a continuous and never-ending quality improvement loop. These arrows show that quality assessment has to be linked to quality improvement strategies, i.e. evaluate to learn and improve. In this context, self-assessment of the different components of the model and use of Total Quality Management tools can play a key role.

Conclusions

This paper made three main contributions to RE&AS. First, it summarized and discussed scholarly bibliography on RE&AS quality. Surprisingly, and despite the relevance of the topic, no one has written a single article synthesizing and systematically discussing the current literature on the topic until now. Second, some of the most relevant debates on service quality were presented and discussed from the point of view of extension services. Finally, the third and foremost contribution of this paper was its proposal for an integrative theoretical framework to address, manage and improve RE&AS quality.

This paper also led to several interesting reflections and conclusions. First, RE&AS quality has to be conceptualized from different points of view in order to grasp its complexity and multidimensionality. On the one hand, what is described in terms of extension service quality has to be addressed from a perspective of process. Enablers make quality possible. Then, there is the production and delivery of a quality service. And, finally, RE&AS quality also means reaching desired results, including the satisfaction of farmers and other stakeholders. Thus, it is necessary to acknowledge and pay attention to all three of these elements to provide a high-quality extension service, and not merely to one of them. On the other hand, different stakeholders (including farmers) have different expectations, interests and goals, which leads to different perspectives on quality. In consequence, what RE&AS quality is, effectively, will be the result of an agreement or compromise between these different perspectives. Interestingly, it implies that establishing what extension service quality is, is not a technocratic procedure, but a social and complex one that involves negotiation and power issues.

Secondly, these reflections also imply that what defines quality in terms of RE&AS will depend on the particular context at hand, due to the existence of stakeholders with different expectations, interests and goals. Thirdly, following this perspective, extension service quality also ends up being an inter-subjective, socially constructed concept, which implies that a definition of extension service quality cannot be reached without considering the point of view of different stakeholders. Finally, reflections also lead to acknowledging the importance of assessing the quality of extension services as a means for organizational learning and for the implementation of innovative improvement initiatives.

This paper contains multiple statements on RE&AS quality. Nonetheless, it is a simple proposal that requires further scholarly discussion, proving practical usefulness in concrete contexts. In this sense, it seems to be a first step in the right direction, inviting its audience to seriously and systematically discuss RE&AS quality from an integrative perspective.

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SCIENTIFIC NOTE

Biomass of *Crotalaria juncea* as a function of plant densities in the semiarid region of Northeastern Brazil

Biomasa del *Crotalaria juncea* en función de la densidad de plantas en la región semiárida del Noreste de Brasil

Neyton de Oliveira Miranda[1]*, Giulliana Mairana Morais de Sousa Vanomark[2], Isabel Giovanna Costa e Melo[3], and Glêidson Bezerra de Góes[4]

ABSTRACT

An increase in the production per area of sunn hemp (*Crotalaria juncea* L.) biomass in order to expand its beneficial effects as green manure is an objective for the agronomic management of this species. Three experiments were performed during consecutive years to test the following plant densities per m^{-2}: 10, 20, 30, and 40; 25, 35, 50, and 100; 25, 50, 75, and 100. For each density the spacing between the sowing rows and within the rows was equal. The experiments were conducted at Mossoro, RN, Brazil, with a randomized complete block design of four replicates. For each experiment, we determined the shoot dry mass and root dry mass per hectare and the ratio between the values per hectare of shoot dry mass and seed rate. Data were subjected to analysis of variance (F test) and regression analysis. The increasing linear behavior of shoot and root biomass of sunn hemp as a function of plant density establishes a recommendation of 100 plants m^{-2} with equal spacing in the sowing row and between rows. However, the amount of dry mass produced by each kg of seeds decreased to the density of 90 plants m^{-2}. The amount of biomass produced was limited by the length of the vegetative phase of sunn hemp during day length conditions at Mossoro.

Key words: sunn hemp, green manure, cover crops, soil management.

RESUMEN

El incremento de la producción por área de biomasa de cáñamo de la India (*Crotalaria juncea* L.) para aumentar sus efectos beneficiosos como abono verde es un objetivo en el manejo agronómico de esta especie. En este sentido, se realizaron tres experimentos en años consecutivos para probar las siguientes densidades de plantas por metro cuadrado: 10, 20, 30 y 40; 25, 35, 50 y 100; 25, 50, 75 y 100. En cada densidad, el espaciado entre las filas de siembra y dentro de las filas fue igual. Los experimentos se realizaron en Mossoró, RN, Brasil, en un diseño de bloques completos al azar con cuatro repeticiones. En cada experimento se determinaron la materia seca de la parte aérea y la materia seca de la raíz, ambas por hectárea, y la relación entre los valores por hectárea de materia seca de la parte aérea y la tasa de semillas. Los datos se sometieron a análisis de varianza (prueba F) y análisis de regresión. El comportamiento lineal creciente de la biomasa de la parte aérea y raíces de *C. juncea* en función de la densidad de plantas permite la recomendación de 100 plantas m^{-2} con el mismo espacio en la hilera de siembra y entre hileras, pero la cantidad de materia seca producida por cada kg de semillas disminuyó hasta la densidad de 90 plantas m^{-2}. La cantidad de biomasa producida se vio limitada por la duración de la fase vegetativa de *C. juncea* en las condiciones de duración del día en Mossoró.

Palabras clave: cáñamo de la india, abono verde, cultivos de cobertura, manejo del suelo.

Introduction

The need for intensifying food production together with inadequate agricultural practices cause soil degradation. This affects the soil's chemical, physical and biological attributes, accelerates the mineralization of soil organic matter (SOM) and causes a decline in the soil productive potential. These processes occur throughout the season of agricultural production and are of concern in small decapitalized farms (Xavier *et al*., 2017).

In the Northeast region of Brazil, this problem is aggravated by unfavorable chemical characteristics of soils, such as acidity, deficiency of some nutrients and low cation exchange capacity, as well as the sandy texture of many soils (Cavalcante *et al*., 2012; Teodoro *et al*., 2018). Because

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[1] Department of Agronomic and Forest Sciences, Federal Rural University of the Semiarid Region (UFERSA), Mossoro, RN (Brazil).
[2] National Institute for Colonization and Agrarian Reform (INCRA), João Pessoa, PB (Brazil).
[3] Company for the Management of Hydric Resources of the State of Ceará (COGERH), Quixeramobim, CE (Brazil).
[4] Center for Technological Education, College of Technology (CENTEC-FATEC), Quixeramobim, CE (Brazil).
* Corresponding author: neyton.miranda@ufersa.edu.br

of this, obtaining a satisfactory crop yield requires large amounts of expensive fertilizers. Low-income farmers tend to substitute chemical fertilizers with manure, but the amount their animals produce is insufficient for the needs of the crops (Silva *et al*., 2007; Cavalcante *et al*., 2012).

Mitigation of soil degradation can be achieved by more sustainable management practices, such as crop rotation and cultivation of species for green manuring and soil cover (Lima *et al*., 2010; Eiras and Coelho, 2012). Green manures, incorporated into soil management or used to cover the soil (Espíndola *et al*., 2005) protect and enrich the soil with SOM and nutrients and reduce the use of industrialized inputs while mitigating environmental degradation (Mateus and Wütke, 2006). This makes agriculture more socially, economically, and environmentally sustainable, and benefits crop productivity and farmers' income (Cavalcante *et al*., 2012; Xavier *et al*., 2017; Teodoro *et al*., 2018).

Among the species used for green manuring and soil cover, the legumes (Fabaceae) stand out because they form a symbiotic association with bacteria that perform biological nitrogen fixation. As a result, after the cultivation of legumes the plants provide a substantial amount of nitrogen for crops in succession (Espíndola *et al*., 2005). An important characteristic of plants that improve the soil (Xavier *et al*., 2017) is that they promote the economic viability and sustainability of production systems by reducing the use of synthetic nitrogen fertilizers (Perin *et al*., 2010).

After cutting and decomposition, green manures release to the successor crop the nutrients absorbed by their roots from the deep soil layers and that have been accumulated in their shoots (Espíndola *et al*., 2005). This nutrient cycling reduces the use of chemical fertilizers in the main crop and preserves the environment. Other consequences are the increase in soil cation exchange capacity (Cavalcante *et al*., 2012), and higher availability of nitrogen (nitrates) and phosphorus, because the increased SOM can decrease soil phosphorus retention (Espíndola *et al*., 2005).

Among the benefits of biomass added to the soil by green manures are weed control, maintenance of soil moisture and temperature, the promotion of biological diversity, and remediation of degraded areas. An increase in SOM improves soil aggregation, which benefits soil density, soil porosity and water infiltration, while soil cover slows rainwater runoff, reducing soil and water losses by erosion (Espíndola *et al*., 2005; Teodoro *et al*., 2016; Pereira *et al*., 2017).

Sunn hemp (*Crotalaria juncea* L.) is one of the main species of green manure and is adapted to the different soil and climatic conditions in Brazil. It stands out for its large capacity of biomass production, nutrient accumulation and the high quality of its residues, which are important for protecting the soil from erosion and for enriching the soil with nutrients (Xavier *et al*., 2017). Sunn hemp is characterized by rapid growth, tolerance to water stress, low soil fertility requirements, and it is able to fix from 78 to 183 kg ha^{-1} of nitrogen into nitrogen oxides that produce ammonia, nitrites and nitrates. In addition, it can control nematodes and weeds (Espíndola *et al*., 2005; Silva *et al*., 2007; Teodoro *et al*., 2016; Facco *et al*., 2018). Sunn hemp is indicated for remediating degraded areas, including compacted soils (Pacheco *et al*., 2015; Pereira *et al*., 2017).

The increase in sunn hemp biomass production can improve its benefits as green manure such as in weed control and SOM accumulation that are directly related to biomass production per area. Additionally, nitrogen accumulation in the shoots accompanies an increase in biomass (Pereira *et al*., 2005). Perin *et al*. (2010) and Teodoro *et al*. (2016) show that phosphorus and magnesium accumulation by sunn hemp is more influenced by the amount of biomass produced than by the content of these nutrients in the biomass, while calcium accumulation is influenced by both higher biomass production and its higher content in biomass.

The objective of this work was to test increasing sunn hemp plant densities to identify the densities producing maximum shoot and root biomass and to determine sunn hemp densities that provide the maximum biomass production per kilogram of seed used.

Materials and methods

This research was carried out in the vegetable garden of the Federal Rural University of the Semi-arid Region (5°12'26" S, 37°24'06" W), in the municipality of Mossoro, located in the state of Rio Grande do Norte, in northeastern Brazil. The climate of the region, using the classification of Thornthwaite, is semiarid, megathermal with a water deficit during the year. The mean annual precipitation is 673.9 mm of which about 550 mm occur in the period from February to May.

Sowing was carried out during the months of August, September and October of the years 2009, 2010 and 2011. The climatological normals for these months for the municipality of Mossoro are presented in Table 1.

TABLE 1. Climatological normals for months of the second semester in the municipality of Mossoro, RN, Brazil.

Parameter	August	September	October	November	December
Potential evapotranspiration (mm)	186.1	202.2	223.7	218.2	234.7
Mean temperature (°C)	27.7	28.3	28.7	28.7	29.0
Maximum temperature (°C)	34.3	35.0	35.1	34.9	34.8
Minimum temperature (°C)	21.7	22.6	23.5	23.7	24.2
Relative humidity (%)	61.7	60.1	61.7	64.5	65.1
Monthly sunshine duration (h)	8.9	9.6	9.9	9.8	8.8
Rainfall accumulated (mm)	7.2	1.8	2.4	1.7	14.3
Wind speed (m s^{-1})	4.7	5.2	5.3	5.1	5.0

Source: INMET (2018).

The soil of the area is classified as an eutrophic red-yellow argisol (Santos *et al*., 2013) whose main characteristics are: pH - 6.22; CE - 0.07 dS m^{-1}; Ca - 1.42 cmolc dm^{-3}; Mg - 0.93 cmolc dm^{-3}; K - 21.57 mg dm^{-3}; Na - 3.85 mg dm^{-3}; P - 22.73 mg dm^{-3}; N - 0.27 g kg^{-1}; Sand - 900 g kg^{-1}; Silt - 70 g kg^{-1}; Clay - 30 g kg^{-1}.

The experiments were set up as a randomized complete block design with four replicates. The experimental plots had a useful area of one m^2. The treatments consisted of plant densities of sunn hemp m^{-2} that changed each year according to the previous results. The plant densities in each year were: 10, 20, 30, and 40; 25, 35, 50, and 100; 25, 50, 75, and 100 plants m^{-2}. For each plant density, the hills were arranged at equal distances in the row and between rows as follows: 10 - 31.6 x 31.6 cm, 20 - 22.4 x 22.4 cm, 25 - 20.0 x 20.0 cm, 30 - 18.3 x 18.3 cm, 35 - 16.9 x 16.9 cm, 40 - 15.8 x 15.8 cm, 50 - 14.1 x 14.1 cm, 75 - 11.5 x 11.5 cm, and 100 - 10.0 x 10.0 cm. This arrangement was made possible by a cardboard template in which holes were drilled according to each plant density.

Seeds were purchased from a commercial seed producing company and the mass of 1000 seeds was 45 g. The second experiment was fertilized with one kg of chicken manure m^{-2}, mixed superficially. The manure had N, P and K contents of 9.19, 1.85 and 3.04 g kg^{-1}, respectively.

Control of weeds was performed manually whenever necessary, and irrigation was performed daily using a microsprinkler system. Irrigation depths applied were 270 mm in the first experiment, 310 mm in the second, and 330 mm in the third experiment.

Biomass production was determined at full flowering, at which time all plants in the plot were removed with the aid of a cutting shovel to a depth of 30 cm, in order to promote minimum loss of the root system. Posteriorly, the plants were cut at the stem base to separate the shoot from the root system, which were weighed separately. Five plants were randomly withdrawn from each plot. These were dried in a forced air circulation oven (model TE-394/500L, Tecnal Equipamentos Cientificos in Piracicaba, SP, Brazil) at 65°C until a constant weight was obtained. The calculated dry mass per plot of roots and shoot was expressed in t ha^{-1}.

The ratio between the shoot dry mass and the seed rate, was determined in kg ha^{-1} considering the following densities in plants m^{-2} and seed rates: 10 - 4.50 kg ha^{-1}, 20 - 9.00 kg ha^{-1}, 25 - 11.25 kg ha^{-1}, 30 - 13.50 kg ha^{-1}, 35 - 15.75 kg ha^{-1}, 40 - 18 kg ha^{-1}, 50 - 22.50 kg ha^{-1}, 75 - 33.75 kg ha^{-1}, and 100 - 45 kg ha^{-1}.

Data of the shoot dry mass (SDM) and root dry mass (RDM), both expressed in t ha^{-1}, and the ratio between shoot dry mass and seed rate were subjected to an analysis of variance. The significance of the effect of plant densities was determined by the F test. In case of a significant effect, the data were subjected to a regression analysis, whose model was chosen by the highest coefficient of determination. The statistical software used was SISVAR (Ferreira, 2011).

Results and discussion

The flowering of sunn hemp occurred at 49, 55, and 63 days after plant emergence (DAE) in the first, second, and third experiments respectively. The plant density per area promoted a significant effect ($P<0.01$) on sunn hemp shoot dry mass (SDM) in all three experiments. The averages obtained were 3.39 t ha^{-1} in the first experiment, 4.92 t ha^{-1} in the second and 2.07 t ha^{-1} in the third experiment. These results are much lower than the biomass production potential mentioned by Wutke *et al.* (2014), which is greater than 15 t ha^{-1}.

The time to flowering and the SDM observed in our study were closer to those obtained in studies conducted in northeastern Brazil, where the day length differs little from that observed in Mossoro. In the state of Alagoas (9°45’ S; 35°38’ W), sunn hemp flowering occurred at 65 d and 3 t ha^{-1} of SDM were obtained in a cycle between May and September (Cavalcante *et al*., 2012). In the state of Paraiba (7°19’ S; 33°51’ W), flowering occurred between 50 and 55 d and the highest SDM obtained ranged from 5.4 to 6.7 t ha^{-1} during five consecutive years with sowings between February and March (Silva *et al*., 2007). In the state of Piaui (3°05’ S; 41°46’ W), Teodoro *et al*. (2018) observed a period of 67 d until flowering, but the SDM of 9.8 t ha^{-1} was obtained because the plants were cut at 100 DAE. The higher SDM (12.82 t ha^{-1}) obtained by Pereira *et al*. (2016) in the state of Ceara (5°07’ S; 37°05’ W), at a distance of 90 km from Mossoro, is due to the harvesting of plants at the end of the cycle (78 DAE).

The previous discussion is confirmed by the fact that our results were closer to those obtained when the sowing was carried out in the autumn-winter season in southern regions of the country. The period until sunn hemp flowering in our study (49 to 63 d) was similar to the sowings in the month of March in São Paulo (24°35’ S, 47°50’ W), when days are decreasing in duration (Lima *et al*., 2010). The SDM obtained by us was smaller than that obtained when the sowing occurred in November in that state (15.6 t ha^{-1}), and the time to flowering was 116 d. According to Lima *et al*. (2010), the greater day length in São Paulo between November and January delays flowering and allows greater accumulation of SDM. There is no time for further accumulation of SDM under the photoperiod conditions of the Northeast region, where flowering occurs earlier.

The effect of photoperiod variation on sun hemp development is confirmed by differences in SDM and time to flowering due to the sowing date in the state of Rio de Janeiro (22°46’ S, 43°41’ W). In this location, sowing in autumn-winter or spring-summer resulted in flowering at 60 or 125 d and SDM of 6.8 t ha^{-1} or 10.7 t ha^{-1}, respectively (Pereira *et al*., 2005). The variation was also seen in the state of Goias (16°43’ S, 49°07’ W), when sowing in the months of November or March was compared (Amabile *et al*., 2000), which resulted in decreasing the time until flowering from 118 to 67 d, with a consequent decrease in SDM from 17 t ha^{-1} to 6 t ha^{-1}. This was slightly higher than that obtained in our study.

The possibility that the low sunn hemp SDM values in our study are due to the anticipation of flowering in our region is confirmed by studies with sunn hemp in the state of Rio Grande do Sul. In one locality (29°42’ S, 53°49’ W), Facco *et al*. (2018) observes that the time until flowering increased from 97 to 110 d, when sowing is performed in October or December, months in which there is an increase in day length. In another location (29°05’ S, 53°12’ W), Pereira *et al*. (2017) observes 120 d until flowering and obtains SDM of 22.71 t ha^{-1} when sowing is performed in December.

The importance of the length of day and night in determining vegetative growth and induction of sunn hemp flowering is stated by Teodoro *et al*. (2018). The development of species like this one is influenced by the interaction between photoperiod and temperature, the sowing date and the latitude (Amabile *et al*., 2000). Sunn hemp is a photoperiod-sensitive species and its flowering is induced by the shortening of day length, resulting in a decrease in biomass production (Lima *et al*., 2010; Eiras and Coelho, 2012). In Mossoro, the length of the day varies slightly, ranging from 11.8 h in June to 12.4 h in December, which is the photoperiod range in which Santos and Campelo Júnior (2003) observe the shortest time until sunn hemp flowering.

The SDM of sunn hemp presented positive linear behavior ($P<0.01$) as a function of plant density per area (Fig. 1). This result is corroborated by other studies, like that of Eiras and Coelho (2012), who observe an increase in SDM as plant density increased from 20, 30, 40, 50 and 60 plants m^{-2}, keeping 50 cm between rows. With the same spacing, Teodoro *et al*. (2016) obtain an increase in SDM with higher densities of 20, 40 and 60 plants m^{-2}. In the study by Pereira *et al*. (2005), the highest SDM is obtained both by increasing the number of plants in the row (5, 10, 20 and 40) as well as by decreasing the row spacing (120, 60 and 30 cm). As plant densities tested by Eiras and Coelho (2012) result in similar dry mass per plant, the increase in plant density per area is determinant for the increase in SDM, as stated by Teodoro *et al*. (2016). Similarly, in our study the SDM increased with higher plant density per area, but SDM per plant decreased.

The fact that the highest SDM observed by Pereira *et al*. (2005) in autumn-winter (6.8 t ha^{-1}) is obtained with 133 plants m^{-2}, while in spring-summer the highest SDM (10.7 t ha-1) is obtained with 100 plants m^{-2}, indicates that in spring-summer the plants develop more, and from the density of 100 plants m^{-2} the competition among them for water, light and nutrients limits the development. This is explained by Lima *et al*. (2010), who point out that the interception of solar radiation by the leaves of the crop canopy and the use of this intercepted radiation are the main

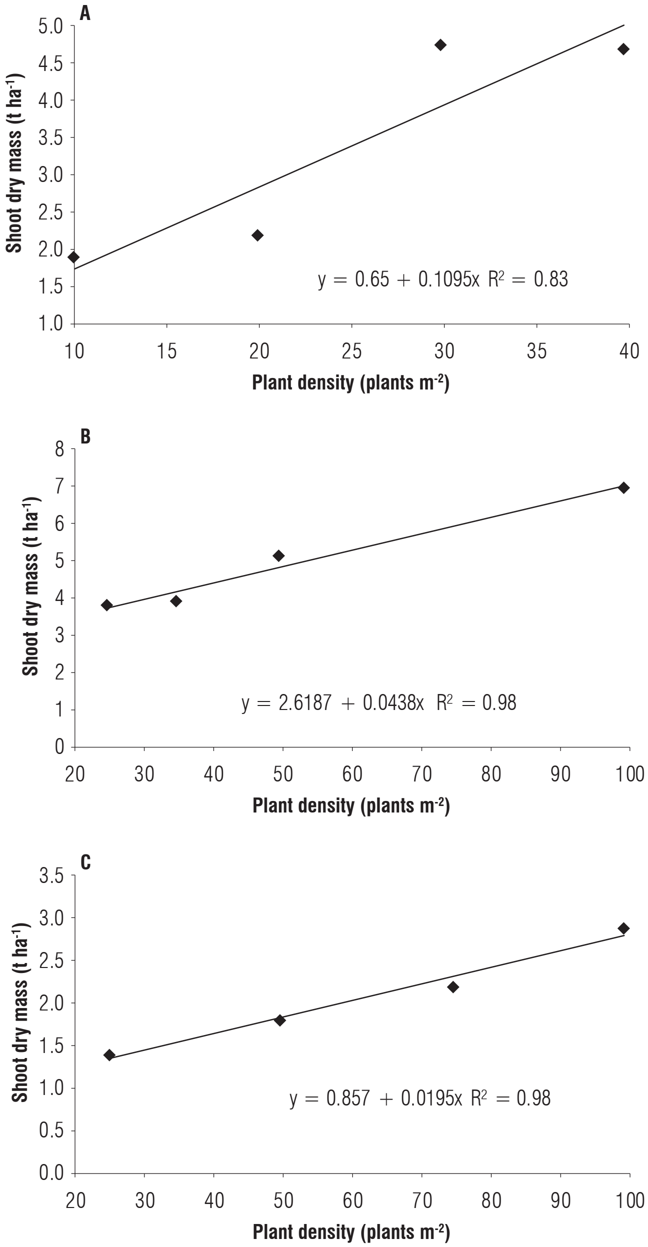

FIGURE 1. Shoot dry mass of sunn hemp as a function of the plant density per area in the first (A), second (B), and third (C) experiments.

factors determining the accumulation of SDM. According to Pereira *et al*. (2005), a higher SDM can be obtained with higher plant density in the row and lower row spacing. This supports the results obtained in our study, in which the row and in-row spacing were equal. In addition, the early flowering may have limited plant growth, so that in none of the densities studied by us did the plant development cause competition for solar radiation or impaired its interception.

In relation to our work, in which the increase in plant density per area promoted an increase in SDM, the importance of maintaining equal spacing in the row and between rows was demonstrated in two papers in which densities of 50 or 62.5 plants m^{-2} were tested, which were obtained with a row spacing of 50 or 40 cm and 25 plants m^{-1} of row. In the study of Lima *et al*. (2010), the increase in plant density per area causes a reduction in SDM, while no effect of increasing density is observed by Amabile *et al*. (2000), who explain that the arrangement of plants studied did not influence competition between plants for light, water and nutrients.

The even interception and use of solar radiation throughout the sunn hemp canopy in our study may have played an important role in dry mass accumulation, even with increasing plant densities. In this sense, Lima *et al*. (2010) argue that the smaller row spacing favors shading within the canopy affecting leaf area. This may impair the accumulation of dry mass by sunn hemp plants. These authors reduce row spacing to obtain an increase in plant density from 50 to 62.5 plants m^{-2} and obtain a reduction in SDM from 15.6 t ha^{-1} to 14.3 t ha^{-1}. However, they do not obtain differences in SDM when the same density (50 plants m^{-2}) is obtained by varying the row spacing (50 or 40 cm) and the number of plants m^{-1} of row (25 or 20).

Plant density per area also promoted a significant effect (P<0.01) on sunn hemp root dry mass (RDM) in all three experiments. The averages obtained were 0.91 t ha^{-1} in the first experiment, 0.83 t ha^{-1} in the second, and 0.26 t ha^{-1} in the third. The values of RDM obtained in our study were smaller than those obtained by Teodoro *et al*. (2016), which range from 1.0 to 1.16 t ha^{-1} at the end of the cycle. In the study by Teodoro *et al*. (2018), the RDM that they obtain (1.70 t ha^{-1}) with a density around 50 plants m^{-2} is considered underestimated because roots are removed only up to 20 cm due to the difficulty of collecting the entire root system. In our research, the excavation sought to reach the depth of greater root concentration, around 30 cm, where 79% of the sunn hemp roots are located, according to Teodoro *et al*. (2018).

The results obtained in the three experiments indicated a linear increase in sunn hemp root dry mass (Fig. 2) with the increase in plant density per area. Similarly, although not statistically significant, Teodoro *et al*. (2016) obtains an increase in RDM as they progressively increase the density of 10, 20 and 30 plants m^{-1} of row, maintaining a row spacing of 50 cm.

Besides the shoot dry mass, it is important to consider the root system of green manure species, which is rarely quantified because even species that do not stand out for

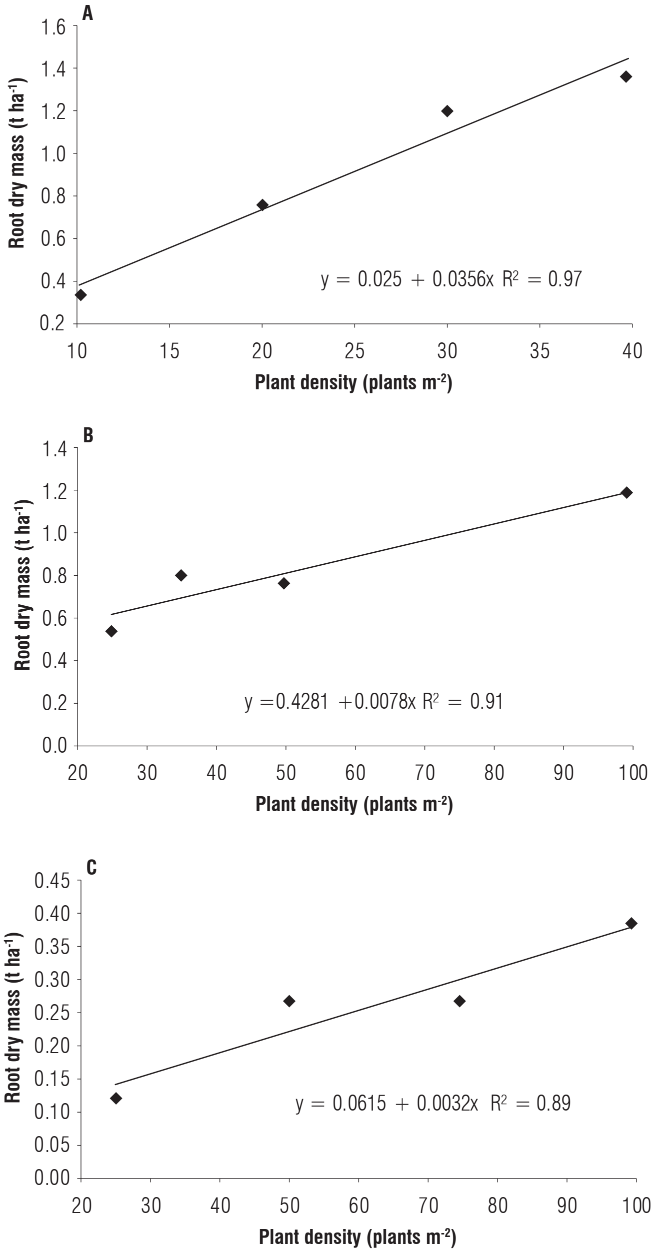

FIGURE 2. Dry mass of roots of sunn hemp as a function of the plant density per area in the (A) first, (B) second and (C) third experiments.

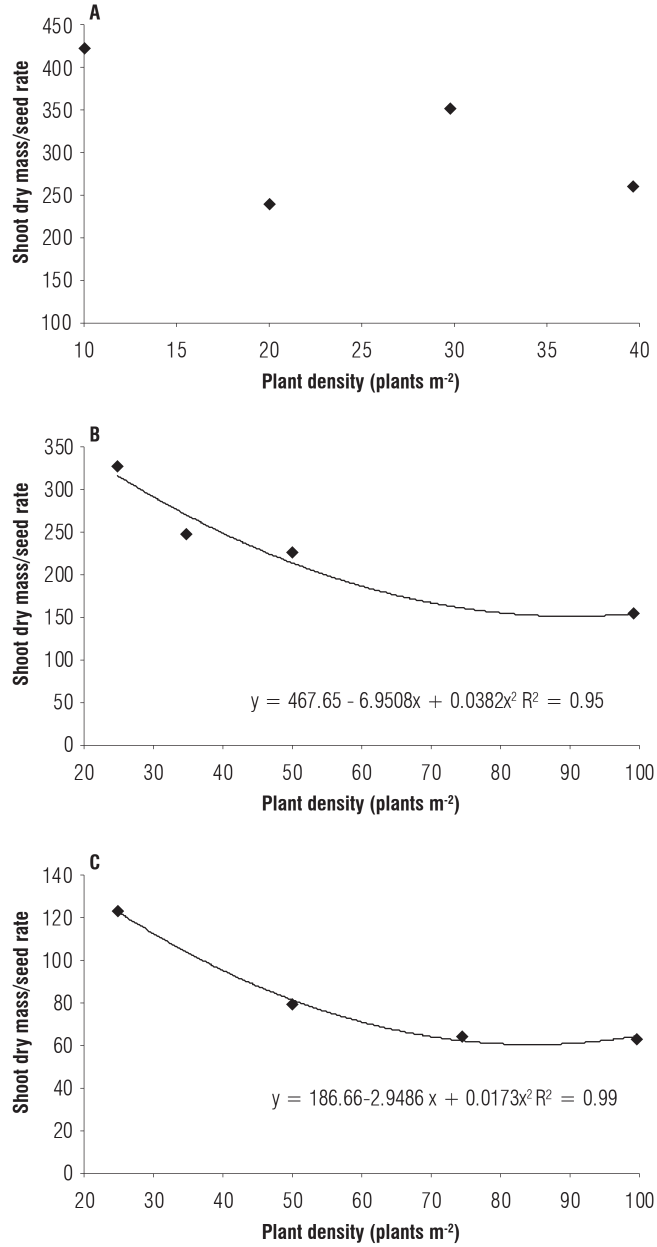

FIGURE 3. Ratio between shoot dry mass and the seed rate of *Crotalaria juncea* in the (A) first, (B) second and (C) third experiments.

the production and quality of the shoot biomass can be very efficient in improving the physical quality of the soil due to a well-developed root system (Xavier *et al*., 2017). Species with high root growth benefit agricultural production in compacted soils because they form channels in the soil profile that favor the root growth of crops in succession (Pacheco *et al*., 2015).

When analyzing the ratio between the shoot dry mass and seed rate in the second and third experiments, we observed a significant effect of plant density per area (P<0.01) and a non-significant effect in the first experiment. Each kilogram of seed produced an average of 319.75, 239.90, and 83.54 kg of dry mass in the first, second and third experiments, respectively.

Analyzing Figure 3, in which the seed rate is represented by the corresponding number of plants m^{-2}, we observed that the shoot dry mass produced by each kg of seeds decreased in a quadratic way as a function of the plant density in the second and third experiments. The point of inflection is at 90 plants m^{-2}. Eiras and Coelho (2012) observe that, in spite of the increase of dry mass per area of sunn hemp due to the increase in plant density, the dry mass of each plant is similar among the densities, while in our work there was a decrease in dry mass of each plant as the density increased up to 90 plants m^{-2}. Therefore, taking into account the possibility of increasing biomass production by increasing plant density, it is important to consider seed availability and cost (Mateus and Wutke, 2006).

The small return in biomass production relative to the higher seed cost when increasing sowing density can discourage small and medium farmers from using higher sunn hemp plant densities. Therefore, it would be important for farmers to produce their own seed (Eiras and Coelho, 2012). In this sense, Pereira *et al*. (2011) comment that the practice of green manuring, despite its advantages, has been little-used by farmers. The lack of immediate economic return and the area that must be occupied by these crops are among the main causes for the lack of adoption.

Conclusion

The increasing linear behavior of shoot and root biomass of sunn hemp as a function of plant density supports a recommendation of 100 plants m^{-2} with equal spacing in the sowing row and between rows.

The amount of shoot dry mass produced by each kilogram of seeds decreased up to the density of 90 plants of sunn hemp m^{-2}.

The amount of biomass produced was limited by the length of the vegetative phase of sunn hemp at day length conditions found at Mossoro, RN, Brazil.

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Editorial policy

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Issued as a four monthly journal, it is intended to transfer research results in different areas of tropical agronomy. Original unpublished papers are therefore accepted in the following areas: physiology, crop nutrition and fertilization, genetics and plant breeding, entomology, phytopathology, integrated crop protection, agro ecology, weed science, environmental management, geomatics, soils, water and irrigation, agroclimatology and climate change, post-harvest and agricultural industrialization, rural and agricultural entrepreneurial development, agrarian economy, and agricultural marketing.

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In a preliminary review stage, two aspects, English grammar and scope, will be assessed in order to ensure minimum requirements of any manuscript to be peer reviewed. If the manuscript is rejected in the preliminary review because English grammar aspect, authors are encouraged to edit for language their manuscript using professional services and submit the manuscript again. When reason for preliminary reject is a mismatch of the scope between the manuscript and journal interests, resubmission should be avoided.

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- ***Scientific and technological research papers:*** those documents presenting detailed original research results. The most generally applied structure has four main parts: introduction, materials and methods, results and discussion, and conclusions.
- ***Scientific note:*** brief document introducing original preliminary or partial results of a scientific or technical research, which usually need immediate publication.

Format and organization of the text

Research article length should not exceed 5,200 words, whereas scientific notes should not have more than 4,000 words. Tables and figures, that is to say, diagrams, drawings, schematic and flow diagrams, pictures and maps, should be consecutively numbered (Table 1 ...Table n; Figure 1... Figure n, etc).

Texts and tables should be prepared using an MS-Word® processor. Double-space all text including table head, figure captions and cited literature. All pages must be numbered consecutively and line numbering on each page is mandatory, Tables and diagrams of frequency (bar and circular diagrams) should be included in the mentioned Word file, as well as in their original MS-Excel® or any different format. Other figures, including photographs and drawings should be submitted in digital JPG (or JPEG) digital compression format, with a minimum resolution of 300 dpi.

As a general rule, tables and figures should only be submitted in black and white, except for those intended for the cover page of the Journal, for those cases in which it is absolutely necessary to present them in color (at the judgment and discretion of the editor), or when the costs of color publication are covered by the authors.

Languages, units, and style

The journal's official language is English. Regarding measurement units, the metric system (SI) should be consistently used through the manuscript, unless the need is seen to apply any specific units that are of frequent use by the scientific community. Multiplication followed by negative superscript (e.g., kg ha-1) can only be used with SI units. The slash (/) is a mathematical operation symbol that indicates "divided by". Anyway, in sciences it is used as a substitute of the word "per", and it is used to indicate rates. Use the slash to connect SI to non-SI units (e.g., 10°C/h or 10 L/pot).

All abbreviations should be explained in full length when first mentioned in the manuscript.

With regards to the tenses, the most commonly used ones are the past, for the introduction, procedures and results; and the present, for the discussion.

Title and authors

The title in English, as well as its corresponding Spanish translation, shall not exceed 15 words. The scientific names of plants and animals shall be italicized and lower cased, except for the first letter of the genus (and of the species author), which must be upper cased.

The authors (including first and second names) shall be listed in order of their contribution to the research and preparation of the manuscript, in completely justified text format (filling the whole line, or, if necessary, the next one below) under the translated version of the title. At the bottom of the article's first page include only the name and city location of the employer or supporting institution(s), and the e-mail address of the corresponding author.

Abstract, resumen, and key words

The abstract should be written in English with Spanish translation for the Summary. Both texts should contain brief (no longer than 200 words in a single paragraph) and accurate descriptions of the paper's premise, justification, methods, results and significance. Both language versions shall be mandatorily provided with a list of (maximum six) key words that have not appeared in the title or abstract, and included in the Agrovoc thesaurus by Agris (FAO).

Introduction

In the introduction, include the delimitation and current status of the problem, the theoretical or conceptual basis of the research, the literature review on the topic, and the objectives and justification of the research. Common names must be accompanied with the corresponding scientific ones, plus the abbreviation of the species author surname when mentioned for the first time.

Materials and methods

Besides a clear, precise and sequential description of the materials used for the research (plant or animal materials, plus agricultural or laboratory tools), this section illustrates the procedures and protocols followed, and the experimental design chosen for the statistical analysis of the data.

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Results and discussion can be displayed in two different sections or in a single section at the authors convenience. The results shall be presented in a logical, objective, and sequential order, using text, tables (abbreviated as Tab.) and figures (abbreviated as Fig.). The latter two should be easily understandable and self-explaining, in spite of having been thoroughly explained in the text. The charts should be two-dimensional and prepared in black and white, resorting to a tone intensity degradation to illustrate variations between columns. Diagram curves must be prepared in black, dashed or continuous lines (- - - - or ———), using the following conventions: ■, ▲, ◆, ●, □, △, ◇, ○. The tables should contain few columns and lines.

Averages should be accompanied by their corresponding Standard Error (SE) values. The discussion shall be complete and exhaustive, emphasizing the highlights and comparing them to the literature.

This section should briefly and concisely summarize the most important findings of the research.

Conclusion (optional)

A short conclusion section is useful for long or complex discussion. It should provide readers with a brief summary of the main achievements from the results of the study. It also can contain final remarks and a brief description of future complementary studies which should be addressed.

Acknowledgements

When considered necessary, the authors may acknowledge the researchers or entities that contributed - conceptually, financially or practically - to the research: specialists, commercial organizations, governmental or private entities, and associations of professionals or technicians.

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  E.g., Dubey, N.K. (ed.). 2015. Plants as a source of natural antioxidants. CABI, Wallingford, UK. Doi: 10.1079/9781780642666.0000.

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AGRONOMIA COLOMBIANA

P.O. Box 14490, Bogota-Colombia

Phones: (571) 316 5498 / (571) 316 5000 ext. 19095

Fax: (571) 316 5176

E-mail: agrocol_fabog@unal.edu.co

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