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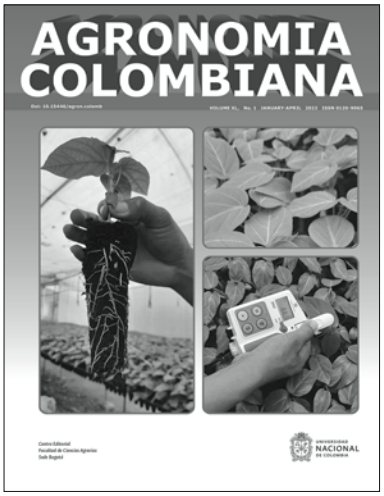

Our cover:

Effect of biochar use as a substrate on granadilla (*Passiflora ligularis* Juss.) growth parameters

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Selection of *Myrciaria dubia* clones under conditions of the savanna/forest transition of Roraima through multivariate analysis

Selección de clones de *Myrciaria dubia* en condiciones de transición sabana/bosque en Roraima mediante análisis multivariado

Roberto Tadashi Sakazaki[1], Edvan Alves Chagas[2], Carlos Abanto-Rodríguez[3*], Pollyana Cardoso Chagas[1], Maria da Conceição Rocha de Araujo[1], João Luiz Lopes Monteiro Neto[1], Maria Luiza Grigio[1], Luiz Guilherme Carvalho Zborowski[1], Raphael Henrique da Silva Siqueira[4], and Jorge Zamir Erazo Amaya[5]

ABSTRACT

Camu-camu (*Myrciaria dubia*), a fruit from the Amazon region, has received attention due to its high content of antioxidant compounds such as ascorbic acid and polyphenols. This study's objective was to select camu-camu clones according to their morphological characteristic using multivariate analyses. We evaluated 56 camu-camu clones distributed in three blocks with two plants per experimental plot. The variables measured were plant height (H), number of basal branches (NBB), number of terminal buds (NTB), basal stem diameter (BSD), chlorophylls a (Chl a) and b (Chl b), and total chlorophyll (Chl a + b). Principal component (PCA) and multivariate clustering analyses were performed using the average linkage mean and Mahalanobis distance algorithms. After 24 months, results showed that the characteristic that least contributed to clone selection was NBB. The plant height (H), number of terminal buds (NTB), basal stem diameter (BSD), chlorophylls a (Chl a) and b (Chl b), and total chlorophyll (Chl a + b) had the most positive contribution towards the initial selection of camu-camu clones at two years of age. The 22 camu-camu clones showed the highest rate of vegetative development or vegetative quality index (VQI) in the transitional savanna/forest area of the northern Amazon, Brazil, after two years of transplanting.

Key words: clonal tests, domestication, genetic improvement, camu-camu, native fruit.

RESUMEN

El camu-camu (*Myrciaria dubia)* es una fruta de la región amazónica que ha recibido atención debido a su alto contenido de compuestos antioxidantes, como el ácido ascórbico y los polifenoles. El objetivo del estudio fue seleccionar clones de camu-camu según sus características morfológicas mediante análisis multivariados. Se evaluaron 56 clones de camu-camu distribuidos en tres bloques con dos plantas por parcela experimental. Las variables medidas fueron altura de la planta (H), número de ramas basales (NRB), número de brotes terminales (NBT), diámetro basal del tallo (DT), clorofila a (Chl a) y b (Chl b) y clorofila total (Chl a + b). Los análisis de agrupamiento de componentes principales (PCA) y multivariados se realizaron utilizando la media de enlace y los algoritmos de distancia de Mahalanobis. Después de 24 meses, los resultados mostraron que la característica que menos contribuyó a la selección del clon fue el NRB. La altura de la planta, número de ramas basales (NRB), diámetro basal del tallo (DBT), clorofila a (Chl a) y b (Chl b) y clorofila total (Chl a + b) tuvieron la contribución más positiva hacia la selección inicial de clones de camu-camu a los dos años de edad. Veintidós clones de camu-camu presentaron la mayor tasa de desarrollo vegetativo o índice de calidad de la vegetación (ICV) en la sabana de transición/área forestal del norte del Amazonas, Brasil, después de dos años de trasplante.

Palabras clave: pruebas clonales, domesticación, mejoramiento genético, camu-camu, fruta nativa.

Introduction

Camu-camu (*Myrciaria dubia* (Kunth) McVaugh) is a perennial species native to the Amazon basin that stands out for having high concentrations of vitamin C (7.36 g/100 g of pulp) (Chagas *et al*., 2015) and other antioxidant compounds, such as ellagic acids, ellagitannins, proanthocyanidins, epicatechin, catechin, delphinidin 3-glucoside, cyanidin 3-glucoside, and rutin (Chirinos *et al*., 2010; Fracassetti *et al*., 2013; Fidelis *et al*., 2020). The pulp can be processed into carbonated drinks, concentrated juices, vinegar, ice cream, candies, pills, energy drinks, creams,

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colognes, among others (Yuyama *et al*., 2011; Grigio *et al*., 2021).

Due to the importance of the species, the Brazilian Agricultural Research Corporation, in partnership with the Federal University of Roraima, has been conducting studies on prospection, selection, collection, cloning, and clonality testing with the purpose of domestication, conservation, and cultivation of the species in ecosystems other than their natural habitat. The search for quality genetic material has been challenging for researchers due to the significant genetic variability of wild populations caused by pollination, with 91% allogamy and 9% autogamy, giving rise to extensive heterogeneity in the agronomic characteristics of interest in production (Vásquez, 2000).

Recommendations suggest working with an adequate number of genetic materials to avoid the reduction of variability that may lead to future problems of vulnerability to pests and diseases. Assessing the plant initial developmental characteristics allows us to determine the growth efficiency and the plant ability to adapt to environmental conditions. However, in most cases, selection based on one or a few characteristics may be inadequate since it must be carried out with superior products selected using several morphological characters (Costa *et al*., 2005; Faleiro & Junqueira, 2011). Thus, the isolated evaluation of growth characters cannot be accepted for assigning a quality index or merit selection, confirmation, or recommendation of quality clones.

Similarly, according to Ventura *et al*. (2012), it is difficult to define which characteristics should be evaluated for the improvement programs. Therefore, the authors suggest using multivariate analysis techniques to determine possible correlations and, thus, identify those responsible for most of the total variation observed. These techniques are useful for evaluating individuals in several aspects, providing a holistic view of each genotype (Cruz *et al.,* 2012).

Another technique used is grouping analysis that aims to gather and classify the genotypes in several groups, following some similarity or dissimilarity criteria based on morphological and agronomic characters (Araújo *et al*., 2002; Alves *et al*., 2003; Martel *et al*., 2003).

The objective of this research was to select camu-camu clones with superior initial development using multivariate techniques at two years of planting under savanna/forest transition conditions of Roraima, northern Amazon.

Materials and methods

The research was conducted at the "Serra da Prata" Experimental Field of the Brazilian Agricultural Research Company, Embrapa-RR, located in the municipality of Mucajaí, at 60°58'40" W and 2°23'49" N, representative of the savanna/forest transition area of the state of Roraima. Historically, the site had been fallowed for four years, with a monthly clearing of spontaneous vegetation.

According to the Köppen classification system, the climate is Am type, with a short dry season. The longest rainy season is from May to July, while the shortest is from October to March, with an annual average of 1,844 mm (Mourão *et al*., 2003).

We used 56 camu-camu clones from 11 native populations characterized and selected from December 2014 to April 2015 by Chagas *et al*. (2015). The clones were produced by rooting piles in sub-irrigation chambers, following the method described by Abanto *et al*. (2014). After 12 months, when the clones had an average height of 80 cm, they were transplanted to the experimental field (June 2016).

The clones used were: 1) AB-04; 2) AB-05; 3) AB-06; 4) AB-07; 5) AB-08; 6) ABU-01; 7) ABU-02; 8) AT-03; 9) AT-07; 10) AT-08; 11) AT-10; 12) AT-13; 13) BQ-03; 14) BQ-04; 15) BQ-12; 16) BQ-18; 17) BQ-26; 18) BQ-27; 19) BQ-28; 20) BQ-29; 21) BQ-32; 22) EV-06; 23) EV-07; 24) EV-09; 25) EV-10; 26) IAB-01; 27) IAB-02; 28) IAB-04; 29) IAB-05; 30) IAB-06; 31) IAB-07; 32) LM-08; 33) LM-26; 34) LM-27; 35) LM-29; 36) LM-30; 37) LM-31; 38) LM-39; 39) LM-47; 40) LR-03; 41) LR-04; 42) LR-05; 43) LR-11; 44) LR-12; 45) MU-06; 46) MU-11; 47) MU-16; 48) MU-17; 49) RQ-04; 50) RQ-11; 51) RQ-14; 52) RUR-01; 53) RUR-02; 54) RUR-03; 55) RUR-04, and 56) RUR-05.

The area chosen for the clonality test was 5,040 m^2. Before the crop was established, the area was cleared, plowed, and gridded so that all the vegetal biomass remaining in the superficial layer of the soil was incorporated. The level of soil fertility was very low, with a low sum of basic exchangeable cations (S) ranging from 0.1 to 0.8 $cmol_c kg^{-1}$ of soil; the cation exchange capacity (CEC) was lower than 2.1 $cmol_c$ kg^{-1} of soil; the low base saturation ranged from 7% to 46%, and the aluminum saturation was greater than 50% in most profiles (Rodrigues *et al*., 2000). Therefore, 1,600 kg ha^{-1} of dolomitic limestone was applied of which 1,550 kg ha^{-1} was applied to the set, and 50 kg ha^{-1} was applied to the pits (150 g/plant). The pits were opened with dimensions of 0.40 m x 0.40 m and received 150 g dolomitic limestone 30 d

before transplanting. At the time of transplanting, 67 g of urea, 110 g of Super Triple fertilizer, 103 g of KCl, and 10 g of slow release micronutrient (FTE BR-12®, Nutriplant, Brazil) were applied per pit. In the second year of cultivation, 111 g of urea, 122 g of Super Triple, and 121 g of KCl were applied.

Drip irrigation was used; the emitters had a flow rate of 3.4 L h^{-1} with a 50 cm spacing. A Rain Bird® controller (Rain Bird, Azusa, CA, USA) automatically activated the irrigation system. Weed control was performed using a brushcutter, manually in the planting rows and attached to a tractor between rows.

The camu-camu clones were transplanted at 5 m x 3 m spacing (5 m between rows and 3 m between plants), arranged in a random block delineation with three blocks and two plants per experimental unit. The evaluation of the vegetative characteristics was carried out 12 months after planting.

The variables measured were as follow: plant height [H (m)], number of basal branches (NBB), number of terminal buds (NTB), basal stem diameter [BSD (mm)], contents of chlorophyll a (Chl a) and b (Chl b), and total chlorophyll (Chl a + b). Chlorophyll a and b were determined with the Falker clofiLOG® chlorophyllometer. The measurement was made on 6 leaves from the middle part of the main branch of all the clones; the measurement was not performed on the leaves of the apical and basal part of the branch.

Principal component analysis was performed (PCA) and conglomerate (cluster) analyses based on Mahalanobis genetic distance (D2), both performed using Infostat software (Di-Rienzo, 2008). Additionally, the vegetative quality index (VQI) of the camu-camu clones was determined using the joint analysis of H, NTB, BSD, and Chl a. The reference parameters were adopted from those reported by Abanto *et al.* (2011) and Abanto-Rodríguez *et al.* (2016). For H, 2.96 m was used as a reference; for NTB, a value of 70 was used; 30 mm for BSD; and 37.15 for Chl a content. The analysis of VQI was an adaptation of the methods proposed by Karlen and Stott (1994), Islam and Weil (2000), Melo Filho *et al.* (2007), and Freitas *et al.* (2012) for the determination of the soil quality index (SQI).

VQI consists of the differences between the evaluated attributes at initial development compared with the baseline of the quality reference attributes existing in the literature that were then calculated and expressed as the means of the individual value deviations for each attribute. The overall mean of each vegetative development attribute's deviations represents its deterioration from the reference (Freitas *et al.*, 2012).

VQI was calculated in two steps, according to equations 1 and 2,

$$Qv = \frac{\left(\frac{w1-k1}{k1}\right)+\left(\frac{w2-k2}{k2}\right)+\left(\frac{w3-k3}{k3}\right)+\left(\frac{wn-kn}{kn}\right)}{n} \quad (1)$$

$$VQI = 1 - (Qvq) \quad (2)$$

Where *Qv* refers to the mean of the deviation of the indicators of each attribute from the reference attributes present in literature; *w* refers to the value of the indicator measured in the systems under study; *k* refers to the value of the indicator measured in the reference system; *n* is the number of indicators that compose each set of attributes that in this study is 4; and *Qvq* is the mean of the deviations of the morphological characteristics of plants.

Results and discussion

Principal component analysis (PCA)

For the PCA, we initially considered the seven characters evaluated for the 56 camu-camu clones. However, after the first analysis, we found that PC1 and PC2 represented 74% of the data total variability. Therefore, seeking to improve the value of variability according to the criteria of Cliff (1998), we chose to discard variables following the methods described by Jolliffe (1972) and Mardia *et al.* (1979).

The criterion adopted for character discrimination was based on the correlation input (Mardia *et al.,* 1979), where characters with minimal correlation are discarded. Thus, the NBB variable was discarded since it was minimally correlated with the other variables and principal components CP1 and CP2 (Tab. 1).

After discarding NBB, a new PCA was performed. Table 2 explains the total variance for all the principal components generated. The variance associated with each principal component was different and decreasing. The first principal component (PC1) accounted for 68.0%, while the second component (PC2) accounted for 18.2% of the total variance, with a persistent downward trend until all the variability was distributed among the six principal components generated. Thus, CP1 and CP2 represented 86.2% of the total data variability.

TABLE 1. Correlation matrix of original core variables and principal components of vegetative development of clones from different populations of transplanted camu-camu in the savanna/forest area of the northern Amazon, Brazil, 2018.

Variables	Component	
	CP 1	CP 2
Plant height	0.82	0.09
Number of basal branches	-0.11	0.16
Number of terminal buds	0.65	0.65
Basal stem diameter	0.79	0.52
Chlorophyll a	0.9	-0.22
Chlorophyll b	0.83	-0.44
Total chlorophyll	0.92	-0.36
% Variance	58.00	16.00
Cumulative % Variance	58.00	74.00

Cophenetic correlation=0.92.

TABLE 2. Core values and proportion of variance explained by PCA analysis of vegetative development characters of camu-camu clones of different populations.

Principal componentes	Core values	The proportion of total variance	
		Absolute (%)	Cumulative (%)
1	4.08	0.68	0.68
2	1.09	0.182	0.862
3	0.43	0.07	0.93
4	0.23	0.04	0.97
5	0.17	0.03	1.00
6	0.00	0.00	1.00

The distribution of coefficients in the principal components (Tab. 3) indicated that H, NTB, BSD, Chl a and b, and Chl a + b had the most positive contribution to CP1 with adequate correlation levels; therefore, these variables were the most responsive to camu-camu clone selection. However, the variables contributing positively to the principal component 2 (PC2) were NTB and BSD.

TABLE 3. Correlation matrix of the original core variables and principal components of vegetative development of camu-camu clones from different populations transplanted for clonality testing.

Variables	Component	
	CP 1	CP 2
Plant height	0.82	0.09
Number of terminal buds	0.65	0.65
Basal stem diameter	0.79	0.52
Chlorophyll a	0.90	-0.22
Chlorophyll b	0.84	-0.45
Total chlorophyll	0.92	-0.37
% Variance	68.0	18.0
% Cumulative variance	68.0	86.0

Cophenetic correlation=0.923.

Figure 1 shows a biplot with the two principal components explaining a significant proportion of the variability of the 56 camu-camu clones recorded data. The clones were scattered, demonstrating variability, although some were of the same origin. The first principal component (PC1) contributed 68.0% of the total variance while the second component (PC2) accounted for 18.2%, bringing the total variability to 86.2%**,** an acceptable value given that it surpasses the 70% threshold put forward in the criteria of Cliff (1998).

PC1 associated the clones of the Branco River populations [Água Boinha (AB-07, AB-06), Lago da Morena (LM-47, LM-29), Lago Muçum (MU-17), Bem Querer (BQ-04, BQ-18, BQ-26, BQ-29, BQ-28), Açaí Tuba (AT-10, AT-13, AT-03), Lago do Rei (LR-03, LR-11, LR-05) and Estirão do Veado (EV-09)); Mucajaí River [Igarapé Água Boa (IAB-01, IAB-02, IAB-07)], and Quitauaú River [Quitauaú (RQ-14)] with the variables that had the highest correlation coefficients.

We infer that all these clones presented desirable results since they positively correlated with all variables associated with PC1. Thus, the increase in the height of the camu-camu plant caused an increase in the BSD as well as a greater number of secondary branches, allowing significant development of leaf area and, consequently, greater exposure to light, photosynthetic activity, and vigor, reflected by the considerable content of Chl a, b and Chl a + b. Accordingly, there were camu-camu clones that showed a differentiated response to the application of nitrogen fertilization, reflected in the significant gains in Chl a and b and Chl a + b content.

The lack of N absorption and low Chl production by some camu-camu clones, possibly, led to inadequate utilization of sunlight for photoassimilate production that may have considerably limited development (Larcher, 2004). Similar observations were made by Colodetti *et al.* (2014), who reported that the significant genetic variability of coffee (*Coffea canephora*) allowed for the identification of genotypes with a greater capacity to use nutrients under different soil conditions.

The principal component PC2 explained 18.2% of the total variance (Fig. 1), where NTB and BSD had a significant positive contribution, associating with clones from Branco River [Lago da Morena (LM-26), Água Boinha (AB-04, AB-05), Lago Muçum (MU-11, MU-16), Lago da Morena (LM-39), Bem Querer (BQ-12), Açaí Tuba (AT-13)], and Mucajaí River [Igarapé Água Boa (IAB-05)].

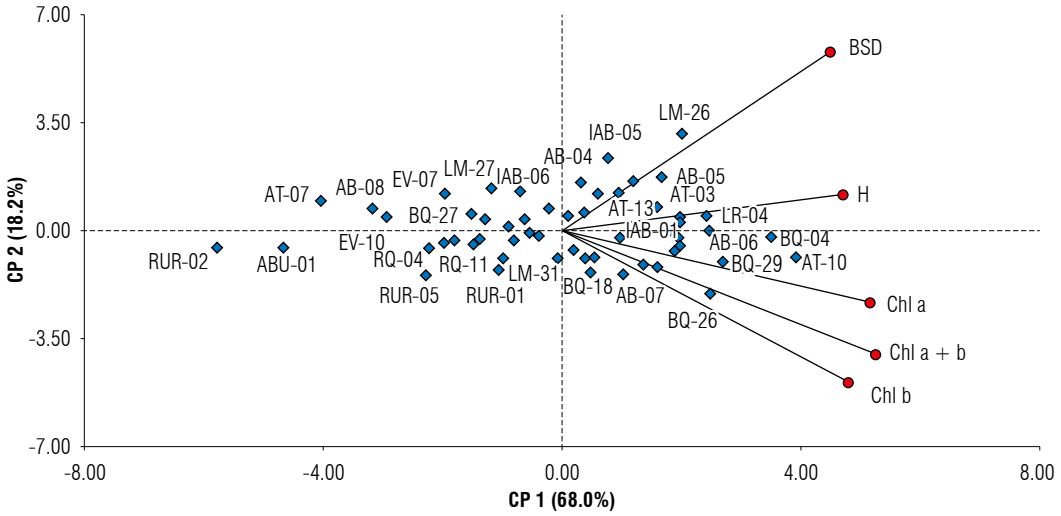

FIGURE 1. Distribution of clone variables from different populations of transplanted *Myrciaria dubia* in a clonality trial in the transitional savanna/forest area of the northern Amazon. Plant height (H), number of terminal buds (NTB), basal stem diameter (BSD), chlorophylls a (Chl a) and b (Chl b), and total chlorophyll (Chl a + b).

Hierarchical cluster analysis

The cluster analysis of the 56 camu-camu clones (Fig. 2) distinguished 13 clone groups according to the dissimilarity of the vegetative characteristics assessed during the first two years after transplantation. In this analysis, the dendrogram's cut-off point was assumed to be a genetic dissimilarity of 1.85 (50%) among all the clones evaluated. Thus, using the six characteristics of initial development as dissimilarity patterns, we have the formation of two larger groups, groups G8 and G9.

The largest group (G9) comprised 20 clones (from LM-27 to AB-05, not in sequential order), that is, 35.7% of all clones that came from the Branco River [Lago da Morena (LM-07, LM-08, LM-30, and LM-39), Bem Querer (BQ-03, BQ-12, and BQ-27), Estirão Veado (EV-06 and EV-10), Lago Muçum (MU-06), Açaí Tuba (AT-03, AT-07, and AT-08), Água Boinha (AB-05 and AB-06), Lago do Rei (LR-04)]; Mucajaí River [Igarapé Água Boa (IAB-04 and IAB-06)], and Urubú River [Urubú (RUR-03 and RUR-04)].

The second group (G8) consisted of 15 clones (from RQ-11 to AB-06, not in sequential order), representing 26.8% of the clones evaluated, coming from the following rivers and populations: Quiatuaú River [Quiatuaú (RQ-11 and RQ-14)], Urubú River [Urubú (RUR-01)], Unimirim River [Água Boa (ABU-02)], and Branco River [Bem Querer (BQ-04), BQ-18, BQ-26, BQ-28, and BQ-29), Good Water (AB-07 and AB-06), Lago do Rei (LR-05 and LR-11), Lago da Morena (LM-31), and Lago Muçum (MU-17). This group was formed by eleven clones of the Branco River population, two clones of the Quitauaú River, and one clone of the Unimirim and Urubú Rivers.

Thus, the G8 and G9 contained 35 of the 56 clones (62.5%). The larger groups represent materials whose dissimilarity could not be identified. Group 12 (G12) consisted of six clones from the Branco River and the following populations: Lago Muçum (MU-11 and MU-16), Lago da Morena (LM-29 and LM-47), Lago do Rei (LR-03), and Açaí Tuba (AT-13)). Group 6 (G6) was formed by three clones from the following rivers and populations: Mucajaí River [Igarapé Água Boa (IAB-01 and IAB-02)], and Branco River [Açaí tuba (AT-10)]. It should be noted that this group had two clones from the Mucajaí River and one from the Branco River. Group 3 (G3) was formed by two clones from the rivers and populations of the Urubú [Urubú (RUR-04)] and Branco Rivers [Lago da Morena do Rei (LR-12)]. Group 4 (G4) was formed by clones from the Urubú [Urubú (RUR-05)] and Unimirim [Agua Boa (ABU-01)] Rivers, and Group 13 (G13) by clones from the Branco [Agua Boinha (AB-04)] and Mucajaí [Igarapé Água Boa (IAB-05)] Rivers.

Finally, groups G1, G2, G5, G7, G10, and G11 were formed in isolation by a clone each from the rivers and populations of the Branco River [Estirão do veado (EV-07)]; Quitauaú River [Quitauaú (RQ-04)]; Branco River [Estirão do veado (EV-09)]; Branco River [Bem Querer (BQ-32)]; Mucajaí River [Igarapé Água Boa (IAB-07)] and Branco River [Lago da Morena (LM-26)], respectively.

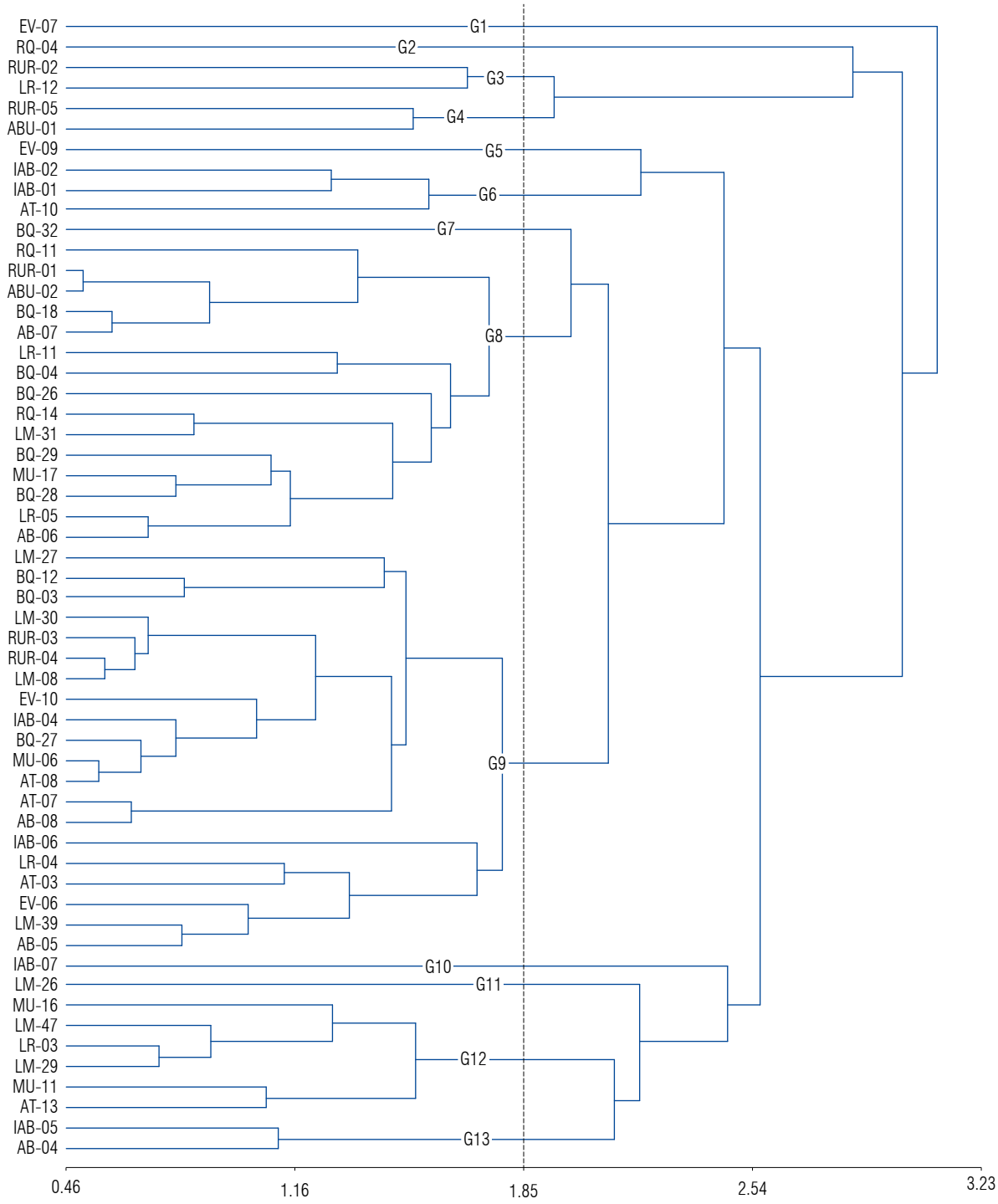

FIGURE 2. Classification dendrogram based on the initial development characteristics of the 56 clones of the different *Myrciaria dubia* populations transplanted in a clonality test using the average linkage mean and Mahalanobis distance algorithm (Cophenetic correlation=0.702).

These results confirm the existence of variability in the genotypes evaluated based on all the characteristics evaluated together. Similar results were obtained by Yokomizo *et al.* (2017) when determining the genetic diversity among the progeny of açai plants from Anajás, Pará, Brazil. This diversity collection occurs due to obtaining materials from native populations (Yokomizo *et al.*, 2017). Thus, the presence of genetic variability could be explored in genetic improvement programs to obtain superior genetic combinations and eventually be used in future controlled

crossings avoiding the use of clones belonging to the same group.

Souza *et al.* (2010) reported that estimating genetic distances between clones is essential; from our results, it is possible to suggest crosses or follow up potential crosses to explore hybrid vigor between progenies. Souza *et al.* (2010) and Cruz *et al.* (2011) highlight that the dissimilarity analysis (distance) allows evaluating possible redundancies in a set of individuals. However, crosses between more distant individuals make it possible to uncover greater segregation in future improvement cycles and, therefore, enrich their genetic basis.

The cophenetic correlation in the present study was 0.702, indicating that the groups formed are reliable. Alvarado *et al.* (2016) obtains similar results, working with the agronomic characterization of 95 coffee accesses, and Santos *et al.* (2014), reports a 0.69 cophenetic correlation in a group analysis of cupuaçu plant clones. Cruz *et al.* (2011) indicate that the estimated coefficients range from zero to one, and the higher the value obtained for this coefficient, the higher the representativity of the dendrogram in relation to the genetic distance matrix.

Vegetative quality indices (VQI)

Figure 3 shows the clones with the highest VQI, resulting from the joint analysis of the initial development variables evaluated in the first two years of age. In this type of analysis, the position in order of merit can be determined more precisely according to all the evaluated attributes. Thus, clones with higher VQI were from the following rivers and populations in descending order: Branco River [Água Boinha (AB-05)]; Mucajaí River [Igarapé Água Boa (IAB-05)]; Branco River [Lago Muçum (MU-11), Bem Querer (BQ-04), Lago da Morena (LM-26), Açaí Tuba (AT-10), Estirão do Veado (EV-06), Lago do REI (LR-04), Água Boinha (AB-06), Lago da Morena (LM-29), Água Boinha (AB-04), and Açaí Tuba [(AT-13), (AT03)]; Lago da Morena (LM-39); Lago do Rei [(LR-03, LR-11, and LR-05)]; Lago Muçum (MU16); Bem Querer (BQ-12); Mucajaí River [Igarapé Água Boa (IAB-06)]; Branco River [Bem Querer (BQ-29)]; Lago da Morena (LM-47)]. The clones with the smallest VQI were: RUR-05, RUR-02 and ABU-01, belonging to the rivers and populations of the Urubú (Urubú) and Unimirim Rivers (Água Boa).

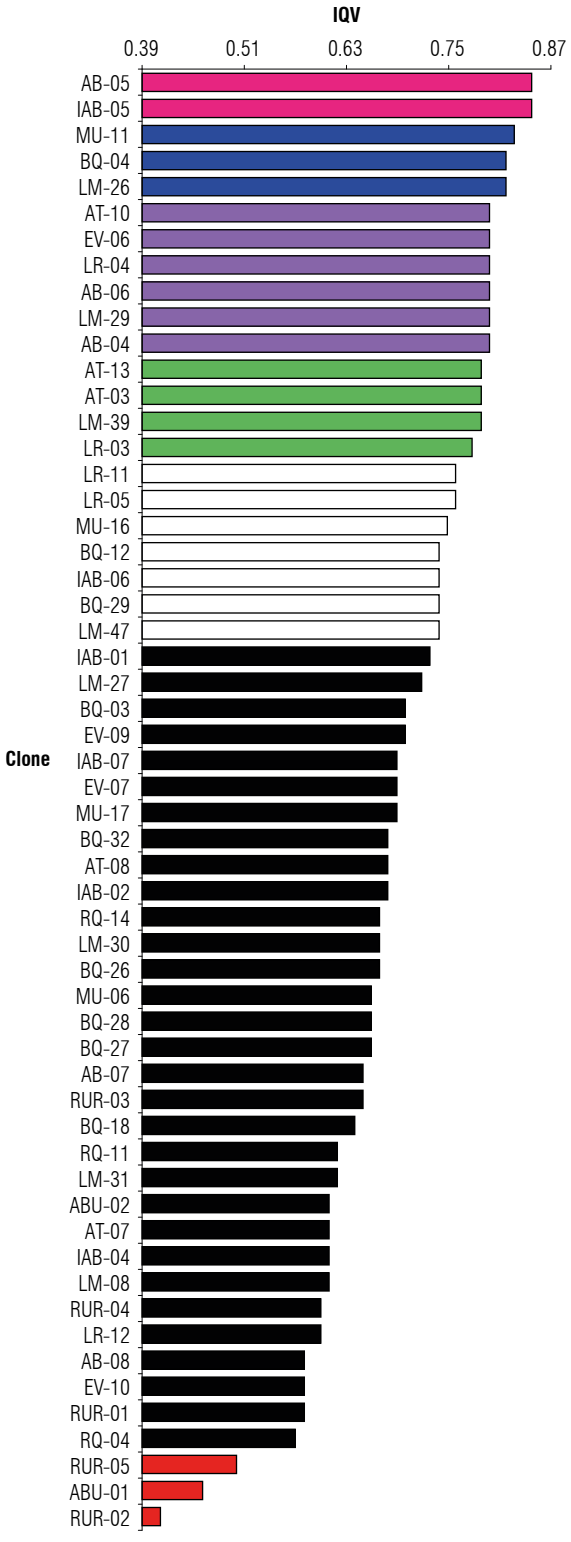

FIGURE 3. Vegetative quality index (VQI) of 56 *Myrciaria dubia* clones according to initial developmental characteristics in the first two years of transplanting.

Conclusions

Plant height, number of terminal buds, basal stem diameter, chlorophylls a and b, and total chlorophyll contributed the most to the camu-camu clone selection in the savanna/forest area of the northern Amazon, Brazil. We verified that 22 clones of camu-camu were better adapted to soil conditions out of the floodplain. In this sense, this study represents a great advance for the domestication of the species, since there are more clones available to be included in the camu-camu genetic improvement program of the Brazilian Agricultural Research Corporation-EMBRAPA Roraima.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

RTS, EAC and PCC formulated the overarching research goals and aims, MCRA and MLG carried out activities to annotate draft data and maintain research data for initial use and later re-use. JLLMN, RHSS and CAR applied statistical, mathematical, computational, and other formal techniques to analyse study data, LGCZ and JZEA conducted the research process, specifically performing the experiments or data/evidence collection. All authors reviewed the manuscript.

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Genetic variability of yam (*Dioscorea trifida*) genotypes in the Ucayali region, Peru

Variabilidad genética de genotipos de ñame (*Dioscorea trifida*) en la región Ucayali, Perú

Lady Laura Tuisima-Coral[1, 2*] and Wilfredo Felipe Guillén Huachua[2]

ABSTRACT

The aim of this research was to assess genetic variability of yam *Dioscorea trifida* genotypes using morphological descriptors for the germplasm collection conserved in the Agricultural Experiment Station in Ucayali, Peru. Thirty-eight morphological traits were evaluated for 30 *D. trifida* genotypes over ten years; from the data we estimated the Shannon-Weaver diversity index (H') and the coefficient of variation and performed principal component analysis and cluster analysis. Qualitative traits with high phenotypic diversity index were: petiole anthocyanin (0.86), internal tuber color (0.86), petiole color (0.81) and stem color (0.80). The quantitative traits with the highest coefficient of variation were: tuber weight per plant (33.01) and tuber yield (32.99). Seventy-six percent of the morphological variability is explained by four principal components, the first component is constituted by the characters tuber width, tuber weight per plant, and tuber yield (29%). Five groups of genotypes were also identified with statistically significant differences, where group B stands out for its higher yield in fewer days to harvest. This research reveals wide morphological diversity in genotypes of *D. trifida*; these results can be used to strengthen the conservation, management, and genetic improvement initiatives of this important species in the Peruvian Amazon.

Key words: ethnic groups, morphological descriptors, sachapapa, genetic diversity index, tropical tuber.

RESUMEN

El objetivo de esta investigación fue evaluar la variabilidad genética de genotipos de ñame *Dioscorea trifida* usando descriptores morfológicos a fin de conocer la variabilidad de la colección conservada en la Estación Experimental Agraria en Ucayali, Perú. Se evaluaron 38 caracteres morfológicos para treinta genotipos de *D. trifida* durante diez años, para obtener el índice de diversidad genética de Shannon-Weaver (H'), el coeficiente de variación, y análisis de componentes principales y análisis de agrupamiento. Los caracteres cualitativos con mayor índice de diversidad fueron: antocianina del peciolo (0.86), color interno del tubérculo (0.86), color del peciolo (0.81) y color del tallo (0.80). Los caracteres cuantitativos con mayor coeficiente de variación fueron peso de tubérculos por planta (33.01) y rendimiento de tubérculos (32.99). El 76% de la variabilidad morfológica se explica a través de cuatro componentes principales, el primer componente está constituido por los caracteres ancho de tubérculo, peso de tubérculos por planta y rendimiento de tubérculos (29%). También se identificaron cinco grupos de genotipos en los cuales se encontraron diferencias estadísticamente significativas, donde se destaca al grupo B por su mayor rendimiento en menor número de días a cosechar. Por lo tanto, esta investigación revela amplia diversidad morfológica en genotipos de *D. trifida,* y los resultados pueden ser usados para fortalecer la conservación, manejo e iniciativas de mejoramiento genético de esta especie de importancia en la Amazonía peruana.

Palabras clave: grupos étnicos, descriptores morfológicos, sachapapa, índice de diversidad genética, tubérculo tropical.

Introduction

The Amazon region is home to many resources for food; one of these is *Dioscorea trifida* L. f., known as sachapapa (common name in Peru) or yam. It is a tuber plant belonging to the Dioscoreaceae family. Viruel *et al.* (2016) refer to three genera that group the Dioscoreaceae family (*Stenomeris, Trichopus, Rajania,* and *Dioscorea*), and the World Checklist of Vascular Plants (2022) accepts 679 species for *Dioscorea* (15 edible and medicinal approximately), providing substantial food for more than 100 million people in the humid and sub-humid tropics, appreciated for their flavor and fine texture (Price *et al*., 2018; Padhan & Panda, 2020). The *Dioscorea* species grow in Southeast Asia, tropical America, and West Africa, in tropical and subtropical regions of the world (Kumar *et al.,* 2017). According to Montaldo (1991), in the upper and lower jungle of the Peruvian Amazon, it constitutes a main source of energy in the diet of the inhabitants. As a crop, *Dioscorea* species requires much light and humid soils

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with good drainage. Padhan and Panda (2020) reviewed the aggregate nutritional composition of old-world yam and reported that its tuber is a good source of essential nutritional compounds, such as starch, protein, lipids, vitamins, and minerals, which agrees with the description of Pérez *et al.* (2009) for the nutritional composition of three varieties of *Dioscorea trifida* in the Venezuelan Amazon. Moreover, Ramos-Escudero *et al.* (2010) determined the content of total polyphenols, flavonoids, tannins, and anthocyanins for *D. trifida*, finding values around 166.10, 27.63, 9.62, and 21.59 mg/100 g of dry matter, respectively. Furthermore, pigments of yam pulp tuber have the capacity to sequester free radicals. This indicates a good *in vitro* protective effect of the pigments extracted from sachapapa (*D. trifida*). The aforementioned studies reveal the importance of sachapapa for food and health, although there are few studies regarding its agronomic management, origin, geographical distribution, and genetic variability of its populations (Nascimento *et al.*, 2015; Arnau *et al.*, 2017). In the Ucayali region (Peru), the production of sachapapa reaches an approximate average of 612.11 t per year (Astete-Verde, 2019), although it is considered a neglected crop because it is mainly cultivated for subsistence by native communities using traditional agricultural practices. In order to develop strategies for the management and conservation of this crop, the genetic diversity must be agro-morphologically characterized (Ocampo *et al.*, 2021; Thakur *et al.*, 2021; Wada *et al.*, 2021). In this context, the objective of this research was to evaluate the genetic variability using morphological descriptors for 30 *Dioscorea trifida* genotypes. This is a broad germplasm collection maintained and conserved year after year in rotation plots under similar environmental conditions, thus ruling out environment as the cause of observed morphological differences.

Materials and methods

Germplasm collection

In 1997, the National Institute of Agrarian Innovation (INIA), through the Genetic Resources Subdirectorate, began the collection of *D. trifida* germplasm. By 2000, 30 accessions from the four provinces of the Ucayali department (Fig. 1, Tab. 1), were collected to maintain and

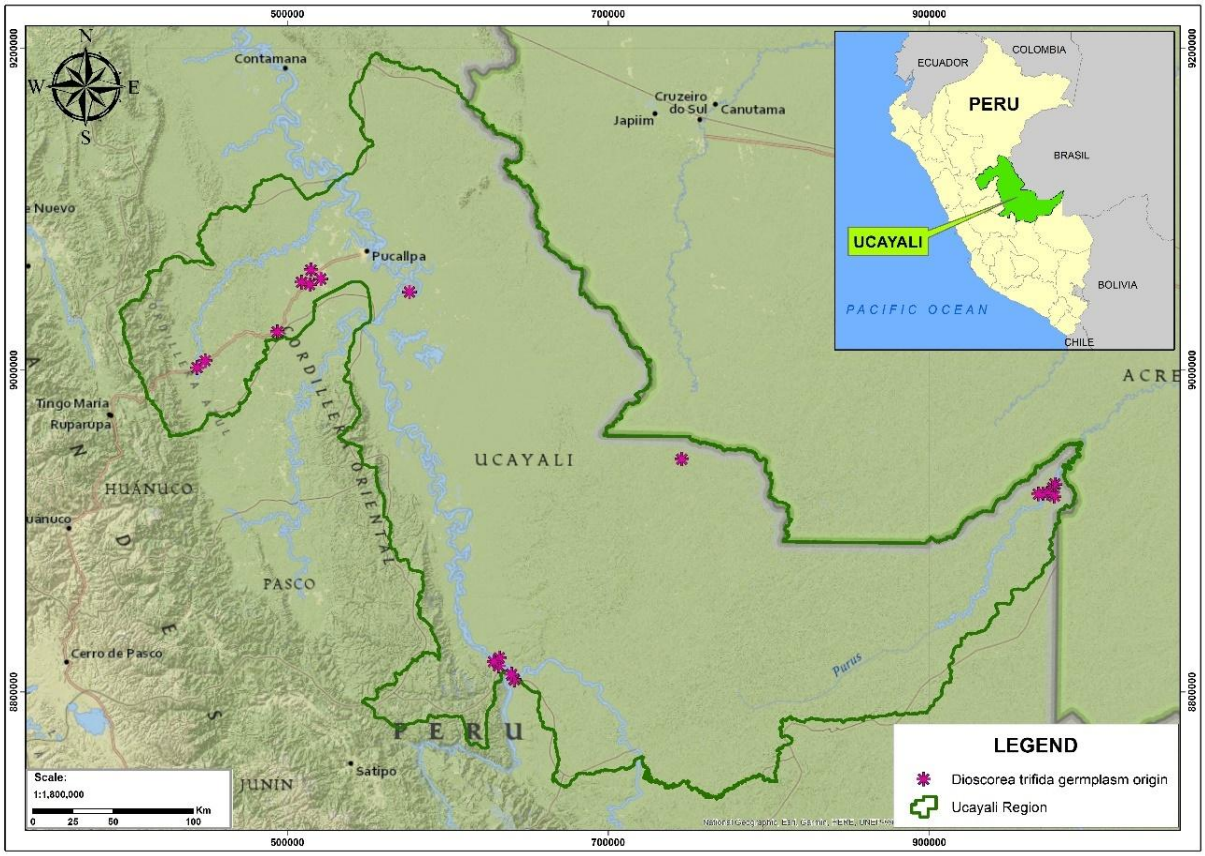

FIGURE 1. Map of *Dioscorea trifida* germplasm origin in the Ucayali region. Each circle represents the geographic origin of the sachapapa germplasm part of the National tropical root and tuber germplasm collection.

TABLE 1. List of *Dioscorea trifida* accessions conserved in a germplasm bank in the Ucayali region, Peru.

N°	Genotype code	Local name	Sampling code	District	Ethnic group	Latitude, S	Longitude, W	Altitude, m a.s.l.
Coronel Portillo Province								
1	IPDT001	Purple SP*	MA001	Masisea	Mestizo	8.605.300	74.306.300	166
2	IPDT002	Purple SP	SP002	Campoverde	Mestizo	8.550.800	74.916.400	208
3	IPDT003	White SP	SP003	Campoverde	Mestizo	8.550.800	74.916.400	208
4	IPDT004	SP	HB004	Campoverde	Mestizo	8.481.300	74.865.000	205
5	IPDT010	Dark purple SP	LP010	Campoverde	Mestizo	8.564.700	74.870.600	224
6	IPDT011	Purple SP	PIM011	Campoverde	Mestizo	8.532.000	74.808.700	210
7	IPDT012	White SP	PIM012	Campoverde	Mestizo	8.532.000	74.808.700	210
Padre Abad Province								
8	IPDT005	White SP	AG005	Padre Abad	Mestizo	9.031.800	75.509.400	313
9	IPDT006	Purple SP	EP006	Padre Abad	Mestizo	8.993.800	75.464.400	307
10	IPDT007	White SP	AVH007	Irazola	Mestizo	8.829.900	75.054.500	234
11	IPDT008	Dark purple SP	AVH008	Irazola	Mestizo	8.829.900	75.054.500	234
12	IPDT009	Light purple SP	AVH009	Irazola	Mestizo	8.829.900	75.054.500	234
Atalaya Province								
13	IPDT013	Purple SP	BR013	Yurua	Mestizo	9.538.900	72.759.100	200
14	IPDT014	SP	BR014	Yurua	Mestizo	9.538.900	72.759.100	200
15	IPDT015	SP	BR015	Yurua	Mestizo	9.538.900	72.759.100	200
16	IPDT017	White SP	CNA017	Raymondi	Ashaninka	10.705.833	73.793.278	241
17	IPDT018	Purple SP	CNA018	Raymondi	Ashaninka	10.705.833	73.793.278	241
18	IPDT019	White SP	CNL019	Raymondi	Ashaninka	10.681.833	73.821.778	235
19	IPDT020	White SP	PLM020	Raymondi	Mestizo	10.784.472	73.704.722	266
20	IPDT021	Purple SP	PLM021	Raymondi	Mestizo	10.783.306	73.706.611	249
21	IPDT022	White SP	JCHM022	Raymondi	Mestizo	10.753.556	73.720.750	248
22	IPDT023	Purple SP	PLCH023	Raymondi	Ashaninka	10.661.056	73.790.583	218
23	IPDT031	SP	CNL031	Raymondi	Ashaninka	10.681.833	73.821.778	235
Purus Province								
24	IPDT024	SP (maona en ashaninka)	CNRA024	Purus	Ashaninka	9.706.222	70.706.222	233
25	IPDT025	SP (maona en ashaninka)	CNRA025	Purus	Ashaninka	9.706.222	70.706.222	233
26	IPDT026	SP (maona en ashaninka)	CNNP026	Purus	Ashaninka	9.726.889	70.702.778	217
27	IPDT027	SP (pua-jushupa)	CNB027	Purus	Cashinahua	9.793.972	70.790.583	241
28	IPDT028	SP (pua-meshupa)	CNNB028	Purus	Cashinahua	9.785.722	70.761.806	235
29	IPDT029	Purple SP	CRLN029	Purus	Mestizo	9.797.222	70.715.472	228
30	IPDT030	Purple SP	PE030	Purus	Mestizo	9.770.417	70.717.694	204

*SP – sachapapa.

conserve in experimental plots a Campoverde Annex of the Pucallpa Agrarian Experimental Station (Federico Basadre Highway, Km. 44, Campo Verde district, Coronel Portillo province, Ucayali department, at coordinates 8°22'00" S and 74°34'80" W, altitude 205 m a.s.l.). The climate of the experimental area is characteristic of the lowland tropical zones; it is humid and warm without marked variations in the annual average temperature and with a defined strong dry season from May to July, with a relative humidity about 70-90%.

Field characterization plots

The 30 accessions of *D. trifida* were sowed at a distance 2 m between rows and 1 m between plants, with a localized application of 800 kg ha^{-1} of island guano and in some cases 10 t ha^{-1} of chicken manure; it was also necessary to install stakes that serve as a support because *Dioscorea trifida* is a climbing species. Field characterization plots were located in the same place as the field germplasm collection described above and plot rotation (50 m of distance between each plot) was applied every year from 2008-2017.

Descriptors

To assess genetic variability, 26 qualitative and 12 quantitative descriptors were evaluated (Tab. 2). Of the 178 descriptors listed by the International Institute of Plant Genetic Resources for *Dioscorea spp* characterization (IPGRI, 1997), 38 descriptors were adapted by the researchers according to the basic morphology of *Dioscorea trifida*.

Experimental design and data analysis

The experimental design was completely randomized with 10 repetitions (years). Rotation plots were evaluated for 10 years. Each year sowing started at the end of October, flowering took place after 5-7 months and harvesting began after approximately ten months. The climatic condition around each plot location was usually characterized by a rainy season around October 15th to April 15th, followed by a dry season from mid-April until the beginning of October. In every plot, we installed thirty accessions with ten plants per accession. For quantitative variables, five plants from the third to the seventh were selected and evaluated. For qualitative variables, we used direct observation to the ten plants and considered the most frequently occurring attribute observed.

TABLE 2. List of agro-morphological descriptors used to assess genetic variability among 30 genotypes of *Dioscorea trifida*.

N	Stage/character	Evaluation/evaluation code
1	**Vegetative phase**	
1.1	Percentage of emergence	%
1.2	Plant type	1. Dwarf, 2. Shrub-like, 3. Climbing
1.3	Twining habit	0. No, 2. Yes
1.4	Stem color	1. Green, 2. Slightly pigmented, 3. Moderately pigmented, 4. Fully pigmented – red, 5. Fully pigmented – purple
1.5	Stem wings	0. Absent, 1. Present
1.6	Stem hairs	0. Absent, 1. Present
1.7	Leaf color	1. Light green, 2. Dark green, 3. Purple green, 4. Purple
1.8	Vein color	1. Light green, 2. Dark green, 3. Purple green, 4. Purple
1.9	Hairiness of upper/ lower surface of leaf	0. Absent, 1. Present
1.10	Leaf type	1. Entire, 2. Very shallowly lobed, 3. Shallowly lobed, 4. Moderately lobed, 5. Deeply lobed, 6. Very deeply lobed
1.11	Position of leaves	1. Verticillate, 2. Alternate, 3. Opposite
1.12	Number of lobes	1. 01 lobe, 3. 03 lobes, 5. 05 lobes, 7. 07 lobes, 9. 09 lobes
1.13	Central lobe shape	0. Absent, 1. Dentate, 2. Semicircular, 3. Semi-elliptical, 4. Elliptical, 5. Lanceolate, 6. Oblanceolate, 7. Linear, 8 Oval, 9. Cordate
1.14	Leaf size	Record the length and width of 5 leaves in cm, with one decimal place, from the base to the apex of the leaf taken from the middle part of the plant
1.15	Petiole color	1. Light green, 2. Dark green, 3. Purple green, 4. Purple
1.16	Petiole pubescence	0. Absent, 1. Present
1.17	Anthocyanin in petiole	0. Absent, 1. Apical part, 2. Basal part, 3. Slightly pigmented, 4. Medium pigmented, 5. Completely pigmented
1.18	Petiole wing	0. Absent, 1 Present

N	Stage/character	Evaluation/evaluation code
2	**Flowering**	
2.1	Flowering	1. Female, 2. Male, 3. Female and male (predominantly female), 4. Male and female (predominantly male)
2.2	Inflorescence type	1. Spike, 2. Raceme, 3. Panicle, 9. Other
2.3	Flower color	1. White, 2. Yellow, 3. Light Green, 4. Purple Green/Pigmented Green
2.4	Number of flowers per inflorescence	For male and female plants
2.5	Flower length	Average of 5 flowers (male or female) per plant in mm (total length from the base of receptacle to the top)
2.6	Fruit formation	0. Absent, 1 Present
2.7	Fruit size	Record length of 5 fruits per plant in mm
2.8	Tuber formation (appearance)	1. Closed/kidney-shaped bunch, 2. Open bunch
2.9	Tuber shape	1. Round, 2. Irregular round (kidney-shaped), 3. Cylindrical, 4. Ovate, 5. Oblong, 6. Oblong oval, 7. Elliptical length, 8. Elongated round, 9. Irregular or curved length, 10. Compact
2.10	Tuber external color	1. White, 2. Cream, 3. Yellow, 4. Light brown, 5. Dark brown, 6. Purple, 7. Gray
2.11	Tuber internal color	1. White or cream, 2. Yellow, 3. Pink, 4. Light purple, 5. Dark purple
2.12	Rootlets	0. Absent, 1. Slight, 2. Intermediate, 3. Abundant
2.13	Pulp color	1. White, 2. Cream, 3. Yellow, 4. Light purple/pink, 5. Light purple, 6. Dark purple, 7. Concentric light purple. 8. Mottled
2.14	Peduncle length (average)	0. Absent or sessile, 1. Very short: < 2 cm, 3. Short: 2-5 cm, 5. Intermediate: 5-8 cm, 7. Long: 9-12 cm, 9 Very long: > 12 cm
2.16	Tuber size	Record 5 tuber length and width
2.17	Number of tubers per plant	
2.18	Weight of tubers/plant in kg	
2.19	Tuber yield in t ha^{-1}	
2.20	Days to harvest	

Simple descriptive statistics were obtained for all variables. The Chi-square test was performed for each qualitative character. The phenotypic diversity for the qualitative variables was determined using the Shannon-Weaver genetic diversity Index (H'). The range of H' is from 0 to 1, where 1 indicates maximum diversity. H' is defined as $H' = \Sigma P_i Log2P_i$, where P_i is the proportion of the total number of genotypes belonging to each category. According to Jamago (2000), H' is classified as low (H'<0.50), intermediate (H'=0.50-0.75), and high (H'>0.75). To determine the similarity between the *D. trifida* germplasm, a hierarchical cluster analysis (CA) was done with Ward's clustering method using Euclidean distance. To determine the occurrence of statistical differences between the groups of the dendrogram, a one-way analysis of variance was performed, and the Tukey test was used ($P \leq 0.05$). A Pearson correlation analysis was added between quantitative descriptors. Likewise, a principal component analysis (PCA) of the twelve quantitative variables under study was carried out. StatGraphics V. 19 software (StatGraphics, 2009) was used for all analyses.

Results and discussion

Qualitative descriptors

Of the 26 qualitative characters evaluated in 30 genotypes of *D. trifida*, eleven (11) were not discriminating (Tab. 3) and allows the species to be described as having a climbing habit with a twisted appearance, with wings, but without pubescence on the stem. The leaves are alternately arranged, without pubescence and with light green veins. Pubescent wings are recorded on the petiole. In all genotypes, spike-type inflorescences with light green flowers were observed. Nascimento *et al*. (2015) also reported genotypes of *D. trifida* with climbing plant behavior, with winged polygonal stems and green petioles with brown pigments and alternate leaves.

TABLE 3. List of descriptors without variability for 30 genotypes of *Dioscorea trifida.*

N	Descriptors	Attribute
1	Plant type	Climbing
2	Twining habit	Yes
3	Stem wings	Present
4	Stem pubescence	Absent
5	Vein color	Light green
6	Leaf pubescence	Absent
7	Leave position	Alternate
8	Petiole pubescence	Absent
9	Petiole wings	Present
10	Type of inflorescence	Spike
11	Flower color	Light green

About fifty eight percent of the qualitative characters evaluated for 30 genotypes of *D. trifida* resulted discriminant. According to the Chi-square analysis and the Shannon-Weaver Genetic Diversity Index (H') (Tab. 4), the most variable qualitative characters of *D. trifida* are: petiole anthocyanin (0.86), internal tuber color (0.86), petiole color (0.81), and stem color (0.80). While the number of lobes and the color of the mature leaf were the characters with the least variability (0.35), which would be consider as low variability (Jamago, 2000). The morphological variability of *Dioscorea spp.* was also determined using the Shannon-Weaver Genetic Diversity Index (H') of Islam *et al*. (2011). These authors also found high diversity indexes for petiole color and tuber shape in *Dioscorea alata* and *Dioscorea bulbifera*. Moreira *et al*. (2017) evaluated the external color and tuber shape of *Dioscorea cayennensis*, finding a low diversity index, which is expected for descriptors with fewer categories.

The slightly pigmented color predominated for the stems, which are green with purple or brown pigments. The predominant color of the petiole was purple green, characteristics that coincide with the description for *D. trifida* genotypes reported by Nascimento *et al*. (2015) in three states of Brazil. Similarly, the number of lobes per leaf can vary from three to five, although five-lobed leaves were predominant.

This result is in sharp contrast with *Dioscorea alata*, a species characterized by single lobe leaves (Norman *et al*., 2011). The color of the pulp for *Dioscorea* species ranges from white, yellow to dark purple (González, 2012). In this study, white pulp color (50%) was predominant, followed by light purple (36.7%), consistent with the results of other studies (Pérez *et al*., 2009; Nascimento *et al*., 2015). Pérez *et al*. (2009) analyzed the nutritional content of *D. trifida* with three colors of pulp, finding a higher content of crude protein in white pulp (6.8 ± 0.02%) in contrast to the values for purple pulp (4.3 ± 0.54%).

Quantitative descriptors

Values regarding the descriptive statistics analysis for 12 quantitative descriptors evaluated in 30 genotypes of *D. trifida* are shown in Table 5. The descriptors with the highest coefficient of variation are tuber weight per plant (33.01) followed by tuber yield (32.99) and the number of female flowers per inflorescence (27.42%).

TABLE 4. Absolute frequency, proportion, and variability within 16 qualitative descriptors of *Dioscorea trifida*.

N	Descriptors	Attribute	Absolute frequency	Proportion	X^2	***H'
1	Stem color	Green	1	0.033	14.53**	0.80
		Slightly pigmented	14	0.467		
		Moderately pigmented	11	0.367		
		Fully pigmented – purple	4	0.133		
2	Leaf type	Shallowly lobed	1	0.033	30.2**	0.53
		Moderately lobed	5	0.167		
		Deeply lobed	24	0.800		
3	Number of lobes	3 lobes	2	0.067	22.53**	0.35
		5 lobes	28	0.933		
4	Central lobe shape	Semi-elliptical	2	0.048	16.8**	0.73
		Elliptical	20	0.476		
		Oval	8	0.190		
5	Petiole color	Light green	7	0.233	12.6**	0.81
		Purple green	19	0.633		
		Purple	4	0.133		
6	Presence of anthocyanin in petiole	Absent	4	0.133	13.2**	0.86
		Slightly pigmented	16	0.533		
		Medium pigmented	6	0.200		
		Completely pigmented	4	0.133		
7	Flowering	Female	11	0.367	--	--
		Male	19	0.633		
8	Fruit	Absent	15	0.500	0.00	1.00
		Present	15	0.500		
9	Tuber formation (appearance)	Closed/kidney-shaped bunch	4	0.133	16.13**	0.57
		Open bunch	26	0.867		
10	Tuber shape	Irregular round (kidney-shaped)	1	0.033	18.2**	0.68
		Ovate	9	0.300		
		Oblong oval	20	0.667		
11	Tuber external color	Light brown	11	0.367	2.13	0.95
		Dark brown	19	0.633		
12	Tuber internal color	White or cream	9	0.300	8.67*	0.86
		Pink	1	0.033		
		Light purple	12	0.400		
		Dark purple	8	0.267		
13	Rootlets	Absent	2	0.067	19.4**	0.70
		Slight	21	0.700		
		Intermediate	7	0.233		
14	Pulp color	White	15	0.500	28.67**	0.70
		Cream	1	0.033		
		Light purple	11	0.367		
		Dark purple	1	0.033		
		Mottled	2	0.067		
15	Leaf color	Light green	2	0.067	22.53	0.35
		Dark green	28	0.933		

* Significant Chi-square, ** Highly significant Chi-square (X^2), *** Shannon-Weaver Genetic Diversity Index (H').

TABLE 5. Descriptive statistics of 12 quantitative descriptors from the evaluation of 30 genotypes of *Dioscorea trifida*.

Descriptors	Mean n=30	SD	CV	Minimum	Maximum
PE	81.00	8.49	10.48	55.00	93.60
LL	17.59	1.19	6.79	15.30	20.40
LW	17.86	1.47	8.25	14.90	22.00
NFFI	18	4.93	27.42	7	25
NMFI	23	3.58	15.73	18	35
FFS	10.50	1.59	15.19	6.00	11.60
MFS	4.45	0.60	13.42	3.90	6.50
LPed	7.02	1.72	24.51	2.60	10.80
TL	11.16	1.37	12.27	7.60	14.20
TW	4.70	0.73	15.60	3.40	6.80
NTP	7	1.65	23.15	5	11
WTP	0.87	0.29	33.01	0.52	1.71
Yield	4.35	1.43	32.99	2.58	8.56
DH	303	11.73	3.87	292	339

SD: Standard deviation; CV: Coefficient of variation PE: Percentage of emergency; LL: Leaf length; LW: Leaf width; NFFI: Number of female flowers per inflorescence; NMFI: Number of male flowers per inflorescence; FFS: Female flower size; MFS: Male flower size; LPed: Length of peduncle; TL: Tuber length, TW: Tuber width; NTP: Number of tubers per plant; WTP: Weight of the tubers per plant; DH: Days to harvest.

Taking into account the coefficients of variation and the ranges of the quantitative variables, wide genetic variability is suggested. However, it is important to evaluate and consider which characteristics are truly inherited and the edaphoclimatic factors that can influence the agro-morphological variation and the management of the genotypes of *D. trifida* for genetic improvement purposes. For example, the genotypes with the highest yield were BR013 and CNNB028. This yield, in addition to other physicochemical characteristics (Carlos *et al.,* 2020), must be evaluated under different environmental conditions prior to technology transfer to farmers. Figure 2 shows the yield in tons per hectares by each *D. trifida* genotype, an important trait to select genotypes for genetic improvement and food security.

Correlation analysis

Leaf width and length, tuber weight per plant and tuber width, tuber yield per plant and tuber width, correlated in a positive and highly significant way (Tab. 6). Peduncle length and tuber length as well as leaf length and number of tubers per plant showed significant and positive correlations.

Principal component analysis

Four components were selected that may explain 86% of the variability among *D. trifida* genotypes (Tab. 7). The first component is constituted mainly by tuber traits: tuber width, tuber weight per plant, and tuber yield (38%). The second component is made up of the descriptors: leaf length and tuber length (20%). The third component is represented

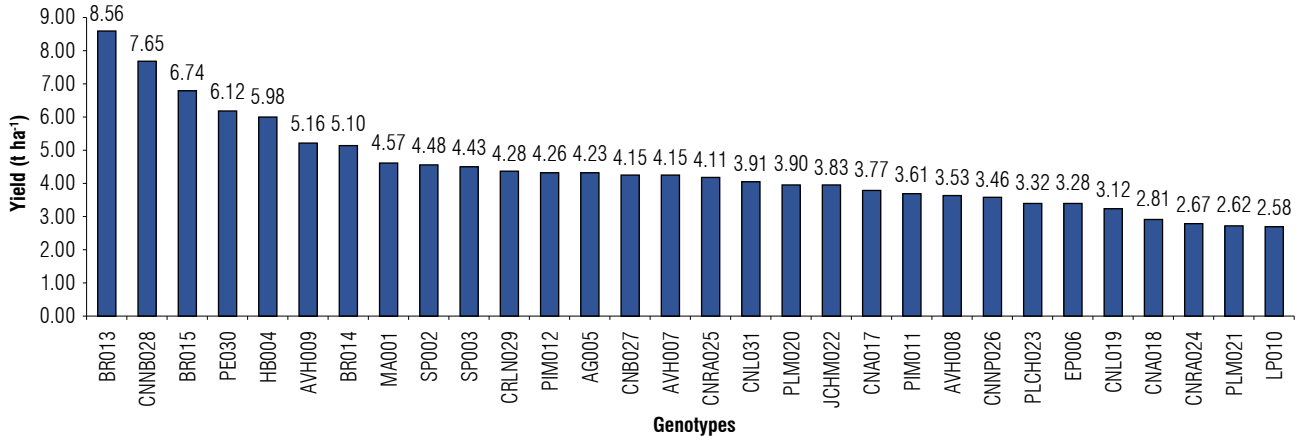

FIGURE 2. Tuber yield for 30 genotypes of *Dioscorea trifida* production.

TABLE 6. Pearson's correlation analysis between nine quantitative characters for *Dioscorea trifida.*

	PE	LL	LW	LPed	TL	TW	NTP	WTP	Yield
PE	1								
LL	0.28	1							
LW	0.34	0.84**	1						
LPed	0.09	0.0005	0.15	1					
TL	0.26	-0.07	0.21	0.51*	1				
TW	0.07	0.34	0.08	0.02	0.03	1			
NTP	0.21	0.41*	0.26	0.06	0.18	0.3	1		
WTP	0.05	0.34	0.27	0.31	0.34	0.69**	0.4*	1	
Yield	0.05	0.34	0.27	0.31	0.34	0.69**	0.4*	1**	1

PE: Percentage of emergency; LL: Leaf length; LW: Leaf width; LPed: Length of peduncle; TL: Tuber length, TW: Tuber width; NTP: Number of tubers per plant; WTP: Weight of the tubers per plant.* Significant at 5% level; ** Significant at 1% level.

by peduncle length and leaf width (17%). Tuber yield and tuber length are included in the set of priority descriptors for characterization of *Dioscorea* genetic resources (Biodiversity International and International Institute of Tropical Agriculture (IITA), 2009), therefore, it is consistent with the principal component analysis.

TABLE 7. Eigenvectors of the principal component analysis for *D. trifida* descriptors.

Descriptors	e1	e2	e3	e4
PE	-0.04	-0.43	-0.32	**0.63**
LL	0.35	**0.48**	0.13	0.21
LW	0.31	0.39	**0.41**	0.26
LPed	0.19	-0.31	**0.48**	-0.03
TL	0.17	**-0.47**	0.46	0.15
TW	**0.38**	-0.01	-0.37	-0.40
NTP	0.30	0.13	-0.33	**0.52**
WTP	**0.49**	-0.21	-0.12	-0.15
Yield	**0.49**	-0.21	-0.12	-0.15

PE: Percentage of emergency; LL: Leaf length; LW: Leaf width; LPed: Length of peduncle; TL: Tuber length, TW: Tuber width; NTP: Number of tubers per plant; WTP: Weight of the tubers per plant.

Clustering analysis

Clustering analysis distributed the 30 sachapapa genotypes in different clusters based on the Euclidean distance in a range from 0 to 20.63. The dendrogram identified five groups using a cut distance of 5.15 (Fig. 3, Tab. 8). Groups B and D comprise five genotypes each, group C has three genotypes, and groups A and E have eight and nine genotypes, respectively. Among the groups, the descriptors with statistically significant differences are emergence percentage, leaf length, leaf width, number of flowers per cluster, number of tubers per plant, and days to harvest (Tab. 8). Group C registered the lowest percentage of emergence, while group B registered the highest percentage of emergence. Likewise, the group with the longest leaf length and width was group C (19.38 cm and 20.34 cm, respectively), while group E was made up of genotypes with the smallest leaf size (16.47 cm and 16.34 cm). Group C registered genotypes with the highest number of flowers per cluster and the longest peduncle length (28.16 cm and 8.58 cm, respectively). Regarding tuber traits, group D registered the highest number of tubers per plant (9), while groups A and E registered the lowest number of tubers per plant. Group

TABLE 8. Comparison of means between the groups formed in the cluster analysis.

Groups	PE (%)	LL (cm)	LW (cm)	LPed (cm)	TL (cm)	TW (cm)	NTP	WTP (kg)	Yield (t ha^{-1})
A	77.74^{ab}	17.16^{cd}	17.16^{bc}	5.73^{a}	10.17^{a}	4.39^{a}	6^{b}	0.60^{a}	3.02^{a}
B	86.84^{a}	17.87^{bc}	17.77^{abc}	7.03^{a}	11.85^{a}	5.72^{a}	8^{ab}	1.30^{a}	6.50^{a}
C	65.62^{b}	19.38^{a}	20.34^{a}	8.58^{a}	11.30^{a}	5.13^{a}	8^{ab}	1.03^{a}	5.13^{a}
D	84.14^{a}	18.74^{ab}	19.16^{ab}	6.95^{a}	10.96^{a}	4.67^{a}	9^{a}	0.91^{a}	4.56^{a}
E	85.62^{a}	16.47^{d}	16.34^{c}	6.89^{a}	11.70^{a}	4.94^{a}	6^{ab}	1.09^{a}	3.95^{a}

PE: Percentage of emergency; LL: Leaf length; LW: Leaf width; LPed: Length of peduncle; TL: Tuber length, TW: Tuber width; NTP: Number of tubers per plant; WTP: Weight of the tubers per plant. Averages with different letters are significantly different at 0.05 *P-value*.

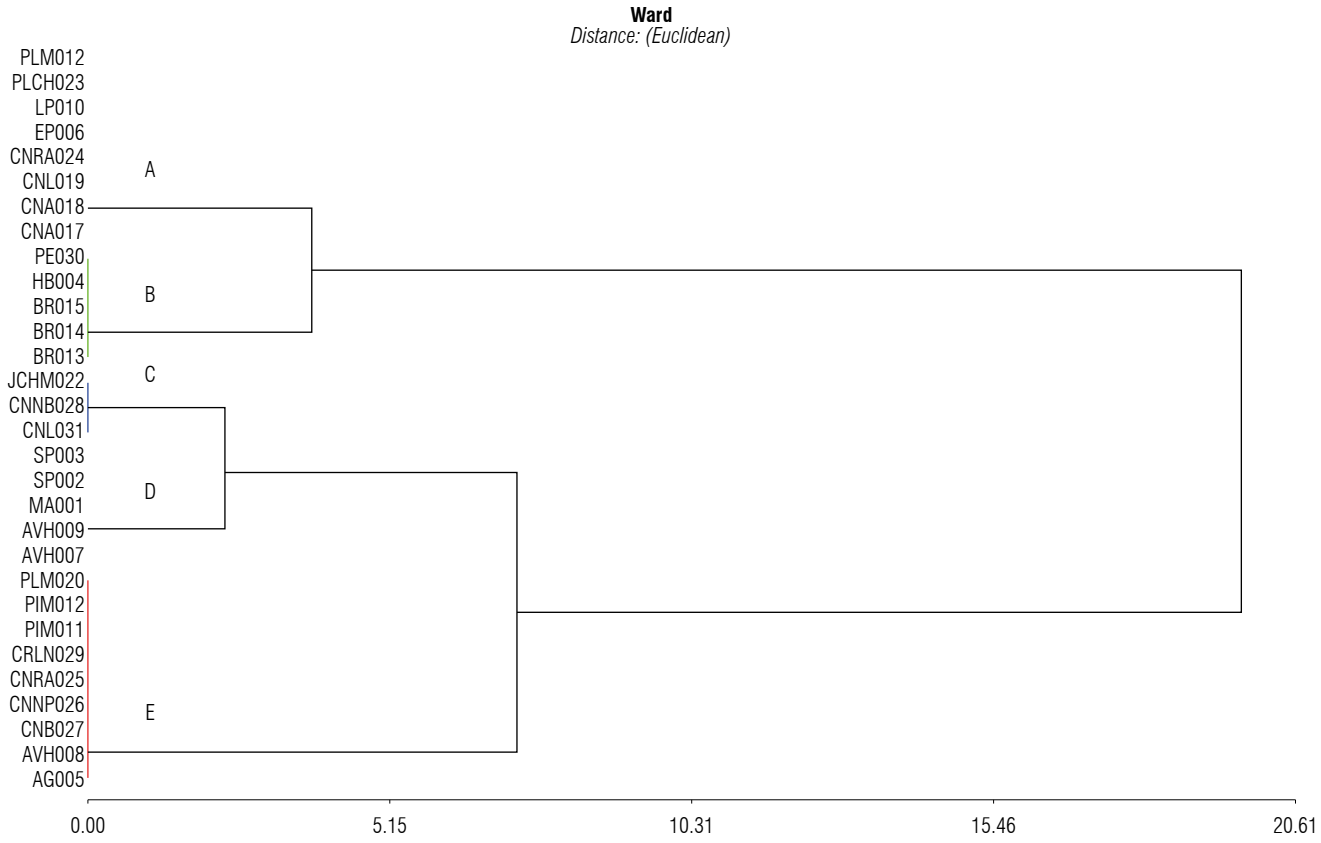

FIGURE 3. Dendrogram generated by the Euclidean distance for thirty genotypes of *Dioscorea trifida*.

B registered greater length and width of tubers (11.70 cm and 5.72 cm, respectively). This group also stands out with higher yield (6.50 t ha^{-1}) in a shorter period (295 d on average) and with light purple pulp. Moreover, groups B and D comprise genotypes of the Mestizo ethnicity, therefore, the cluster analysis may reflect the grouping in relation to the ethnic group to which it belongs. In this context, a conservation strategy within ethnic groups would be to promote the repopulation of the diversity of sachapapa genotypes along with teaching agronomic management techniques applied in the germplasm collection of this study.

Conclusions

The morphological characterization of thirty genotypes of *Dioscorea trifida* using 38 descriptors revealed high variability for the qualitative characters of petiole anthocyanin, internal tuber color, pulp color, and stem color. The quantitative descriptors with the highest coefficient of variation were tuber weight per plant and tuber yield. Four main components can explain 86% of the variability of the 30 genotypes of *D. trifida*. Descriptors with the greatest contribution to variance are tuber width, tuber weight per plant, and tuber yield (38%). It was also possible to distinguish five groups based on the Euclidean distance and Ward hierarchical clustering method, where group B highlights with genotypes HB004, BR013, BR014, BR015, PE030 registering higher tuber yield in fewer days. This is a preliminary study aiming at assessing genetic variability of the germplasm collection rather than high yielding genotypes. For more reliable yield data, replicated trails in different locations would be required. Moreover, complementary studies of nutritional value and biochemical composition of these genotypes are suggested, so that they can be shared with the population and additional molecular analysis must be performed to support the basis for the genetic improvement of this species. In addition, the agro-industrial potential of yam pulp tuber color varieties for food and health should be analyzed.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author´s contributions

WFGH designed the experiments, collected and organized the data and conducted the research. LLTC formulated the research aims and performed the data analysis. LLTC and WFGH wrote and reviewed the manuscript. All authors have reviewed the manuscript.

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Effect of biochar use as a substrate on granadilla (*Passiflora ligularis* Juss.) growth parameters

Efecto del uso de biocarbón como sustrato sobre parámetros de crecimiento en granadilla (*Passiflora ligularis* Juss.)

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ABSTRACT

The impact of biochar on soils has been demonstrated, including its improvements of physical, chemical, and biological properties that promote agricultural production. This study aims to evaluate the effect of biochar on the growth of granadilla (*Passiflora ligularis* Juss.) seedlings. For this research, biochar was obtained from the pyrolysis of agricultural waste in a conical flame curtain reactor at temperatures between 400°C and 500°C for 90 min. The different biomasses used consisted of cholupa (*Passiflora maliformis* L.) fruit shells, residues of guamo (*Inga spuria*) wood, coffee (*Coffea arabica* L.) husks, and rice (*Oryza sativa* L.) husks. The biochar produced was mixed with Jiffy® brand peat in doses of 5%, 10%, and 20% (v/v) for each of the four types of biochar, with a control of 100% peat. For each treatment, 100 seedlings were planted, taking 12 random samples of each at 43, 57, and 71 d after sowing. Data were analyzed using the Kolmogorov-Smirnov and Levene test, followed by a factorial analysis of variance, evaluating variables such as dry weight, root length, leaf number, stem diameter, and chlorophyll index. The biochar obtained from the coffee husk promoted further growth, but its effectiveness decreased at a concentration of 20%.

Key words: biomass, pyrolysis, propagation, seedlings.

RESUMEN

Se ha demostrado el impacto del biocarbón en el suelo, así como sus mejoras en algunas propiedades físicas, químicas y biológicas, promoviendo la producción agrícola. Este estudio tuvo como objetivo evaluar el efecto del biocarbón en el crecimiento de plántulas de granadilla (*Passiflora ligularis* Juss.). En el desarrollo de esta investigación, se obtuvo biocarbón a partir de la pirólisis de desechos agrícolas en un reactor cónico de cortina de llama, a temperaturas entre 400 a 500°C durante 90 min. Las biomasas empleadas fueron: cáscaras de cholupa (*Passiflora maliformis* L.), residuos de madera de guamo (*Inga spuria*), cascarilla de café (*Coffea arabica* L.) y de arroz (*Oryza sativa* L.). Los biocarbones obtenidos se mezclaron con turba marca Jiffy® en dosis de 5%, 10% y 20% v/v por cada uno de los cuatro biocarbones, con un control de 100% turba. Por cada tratamiento se sembraron 100 plántulas, tomando 12 muestras aleatorias de cada uno, a los 43, 57 y 71 d después de la siembra. Los datos fueron analizados empleando la prueba de Kolmogorov-Smirnov y Levene, seguido de un análisis factorial de varianza, evaluando variables de peso seco, longitud de raíz, número de hojas, diámetro del vástago y contenido de clorofila. El biocarbón obtenido de la cascarilla de café promovió mayor crecimiento, pero su efectividad disminuyó a una concentración de 20%.

Palabras clave: biomasa, pirólisis, propagación, plántulas.

Introduction

Throughout 2019, 63 million t of plant products were harvested in Colombia, with a predominantly agro-industrial crop distribution (Fig. 1) with palm oil, sugarcane, and coffee standing out (DANE, 2020). This production inherently generates waste with a negative impact on the environment (González, 2013; Peñaranda *et al*., 2017) and creates high costs for its final disposal (Cury *et al*., 2017), mainly associated with the precarious technologies in the country for waste utilization (González, 2013; Vargas & Pérez, 2018).

There are different biological, chemical, and thermal treatments that could add value to these biomass residues, obtaining benefits such as the development of agricultural substrates and fertilizers, generation of energy, or production of livestock feed supplements, etc. Specifically, one of the benefits is biochar synthesis by heat treatments (Garrido, 2017; Rueda-Ordoñez & Tannous, 2017) implemented as edaphic substrates in agricultural crops.

Studies carried out on wheat, corn, and tomato showed that the amendments of biochar into the soil increase reserves

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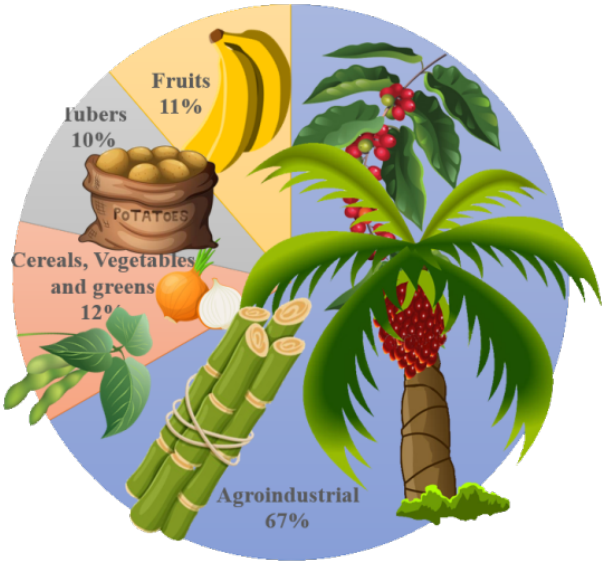

FIGURE 1. Distribution of production by group of crops in Colombia in 2019. Source: DANE (2020).

of carbon, nitrogen, phosphorus (Zhang *et al.*, 2020) and the pH of acid soil (Machado *et al.*, 2018), and increase root growth, improving plant height, stem diameter, and fresh weight of its components and fruits (Agbna *et al.*, 2017) that finally increase crop yields.

Originally, the biochar found in the soil originated from kitchen scraps and wildfire. Farmers add food scrap-derived biochar to the soil of their plantations because they have no access to biochar production technologies, making it pertinent that the project developed a flame curtain kiln for production of biochar, implementing conditions used by farmers (Glaser *et al.*, 2001).

Previous studies promoted the development of this research that focuses on the production and use of biochar to evaluate its effect on the growth of granadilla (*Passiflora ligularis* Juss.) seedlings in the department of Huila in Colombia. This plant has great economic importance and productive interest (Ramírez *et al.*, 2021) with 19,000 t of fruit harvested in 2019 (DANE, 2020). Specifically, this study focuses on an analysis of the growth parameters of granadilla seedlings (dimensions, mass, and chlorophyll index) as a function of the type of biochar and its concentration in the substrate.

Materials and methods

Types of biomass

The choices of biomass used to produce four different biochars were derived from residues from the agro-industrial sector of Huila department: cholupa (*Passiflora maliformis*) fruit shells, residues of guamo (*Inga spuria*) wood, coffee (*Coffea arabica*) husks and rice (*Oryza sativa*) husks. Each type of biomass was gathered in San Agustín (Huila, Colombia), except for the cholupa shells, gathered in Rivera (Huila, Colombia). The pulp and mucilage were removed from the cholupa fruits, and the shells were washed with water. Biomass was subjected to tray drying without convection at room temperature (30°C).

Biochar preparation

A conical flame curtain kiln was used for biochar production. The conical kiln was built by researchers at the Ithaka Institute following a design proposed by Smebye *et al.* (2017) and Stadler and Perteguer (2018). Adjustments to the support structure were made in order to make the equipment mobile, and the thermal insulation system was modified to a sandwich type (Fig. 2).

The conical kiln allowed the compaction of the biochar at the bottom of the structure as well as the maintenance of a

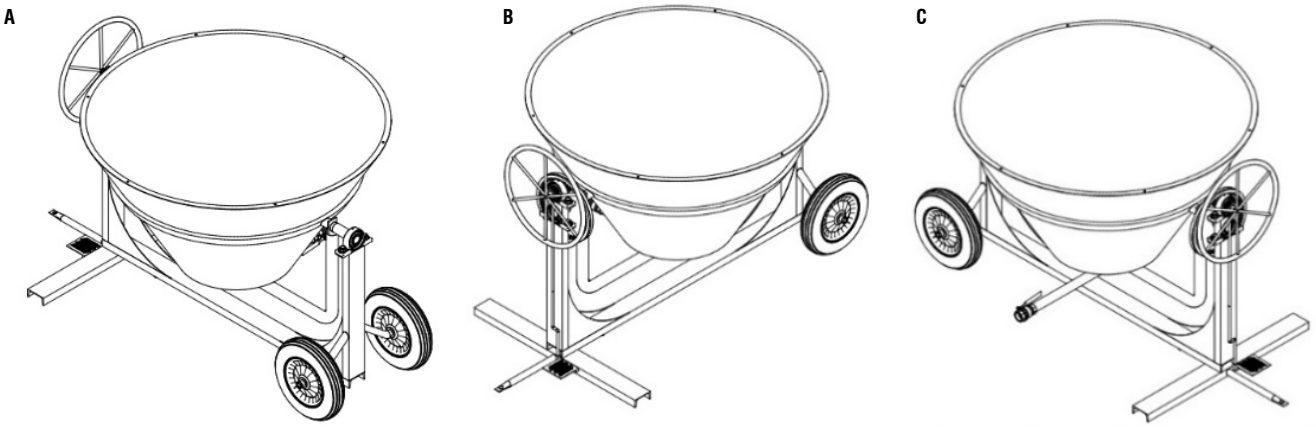

FIGURE 2. Flame curtain conical kiln: A) front right layout view, B) front left layout view, and C) rear left layout view. Cone dimensions: upper base diameter of 135 cm, lower base diameter of 48.4 cm, height of 75 cm, and volume of 500 L.

large surface flame that isolated the pyrolytic process from oxygen (Smebye *et al*., 2017). The conical kiln was loaded with a volume of approximately 10% (25 L) of the biomass to be processed. It was ignited with a match to obtain a steady flame (around 15 min). Then, the leftover biomass (225 L) was introduced to the flame curtain for 90 to 120 min at an average of 400°C to 500°C.

The reaction was finished by adding water. First, the product was dampened; then the water from the reactor was drained in order to obtain the biochar. A sample of each biochar was sent to the laboratory for a saturation extract analysis in soluble phase (Tab. 1).

Plant material and growth conditions

Seeds were obtained from the mature granadilla fruits from a 2-year-old crop located in Huila department. The fruits were disinfected with a mixed solution of Agrodyne SL, 132 g L^{-1} of polyethoxy-polypropoxy iodine and polyethoxy ethanol (Electroquímica West S.A.) with an added dose of 5 cm L^{-1} of water. Subsequently, the fruits were depulped, and the seeds were suspended in Pectinex® Ultra SP-L (3800 PGNU/ml polygalacturonase, Novozymes®) with a dose of 0.5 g L^{-1} per 100 g of pulp added for 150 min and washed with water. Then, the seeds were spread on a tray lined with Kraft paper at room temperature in the dark for 8 d. Finally, the seeds were stored from 15 to 20 d at 4°C.

Plant propagation from seeds was carried out in polystyrene trays with 50 holes (16 cm deep and 0.15 L) filled with Jiffy® peat (Jiffy group, the Netherlands) plus the addition of each biochar corresponding to each treatment (Tab. 2), using two seeds per hole. The trays were then transferred to the germination chamber for 15 d and, subsequently, trays with germinated seedlings were arranged in the nursery according to the treatments. Thinning was carried out over 20 d after sowing, leaving 50 seedlings per tray.

Air temperature was maintained from 15°C to 23°C. Phytosanitary management was carried out with an application of copper oxychloride (equivalent to 35% Cu, Grupo Agrociencias LTDA.) at a dose of 2 g L^{-1} and Mertect 20SL (220 g L^{-1} of Thiabendazole®, Syngenta, Colombia) at a dose of 1 cc L^{-1} at 20 d after sowing, and foliar applications of copper oxychloride and Ridomil Gold® MZ68WP (4% of Metalaxyl-M + 64% of Mancozeb®, Syngenta, Colombia)

TABLE 1. Results of saturation extract analysis in soluble phase for each biochar.

Biochar name		Cholupa shells	Residues of guamo wood	Coffee husks	Rice husks	Method
Parameter	Units					
Na	meq L^{-1}	9.00	0.48	0.08	0.41	Atomic emission spectrophotometry direct flame air – acetylene (APHA, 2012)
K	meq L^{-1}	348.00	48.00	2.57	1.50	
Ca	meq L^{-1}	0.01	48.00	0.63	0.90	Atomic absorption spectrophotometry with direct flame air – acetylene (APHA, 2012)
Mg	meq L^{-1}	0.37	0.32	0.27	0.20	
Fe	mg L^{-1}	0.45	0.38	1.14	0.17	
Mn	mg L^{-1}	0.07	0.11	0.17	0.11	
Cu	mg L^{-1}	0.04	0.03	0.06	0.01	
Zn	mg L^{-1}	0.14	0.09	2.45	0.03	
CEC	meq 100 g	54.00	35.00	33.00	40.00	
$N\text{-}NH_4$	meq L^{-1}	0.26	0.15	0.04	0.09	Colorimetry (APHA, 2012)
$N\text{-}NO_3$	meq L^{-1}	0.36	0.36	0.36	0.36	
S	meq L^{-1}	128.41	10.04	0.15	1.28	Turbidimetry (APHA, 2012)
Chlorides	meq L^{-1}	130.00	4.64	0.38	1.38	Volumetry (APHA, 2012)
Carbonates	meq L^{-1}	74.24	25.52	0.00	0.00	Volumetry (IGAC, 2006)
Bicarbonates	meq L^{-1}	171.68	55.68	1.90	0.90	
P	meq L^{-1}	2.34	1.18	0.01	0.19	Colorimetry with molybdate ammonium vanadate - stannous chloride (APHA, 2012)
B	mg L^{-1}	1.53	0.53	0.47	0.93	Colorimetry with azomethine (IGAC, 2006)
pH		9.87	9.97	6.86	7.92	Electrometric (APHA, 2012)
EC	mS cm^{-1}	47.90	9.75	0.53	0.72	
Water saturation	%	53.00	67.00	13.00	29.00	Calculation
Total porosity	%	75.95	82.37	82.59	86.43	Gravimetry (IGAC, 2006)

with doses at 1.5 g L^{-1}. Mineral nutrition management was scheduled through foliar applications and substrate fertilization starting from 27 d after sowing with applications of Master 13-40-13 (NPK), CRECIFOL® (100 g L^{-1} of total Nitrogen, 300 g L^{-1} of P_2O_5 and 100 g L^{-1} of K_2O, BIOEST®), and Safer Mix® (*Beauveria bassiana* $4x10^8$ spores/g, *Metarhizium anisopliae* $4x10^8$ spores/g, *Lecanicillium lecanii* $1x10^8$ spores/g, *Bacillus thuringiensis* $1x10^8$ spores/g, SAFER Agrobiológicos S.A.S.) with doses dependent on the development of the leaves of the seedlings. The irrigation dose was approximately 100 L m^3 of substrate, with daily irrigation.

Treatments and analyzed variables

For each treatment depicted in table, 2100 seedlings were grown. Twelve samples of each treatment were employed to made destructive observations at 3 perods: 43, 57, and 71 d after sowing.

TABLE 2. Treatments and coding used in the experimental development.

Code	Treatment	Biochar amount added to the peat (% v/v)
C100%	Control (peat, Jiffy®)	0
P-CFS5%	Cholupa fruit shells	5
P-CFS 10%	Cholupa fruit shells	10
P-CFS 20%	Cholupa fruit shells	20
P-CH 5%	Coffee husks	5
P-CH 10%	Coffee husks	10
P-CH 20%	Coffee husks	20
P-RH 5%	Rice husks	5
P-RH 10%	Rice husks	10
P-RH 20%	Rice husks	20
P-RGW 5%	Residues of guamo wood	5
P-RGW 10%	Residues of guamo wood	10
P-RGW 20%	Residues of guamo wood	20

For each sampling, the chlorophyll index was obtained from the last developed true leaf using a portable chlorophyll meter (SPAD-502, Konica Minolta, Inc., Japan)). Subsequently, the seedlings were washed down to the bare roots. The root length (cm) was then taken from the beginning of the main root to the length that marked the longest secondary root, the leaf number was counted, the stem length (cm) was measured from the base of the seedling to the height of the last developing leaf, and the basal diameter (mm) was measured 3 cm above the base of the seedling. The seedlings were dried at 75°C for 48 h and the root dry weight (g), leaf dry weight (g), stem dry weight (g), and total dry weight (g) were measured.

Statistical analysis

The data were analyzed using the Kolmogorov-Smirnov normality test. The distribution of the evaluated variables was normal, then the measurement frequencies of the total dry mass to a histogram was adjusted by deciles, normalizing the area under the curve (equal to 1) to obtain the density function that was compared against a normal density function for the same sample mean and deviation. The homogeneity of variances was analyzed with Levene's test. Subsequently, a factorial analysis of variance and the Tukey's multiple comparison test (α=0.05) were performed.

Results and discussion

After 43 d the sample seedlings still had cotyledons, and at 57 d the plant roots had reached the bottom of the containers (16 cm deep). At 71 d seedling growth was evident by the development in both its length and diameter. On d 43 and d 57 there were no statistical differences between the treatments; and, therefore, the discussion of the results focused on the differences found for d 71.

The morphological parameters for growth evaluation were obtained for the leaves, stem, and roots in each sampled seedling (Fig. 3).

According to the total dry matter, the influence of the type of biochar on the growth of the seedlings showed variations without exceeding the control treatment. However, the addition of 5% of the biochar derived from the cholupa fruit shells showed a superior effect among the four types of biochar (Fig. 4), but at higher concentrations it has an opposite growth effect. The highest concentrations of coffee husk biochar did not generate inefficiency in seedling development.

Biochar derived from coffee husk contains most elements at adequate levels, with a neutral pH. However, according to the results of saturation extract analysis in soluble phase, the levels of zinc in the biochar were high. Zinc in high concentrations is toxic to plants (You *et al*., 2021). The treatments with biochars derived from cholupa fruit shells, coffee husks, and rice husks did not differ from one another; their pH showed a tendency to be alkaline and could reduce the availability of minor mineral nutrients for the plants.

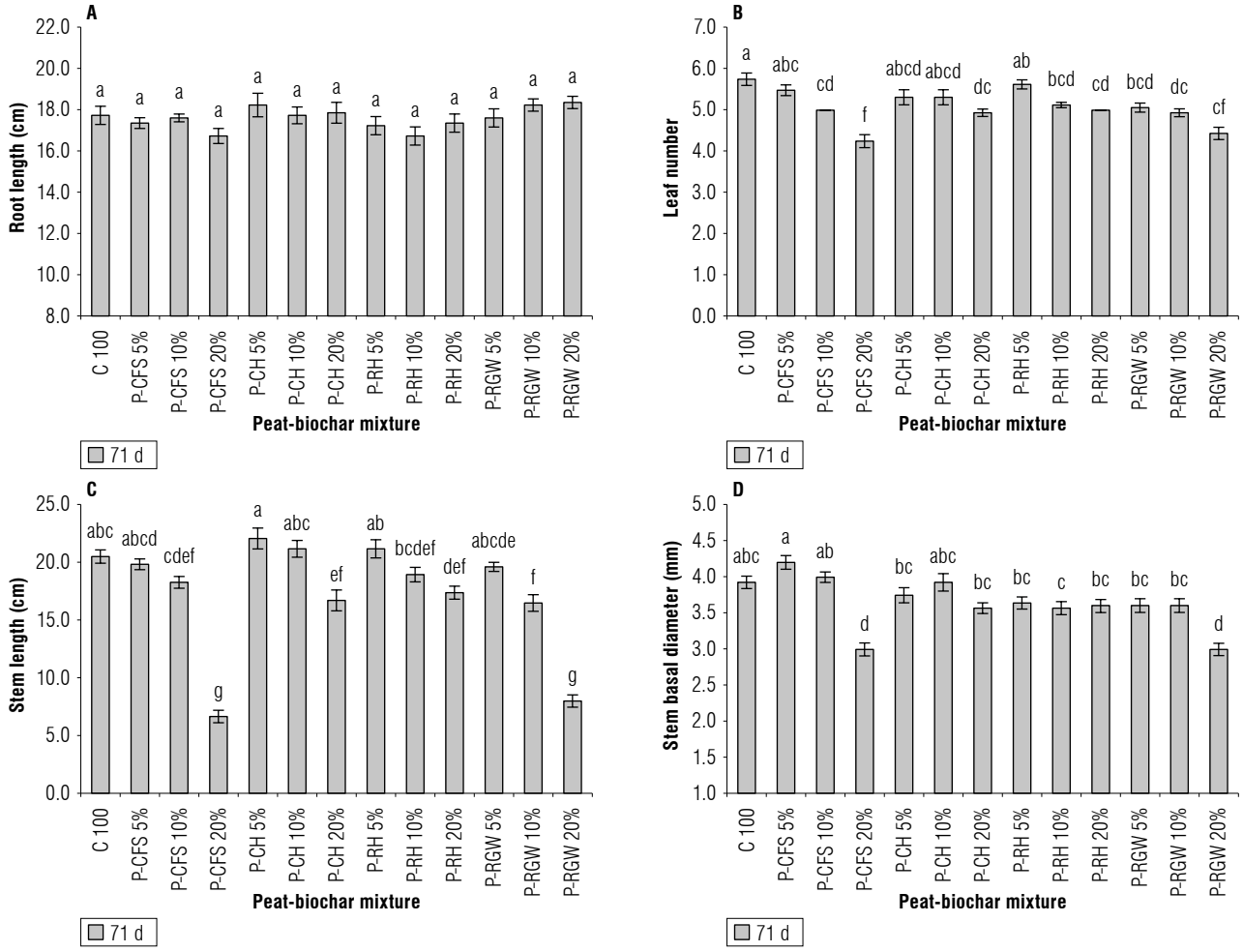

FIGURE 3. A) root length, B) leaf number, C) stem length, and D) stem basal diameter of granadilla seedlings grown on different peat-biochar mixtures at 71 d after sowing. Significant differences ($P<0.05$) among treatments are indicated by different letters according to the Tukey's test. The vertical bars in each average indicate the standard error (n=12 seedlings). See codes of peat-biochar mixtures in Table 2.

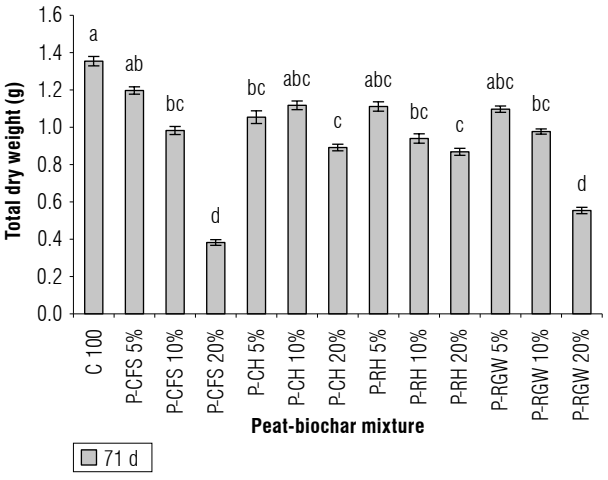

FIGURE 4. Effect of thirteen treatments on dry matter of seedlings of granadilla at 71 d after sowing. Significant differences ($P<0.05$) among treatments are indicated by different letters according to the Tukey's test. The vertical bars in each average indicate the standard error (n=12 seedlings). See codes of peat-biochar mixtures in Table 2.

According to Santos *et al.* (2020), root growth depends on the electrical conductivity and pH of the substrate. Figure 3A shows the effect of the type of biochar on root growth, whose vertical growth stops once the length equaled that of the container, although the development of dry matter of the plant continued. At 71 d, a positive effect on the length of the root was clear due to the biochars derived from rice and coffee husks that have medium levels of electrical conductivity, contrasting to those derived from cholupa fruit shells and guamo wood residues.

The high concentration of biochar in the substrate had negative effects on the granadilla seedlings because of its effects on the plant dry matter. The concentration of 20% of the different types of biochar caused lower growth. In addition, the analysis of the saturation extract of the biochars showed that biochar derived from cholupa fruit shells and guamo wood residues contained excessive levels of potassium, chlorides, and sulfur.

Chrysargyris *et al.* (2019) finds that biochar from wild bamboo and wood sieves at high levels (15 and 20%) negatively affects dry matter and root length of cabbage seedlings. However, at 71 d of the present study, the addition of biochar had positive effects on root length, without exceeding the root dry matter generated by the control treatment.

The addition of 5% of biochar did not lead to an imbalance that would cause negative effects on the seedlings. The means by which seedling propagation is carried out is equivalent to one of the highest production costs, so this study was an approximation to the equivalences in which biochar can be used.

The analysis of the leaf number and chlorophyll index in the granadilla seedlings indicated that the biochar derived from guamo wood residues was the substrate that generated the lowest leaf numbers. The lowest chlorophyll index in the three samplings was obtained from biochar derived from cholupa shells, and the best response was obtained from the coffee and rice husks.

The effect of chlorophyll directly impacts plant growth, and it is related to the leaf area. Likewise, chlorophyll levels are an indicator of the plant´s ability to produce carbohydrates. The results regarding this parameter indicated that the biochar treatments derived from coffee and rice husks did not generate an imbalance in the substrate. According to analysis of the biochars, they have a lower sum of cations than those derived from guamo wood residues and cholupa shells that have cations in excess.

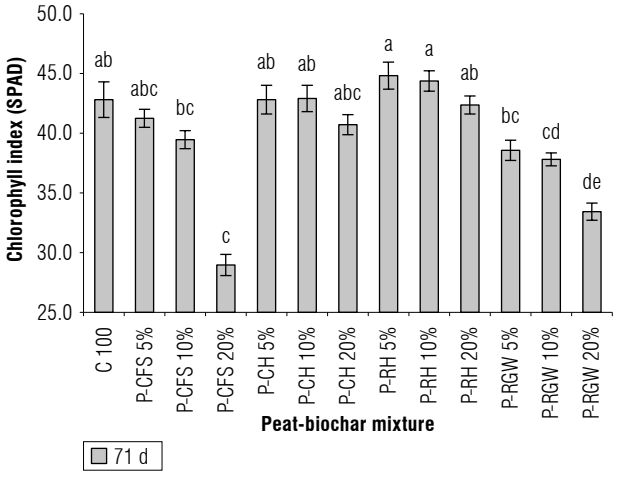

FIGURE 5. Effect of thirteen treatments on chlorophyll index of seedlings of granadilla at 71 d after sowing. Significant differences ($P<0.05$) among treatments are indicated by different letters according to the Tukey's test. The vertical bars in each average indicate the standard error (n=12 seedlings). See codes of peat-biochar mixtures in Table 2.

The results are presented by comparing the chlorophyll index and the leaf number in the three sampling moments. These results agree with the findings of Chrysargyris *et al.* (2019), since the chlorophyll index (SPAD) in the cabbage seedlings is affected differently by the type and proportion of biochar with decreases often more pronounced at higher levels of biochar (15 and 20%). On the other hand, Bommaraju (2016) finds that the substrate constituted by vermicompost and biochar from forest residues (50% biochar and 50% vermicompost) improves photosynthesis in coffee seedlings.

Conclusions

This study showed the effect of the type and different concentrations of biochar as substrate on growth parameters of granadilla seedlings. The analysis of the type of biochar demonstrated that those from materials such as rice husk and coffee husk showed superior results on the growth of the plants, thus, showing that their effect on the substrate generated a positive impact at low concentrations.The biomass that had the greatest results for biochar production as a substrate is coffee husk. The results were also conclusive regarding the concentration of biochar in the substrate, where 5% had the greatest effectiveness, but they did not exceed the control treatment.The control treatment had the best result because the peat used is a substrate of natural origin specially conditioned for seedling propagation, implying that the high concentrations of biochar unbalanced the base conditions of the substrate. However, biochar has effects that should continue to be studied in combination with other organic substrates such as soil and coconut fiber.

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Conflict of interest statement

The authors declare that there are no conflicts of interest regarding the publication of this article.

Author's contributions

TCPE and ARC formulated the evolution of overarching research aims; TCPE and ARC carried out the field and laboratory experiments; TCPE wrote the original draft; ARC and CAL reviewed and edited the writing; CAL did the visualization. All authors reviewed the manuscript.

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Basal rot in carnation (*Dianthus caryophyllus* L.) is caused by *Fusarium verticillioides* (Sacc.) Nirenberg

La pudrición basal en clavel (*Dianthus caryophyllus* L.) es causada por *Fusarium verticillioides* (Sacc.) Nirenberg

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ABSTRACT

The carnation is one of the most important products for export in the floriculture industry of Colombia. Fusariosis (a disease resulting from presence of *Fusarium*) appears on the crops in two forms: vascular wilt and basal rot. The first is caused by *Fusarium oxysporum* f. sp. *dianthi*, which is a well-characterized disease. The second, caused by *Fusarium verticillioides* (previously *Fusarium roseum*), is a non-characterized disease and its development in the plant has not been described in detail. The symptoms of basal rot were differentiated from vascular wilt, through infection of plants in the greenhouse, with isolates of *F. verticillioides* and *F. oxysporum* obtained from lesions of symptomatic plants. The fungi morphological characterization allowed differentiation of the isolates of these two species and their growth habits on different media. The sequencing of 8 different genes with more than 13 amplicons in the 2 species showed genetic differences that grouped the isolates into different taxa. Multilocus sequence typing analysis using DNA sequences of 8 different genetic regions confirmed the presence of *F. verticillioides*. In this study, the role of *F. verticillioides* was demonstrated in the stems of carnation in commercial crops that presented pathogenic lesions. According to the results of the study, *F. verticillioides* is the etiological agent that produces the basal rotting in carnation plants, alone or in association with *F. oxysporum*.

Key words: *Fusarium oxysporum, Fusarium roseum*, morphological characterization, vascular wilt.

RESUMEN

El clavel es uno de los productos más importantes para exportar en la industria floricultora de Colombia. La fusariosis (una enfermedad resultante de la presencia de *Fusarium*) aparece en el cultivo en dos formas: la marchitez vascular y la pudrición basal. La primera es causada por *F. oxysporum* f. sp. *dianthi* y es una enfermedad bien caracterizada. La segunda, causada por *Fusarium verticillioides* (anteriormente *Fusarium roseum*), es una enfermedad no caracterizada y su desarrollo en la planta no ha sido descrito en detalle. Los síntomas de la pudrición basal fueron diferenciados de los producidos por la marchitez vascular, a través de la infección de plantas en el invernadero, con aislados de *F. verticillioides* y *F. oxysporum* obtenidos de lesiones de plantas sintomáticas. La caracterización morfológica de los hongos permitió diferenciar las dos especies de *Fusarium* y se observaron sus hábitos de crecimiento en diferentes medios. Las secuencias de 8 diferentes genes con más de 13 diferentes amplicones en las 2 especies mostró diferencias genéticas que agruparon los aislados dentro de taxones diferentes. Análisis usando tipificación de secuencias múltiples de DNA confirmaron la presencia de *F. verticillioides*. En este estudio se estableció el papel de *F. verticillioides* presente en los tallos de clavel en cultivos comerciales que presentan lesiones patogénicas. De acuerdo con los resultados del estudio, *F. verticillioides* es el agente etiológico responsable de la pudrición basal en plantas de clavel solo o en asocio con *F. oxysporum*.

Palabras clave: *Fusarium oxysporum*, *Fusarium roseum*, caracterización morfológica, marchitez vascular.

Introduction

The global carnation market is projected to reach USD 3.266 billion by 2026, from USD 2.719 billion in 2020 (Global carnation sales market report, 2020). Fusariosis disease is a critical problem for carnation growers in Colombia as in most countries where carnations are produced. *Fusarium oxysporum* (FOX) f. sp. *dianthi* (Prill. and Delacr.) (Snyder & Hansen, 1940) has become the most significant pathogen in fusariosis disease in this crop (Arbeláez, 1987; Arbeláez, 1992; Borrero *et al*., 2009). Large losses can occur due to the easy spread of the fungi across the infected cuttings, the fast dissemination by diverse means, the persistence of the pathogen in the soil, and the high cost of control

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measures. All these causes confer special importance to this disease (Graces *et al*., 2001).

Lopez and Fergus described the carnation basal rot for the first time in 1965. Since then, growers have related the disease to *F. roseum*. More recently, the species *F. roseum* has been replaced by other species of *Fusarium* (Burgess *et al*., 1988). Arbeláez (1992) reported that both FOX and *F. roseum* could occur together in the same crop, with their masking symptoms making their identification difficult.

Fusarium verticillioides (Sacc.) (FVER) (Nirenberg, 1976) is an important phytopathogen in cereal crops that produces significant economic losses around the world. It attacks mainly corn, causing rotting in shoots, roots, and spikes (Bush *et al*., 2004). Filgueira *et al*. (2007) first reported FVER presence on carnation stems with basal rot symptoms. The objective of our study was to identify the etiologic agent that produces basal rot on carnation and to describe the development of the disease in the plants.

Materials and methods

Biological samples

More than 1,500 plants with basal rot symptoms were used to isolate the fungus. These plants were collected between the 2012 and 2018 from 12 different carnation farms, located at the Bogotá plateau in the municipalities of Cajicá, Madrid, Siberia, Chía, Suesca, Sopó, Facatativá, and Tocancipá. All of them were in the Cundinamarca department of Colombia. The commercial carnation varieties sampled were: ´Nelson®', 'Tasman®', 'Delphi®', and 'Pink Nelson®', with basal rot symptoms as previously reported by Arbeláez (1987) and Filgueira *et al*. (2007).

Fungi isolation

All the stem fragments, sampled at the crown level of infected plants, were surface sterilized with 5% sodium hypochlorite for 1 min and then with 70% ethanol for 1 min and rinsed several times with sterile water. Once surface disinfection was completed, the stem was sectioned into discs of 2 mm thickness, which were then grown on Potato Dextrose Agar (PDA) (MERK) and Czapek Dox (OXOID) and incubated at 25°C for 7 d. Once the pathogen colony was visible, it was transferred to liquid Czapek Dox Broth Sigma with agitation for a week at 25ºC. Then, a single-spore culture was obtained from each isolation, three 1:10 serial dilutions of the spore suspension were made and 0.1 ml of the last dilution was taken and scattered on PDA. After incubation, a monosporic growth was selected and isolated on PDA. Finally, the fungi were conserved in glycerol 50% at 4ºC. Colony characteristics such as color and appearance were observed in PDA cultures. The bright field in an Axioskop 2 Plus Carl Zeiss microscope was used to determine the presence, absence, and type of micro and macroconidia, the morphological characteristics of the mycelium, and possible formation of chlamydospores by the isolate. Asexual structures like macroconidia were measured using the Scion Image 4.0 free program.

Symptom characterization

On 53 carnation hybrid lines, the product of the crossing between varieties resistant and susceptible to FOX and one commercial variety 'Nelson®' hybrid lines and the commercial variety were established from seeds *in vitro* using MS basal media (Murashige & Skoog, 1962). One hundred microplants of each hybrid line and 'Nelson' variety were acclimated to outdoor conditions of the Bogotá plateau (maximum temperatures around 19 to 20°C [66 to 68°F] and minimum temperatures from 7 to 9°C [44.5 to 48°F]; the average annual relative humidity of 79%), with a vermiculite-like substrate. Four weeks later, plants of each hybrid line and commercial line were transplanted into pots. This evaluation used a quantitative measure of disease intensity with time (AUDPC) with analysis of variance by ANOVA in three repetitions that showed a normal distribution. The roots of each of the sixty plants were inoculated with 6 ml of a suspension of $1x10^6$ spores/ml (30 plants with FVER and 30 with FOX) using a syringe and injecting the fungus around the plant crown. Seven d after inoculation, the plants were re-inoculated with 200 µl of a suspension of $1x10^6$ spores/ml by puncturing next to the stem base (Filgueira, 2011). As a control, 20 plants of each hybrid line and the commercial variety were inoculated in the same way with distillate water. Finally, 20 plants were used as negative controls without inoculation.

Weekly for 4 months, the plants were observed and a detailed record of the appearance of symptoms was performed. The criterion used for the external symptom registration was yellowing and the discoloration or loss of green color was measured as presence/absence. The wilting of the foliage, stem, and buds was measured according to the following scale: absence 0, wilting of the stem base and cracking 1, wilting that affects the stem and leaves that can be observed in ¾ of plant 2, total wilting of the plant and death 3. Spots, such as stem discoloration, were measured as absence/presence. External necrosis, such as basal disintegration of the stem, was recorded as absence 0, moderate tissue damage 1, brown color in the stem base 2, and possible leaf necrosis, loss of turgor, stem decay, and dehydration 3. Finally, symptomatic plants were collected

at random, and the pathogen was isolated *in vitro* to corroborate the entrance of the pathogen to the plant. The incidence was estimated as the number of plants that are diseased, relative to the total number collected (Campbell & Neher, 1994).

In vitro evaluation of the plant response to *Fusarium* presence

In vitro evaluation consisted of an essay of "dual" cultures, in which undifferentiated cells were obtained from microplants leaf explants of 53 carnation hybrid lines, cultivated on MS basal media supplemented with 1 mg L^{-1} of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Filgueira, 2011). One gram of *in vitro* cells was placed in a Petri dish (diameter 90 mm) border, with the same media as the callus induction, and cultured for a week at 26°C. Next, 10 µl of spore solution of the pathogen (FVER or FOX) at a concentration of $1x10^6$ spores/ml was inoculated on a sterile circle of filter paper on a Petri dish located opposite to plant cells and then left for two weeks. Possible inhibitory (resistant) or not-inhibitory (susceptible) responses by plant cells towards pathogen growth were recorded measuring the growth of the fungi in the direction of the plant cells in centimeters (Filgueira, 2011).

PCR identification

For this purpose, four of the characterized isolates of both FOX and FVER that were placed by rapid growth in the media and did not present other biological contaminants were used. Fungal DNA was extracted, and PCR amplification was performed following the method described by Cenis (1992), with the modification by Abd-Elsalam *et al*. (2003). The fungal conidia were inoculated in 10 ml of liquid Czapek Dox Broth (Sigma®) for 72 h at 25°C; later, the fungal mycelia were centrifuged at 15000 g for 5 min. Next, the samples were washed with 2 ml of Tris Buffer (TE), (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and the mycelia were macerated in liquid nitrogen for 5 min. Three hundred µl of lysis buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added for 5 min, followed by the addition of sodium acetate 3 M (pH 5.2). The fungal samples were put at 20°C for 10 min and centrifuged at 15000 g for 5 min. The supernatant was transferred to a new tube and an equal volume of isopropanol was added and incubated at 4°C for 4 h. Finally, the suspension was centrifuged at 15000 g for 10 min. Ethanol 70% was added to the pellet to wash and then discarded. The DNA was suspended in 100 µl of TE.

The primers used to identify the fungi are summarized in Table 1. The PCR mixture contained 5 to 10 ng of template DNA, 1 µM of each primer, 3 mM of $MgCl_2$, 200 µM of dNTP, 2.5 U of Taq polymerase, and reaction buffer (50 mM KCl, 50 mM Tris-HCl; pH 8.3, 0.1 mg ml^{-1} BSA), in a final volume of 20 µl. Thirty PCR cycles with the following temperature regime were performed at 95°C for 2 min (only in the first circle), at 94°C for 1 min, at the annealing temperature for 0.5 min, and at 72°C for 1 min. The PCR products were analyzed by electrophoresis on 1% agarose gel stained with 0.5 µg ml^{-1} of ethidium bromide. As an external group, DNA of *Botrytis cinerea,* obtained under the same conditions, was used. The outgroup is important as a point of comparison for the in-group and allows the phylogeny to be rooted. Each amplicon was sequenced twice (forward and reverse) using the Sanger *et al*. (1977) method by Macrogen® (Korea). The DNA consensus sequence was compared with the GenBank database. Sequences that presented similar values higher

TABLE 1. Forward and reverse primers employed. Thirteen amplicons belonging to eight different genes or genetic regions were used to identify the fungi taxonomically.

Gene	Primer	Sequences	Sense	Size expected and size obtained	Author
β-Tubulin	Bt1α	5'TTCCCCCGTCTCCACTTCTTCATG3'	Forward	537bp 280bp	Glass and Donaldson, 1995
	Bt1b	5'GACGAGATGGTTCATGTTGAACTC3'	Reverse		
	Bt2a	5'GGTAACCAAATCGGTGCTGCTTTC3'	Forward	495pb 380bp	
	Bt2b	5'ACCCTCAGTGTAGTGACCCTTGGC3'	Reverse		
Histone 3	H31α	5'ACTAAGCAGACCGCCCGCAGG3'	Forward	447pb 540bp	
	H31β	5'GCGGGCGAGCTGGATGTCCTT3'	Reverse		
Calmodulin	Cmd-F	5´GATCAAGGAGGCCTTCTC3´	Forward	672pb 720bp	O'Donnell *et al*., 2000
	Cmd-R	5´TTTTTGCATCATGAGTTGGAC3´	Reverse		
Elongation factor 1-α	EF1H	5'ATGGGTAAGGAAGACAAGAC3'	Forward	678pb 700bp	O'Donnell *et al*., 1998
	EF2T	5'GGAAGTACCAGTGATCATTGTT3'	Reverse		

To be continued

Gene	Primer	Sequences	Sense	Size expected and size obtained	Author
Cytochrome C oxidase	AHyFuF	5'CTTAGTGGGCCAGGAGTTCAATA3'	Forward	568pb	Gilmore *et al*., 2009
	AHyFuR	5'ACCTCAGGGTGTCCGAAGAAT3'	Reverse		
rDNA	ITS FuR	5' GCGACGATTACCAGTAACGA3'	Forward	416pb 398bp	White *et al*., 1990
	ITS FuF	5'CAACTCCCAAACCCCTGTGA3'	Reverse		
	ITS1	5'TCCGTAGGTGAACCTGCGG3'	Forward	250pb 220bp	
	ITS2	5'GCTGCGTTCTTCATCGATGC3'	Reverse		
	ITS1	5'TCCGTAGGTGAACCTGCGG3'	Forward	570pb	
	ITS4	5'TCCTCCGCTTATTGATATGC3'	Reverse		
	ITS4	5' TCCTCCGCTTATTGATATGC3'	Forward	524bp 530pb	
	ITS5	5'GGAAGTAAAAGTCGTAACAAGG3'	Reverse		
Mitochondria small subunit	NMS1	5´CAGCAGTGAGGAATATTGGTCAATG3'	Forward	716pb 720bp	
	NMS2	5´GCGGATCATCGAATTAAATAACAT3'	Reverse		
Intergenic spacer rDNA	VERT1	5' GCGGGAATTCAAAAGTGGCC 3'	Forward	800bp	Patiño *et al*., 2004
	VERT2	5' GAGGGCGCGAAACGGATCGG 3'	Reverse		
Intergenic spacer rDNA	VERTF1	5' GTCAGAATCCATGCCAGAACG 3'	Forward	400pb	
	VERTF2	5' CACCCGCAGCAATCCATCAG 3'	Reverse		

The DNA sequence data obtained in this study have been deposited in NCBI GenBank and the accession numbers are listed in https://www.ncbi.nlm.nih.gov/nuccore/?term=Fusarium%20filgueira.

than 97% with the morphologic species, using the BLAST tool, were used to generate a consensus using CLC DNA Workbench® software. The consensus sequences were compared by multiple alignments with CLUSTAL OMEGA in the MEGA7 software (Molecular Evolutionary Genetics Analysis), which was used to build the phylogenetic tree using the Neighbor-Joining distance method.

Processing of data

All biological materials in this study were randomly selected and the data were handled with descriptive statistics for qualitative dichotomous characteristics by presence or absence (P/A) of symptoms of necrosis, spots, curl, sporulation, stem chlorosis, turgidity loss, rachis, rot, and decay. The data were processed in the MINITAB Release 17 program using a model of logistic regression for the P/A of symptoms. The variance between assay groups with the ANOVA program was evaluated, and then the data adjustment in the model was tested. All the data were studied in time (d). Using the previous method, the tendency of the symptoms in time was observed, and finally, the Tukey's range test was applied.

The information for sequences of the different loci used in this study was processed using the Multi-Locus Sequence Analysis (MLST) tool of the CBS-KNAW Fungal Biodiversity Centre's Fusarium MLST website (https://fusarium.mycobank.org/) and the corresponding Fusarium-ID site hosted at the Pennsylvania State University (http://isolate.fusariumdb.org). Dendrograms were generated by neighbour-joining and split decomposition with bootstrap analysis with 1,000 replications, using MEGAX.

Results

Symptoms in plants

In general, it is possible to distinguish fusariosis symptoms at the base of the plants in the crop field, *e.g.*, FVER produces a dry rot that ascends towards the medial part of the stem, forming caverns-like cracks with or without jumps. These lesions did not compromise the vascular bundle (Fig. 1A-C). In advanced stages, it was possible to detect a white to reddish cottony mycelium inside the lesion (Fig. 1G). On the other hand, the infection with FOX in the basal portion (crown) of the plant was characterized by a lesion in the form of a narrow ring limited to the vascular system that did not compromise the medullary tissue in the first stage of the infection (Fig. 1D-E). In some cases, it was possible to detect both fungi infecting the same plant (Fig. 1F). Other external symptoms of basal stem rot caused by FVER are: partial or complete chlorosis, generalized or partial wilting green, stem turgor loss, cracking in the crown, presence of conidiation structures, necrosis, and finally plant death (Fig. 1H).

The analysis of the fusariosis disease under field conditions showed that the Pink Nelson variety presented a higher FVER incidence of 7.1% (SD=1.2), followed by the Delphi

FIGURE 1. Symptoms of fusariosis in carnation plants. A-C) transversal and longitudinal cuts in the crown of carnation plants with the symptom of basal rot caused by FVER (C initial stage of invasion and B final stage); D-E) Transversal and longitudinal cuts in plants with symptoms of vascular wilt caused by FOX; F) Plant infected by both parasites with the symptom of basal rot (black arrow) and vascular wilt (red arrow); G-H) Carnation plants that present the final phase of the disease caused by FVER with cracking in the crown, the presence of fungal structures, and necrosis.

variety with 3.7% (SD=0.6) and the Nelson variety with 3% (SD=0.5) incidence. In the case of FVER, the maximum incidence observed was 10% (SD=1.6) as opposed to FOX, with a maximum incidence value of 68% (SD=5.7) in the farms.

Fungal isolation and preliminary characterization

The evaluation of 1,500 samples of monoconidial culture showed that, in 68.3% of the samples presenting symptoms of fusariosis, FOX was present, while in 8.2% of the samples, FVER was present, values close to that of the infected plants that were observed in the field. Ten percent of the samples presented both species simultaneously; 11.5% of the plants presented other *Fusarium* species (*F. graminearum, F. foetens, F. culmorum, F. avenaceum*, and *Fusarium* sp.), and, in 2% of the cases, it was not possible to identify which of the parasites was present. Microscopically it was possible to identify septate mycelium in FVER isolates and production of long and slim macroconidia with thin walls, bent apical cells, and basal cells in the form of foot, with three to five septa. The morphological characterization of vegetative and reproductive structures of FOX and FVER is presented in Table 2. Macroconidia of FVER are shown in Figure 2A-B. Figure 2D shows observed simple branched FVER monophialides and abundant ovoid microconidia. The microconidia were only obtained on CLA (carnation leaf agar) media after 45 d of cultivation (Fig. 2B-C) and occurred separated from each other or in short and long chains (Fig. 2C). This last characteristic is important for distinguishing FVER from FOX. In this study, dark yellow *sporodochia* were observed in FVER *in vitro* media with more than 45 d of cultivation.

FIGURE 2. Isolation of FVER and FOX in nutrient media and microscopy recognition. A) Macroconidia of the FVER; B) FVER Macroconidia and microconidia. Bar is one μm; C) Production of FVER microconidia in a chain, 100X; D) FVER branched monophialide, 180X; E) Macroconidia, microconidia, and chlamydospores of FOX. Monophialides of FOX. A, C, D, and F, 1100X. B and E, 750X.

TABLE 2. Morphological characterization of FOX and FVER. The values are measurement averages. Range of the standard deviation in all the measures was between 0.001 and 0.15 μm.

		FOX	FVER
Macroconidia	**Form**	Straight or slightly curved	Long and thin
	Apical cell	Tapered and curved	Curved
	Foot cell	Definite	Definite
	Area (μm)	123	101
	Perimeter (μm)	779.5	911
	Major axis (μm)	290.5	363.5
	Minor axis (μm)	53.75	36
Microconidia	**Form**	Ellipsoidal, kidney-shaped	Oval
	Phialide	yes	no
	Area (μm)	0.11	0.07
	Perimeter (μm)	1.55	1.24
	Major axis (μm)	0.67	0.51
	Minor axis (μm)	0.22	0.17
Chlamydospores		yes	no

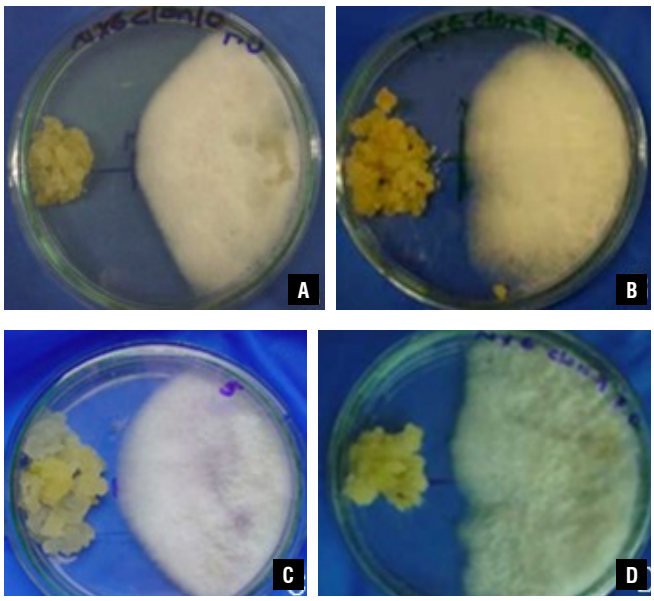

FIGURE 3. "Dual" culture assay *in vitro*. A) In the interaction between susceptible carnation cells and FVER, the fungus grew towards the carnation cells; B) Interaction between carnation-resistant cells and FVER indicating that the fungi stopped its growth by the presence of the resistant plant cells. An assay using FOX with carnation-susceptible cells C) and resistant cells D).

Tests of pathogenicity

In vitro interaction

Through the different tests, we study the processes of recognition and invasion of carnation plants by FVER. The first test was an *in vitro* assay, where the pathogen and undifferentiated cells of the plant can mutually recognize each other. In this case, it was possible to observe that the mycelia of the fungi grew mainly in the direction in which the plant cells were present (Fig. 3A, Fig. 4A). In the opposite case, where the cells present a resistant reaction to the presence of fungi, the fungi mycelia grew more slowly in the direction of the plant cells, as a type of growth inhibition reaction (Fig. 3B, Fig. 4A.). This evidences a host-parasite interaction between carnation cells and FVER. This behaviour was also observed in an *in vitro* assay with FOX (Fig. 3C-D., Fig. 4B).

To determine if the daily growth of the funguses in presence of resistant and susceptible carnation cells differs significantly with the "dual" culture in the Petri dish, variance analysis was done by d using ANOVA program with a $P<0.05$. The results show significant statistical differences between the fungi growth in the presence of resistant cells and susceptible cells (Fig. 4A-B).

Ex vitro interactions in the greenhouse

In the inoculation test of susceptible plants inoculated with FVER in germination trays, the first symptom of dry stem bark at the plant base was observed 15 d after the first inoculation. This symptom became most prominent and ascended between 10 and 15 cm from the stem base by week 10 to 13. The Tukey´s test presented a value of 3.45 (α=0.05, K=3, df = 5) near week 7. It was possible to observe wilt with foliar yellowing on the leaves located near the plant base of the susceptible varieties. In this case, the Tukey's range test showed a value of 2.95 for this symptom by week 5. Additionally, internally in the plant base, a continuous or discontinuous pattern of rots at the same zone on the stem medulla was found, as shown in Figures 1A and C., which was near d 30 after the first inoculation. This symptom reached a value of 3.4 in the Tukey´s test near week 10. The rot spread on the stem base from the modular zone toward the vascular bundles and other stem tissues (Fig. 1B). The rots inside the stems sometimes appeared with red stains located in the modular zone, and wilting was evident from week 3. The Tukey´s test showed a value of 3.95 for this symptom after week 7.

Likewise, the spot symptom in the base stem indicated pathogen colonization, which was evident between weeks 3 and 4 after the inoculation. The Tukey´s test showed a value of 3.85 to 3.95 after week 10. The tissue necrosis symptoms were present posterior to the spot apparition, near week 4 (Fig. 1G). Plant behavior was similar in all the control groups. A comparative study with FOX (data not

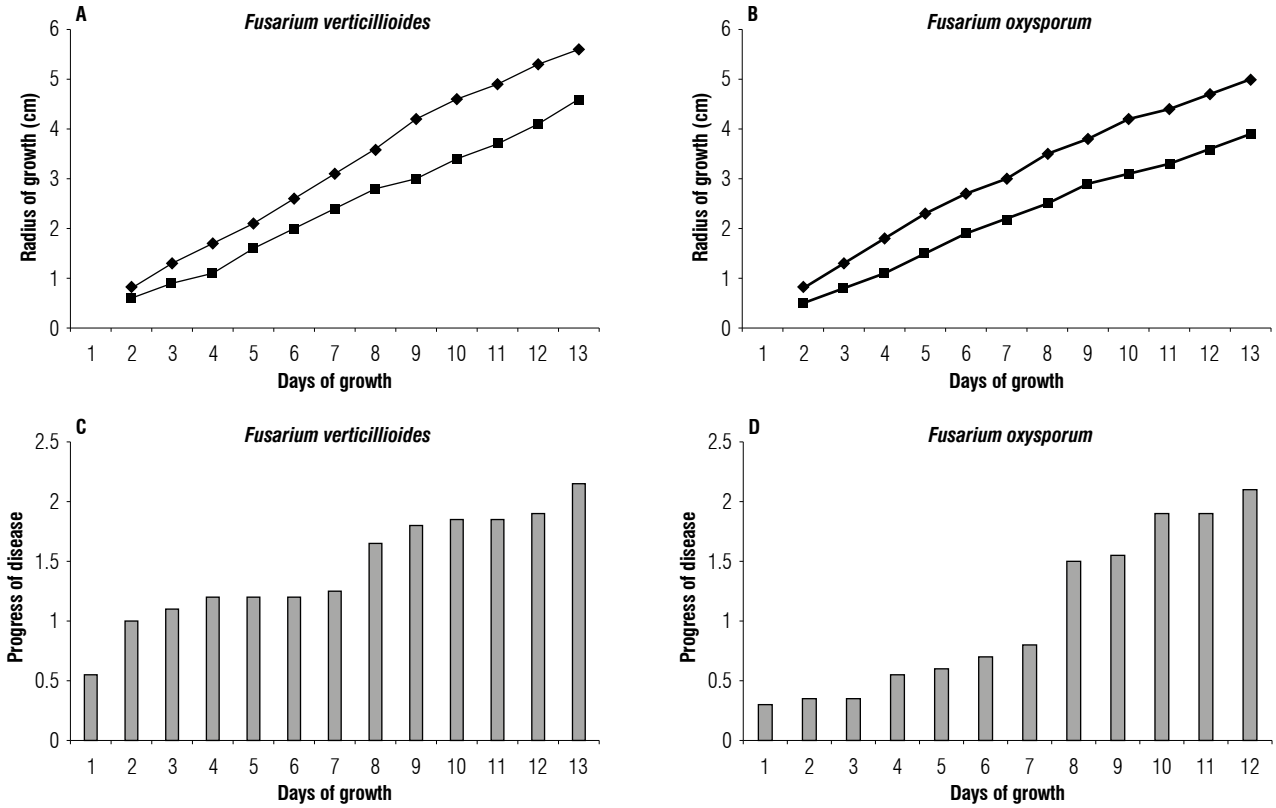

FIGURE 4. Fungi growth *in vitro* (A and B). Disease progress in the *ex vitro* test (C and D); A) FVER growth and B) FOX growth. The fungus growth is shown on *in vitro* assay ("dual" culture), in the carnation resistant cells interaction (■) with the susceptible cells (◆); C) Plants inoculated with FVER and D) plants inoculated with FOX. The plant wilting progress is recorded in days in the assay of plant inoculation in the greenhouse.

presented here) indicated a significant difference in the infection progress of the disease (Fig. 4C-D), such as the plant wilting progress over time. These data determine that FVER is more aggressive in the infection and colonization of the plant stem than FOX.

As mentioned above, we selected symptomatic plants randomly for each inoculation treatment and the re-isolation in vitro of the pathogen to confirm the presence or absence of the isolates, fulfilling Koch's postulates. Twenty plants of the susceptible commercial variety Nelson were re-inoculated with conidia of the pathogen obtained from infected plants from the first inoculation experiment. All these plants presented severe symptoms of basal rot with foliar yellowing, vascular chlorosis, medullar rot, and stem cupping. Fragments of this infected tissue were used to isolate the parasite and determine the presence of the fungus that was used to inoculate the initial plants like FVER.

Molecular identification

An adequate quantity of genetic regions was employed to appropriately identify the two species because of the problems in using morphological characters in the identification of FOX and FVER. First, we used the ITS region for the identification of the genus to resolve the limitation that this genetic region presents for use as barcoding. Next, we used a group of primers to determine with the greater accuracy of the fungal species, especially when the study implicates phylogenetic analysis (Tab. 1). In this sense, the use of MLST is the best option to identify and obtain phylogenetic relationships between species of difficult identification (Matsumura, 2013).

Ten samples of each of the morphological species FVER and FOX were selected. To prevent slants or false positives, a preliminary molecular characterization by PCR using ITSs and EFs 1-α (Tab. 1) was performed to determine if the isolates previously classified as FOX or FVER belong to the *Fusarium* genus. Figure 5A-E shows the amplicons of the primers ITSFuF/ITSFuR, ITS1/ITS2, ITS1/ITS4, ITS4/ITS5, and EF-1H/EF-2T. The accession number (ID) to view the nucleotide sequence in the GenBank of NCBI of these amplicons is listed in Table 3. In most cases, such as amplicons ITS1/ITS2 and Elongation Factor 1-α, the

TABLE 3. GenBank (NCBI) IDs and comparisons with related sequences. The DNA sequences of FVER and FOX obtained in this study were compared using the BLAST tool, and the results show the identity percentage and the E value of the most related sequences.

Locus	Gene	FVER*	FOX*	Related specie	Percentage Identity	E value
β-Tubulin	Bt1	KF467337.1	KF467331	*F. verticillioides*	100	0.0
β-Tubulin	Bt2	KF467337	JF773354	*F. verticillioides*	97	0.0
Histone 3	H3-1	KF467389	KF467383	*F. verticillioides*	100	0.0
Calmodulin	Cmd	KF467350	KF467344	*G. moniliformis*	99	0.0
Elongation factor 1-α	EF	KF467376	KF467370	*F. verticillioides*	100	0.0
Cytochrome C oxidase	AHyFu	KF467363	KF467357	*G. moniliformis*	99	0.0
rDNA	ITS Fu	KF467402	KF467396	*F. verticillioides*	100	0.0
rDNA	ITS1-2	KF467415	KF467409	*F. verticillioides*	100	0.0
rDNA	ITS1-4	KF467428	KF467422	*F. verticillioides*	100	0.0
rDNA	ITS4-5	KF467441	KF467435	*F. verticillioides*	100	0.0
Mitochondria small subunit	NMS	KF467454	KF467448	*F. verticillioides*	100	0.0
Intergenic spacer rDNA	VERT	submitting	submitting	*G. moniliformis*	97	0.0
Intergenic spacer rDNA	VERTF	submitting	submitting	*G. moniliformis*	95	1 e-121

identity of these sequences with different sequences of *F. verticillioides* (*e.g.*, ID XR_001989346.1) in GenBank was 100% and the e-value 0.0.

Other genes used in this study permitted a more adjusted taxonomic classification. The accession numbers of GenBank (NCBI) of the sequences obtained in this study with the different genes used are listed in Table 3. In the same way, Table 3 lists the identity percentages and the E values produced by the comparison between the sequences obtained in this study with the sequences of the closest taxonomic species, using the BLAST tool of NCBI.

Using the sequences obtained with the different amplicons, a MultiLocus Sequence-Typing (MLST) schema was built. The MLST schema used an agglomerative clustering of a complete linkage of eight different genes of FVER (EF1, Cmd, Bt1, VERT, VERTF, ITS1-2, ITS Fu, and NMS). The eight genes of FVERT were compared with thirty-one conserved housekeeping genes to derive a combination of alleles known as a sequence type (ST), using the *Fusarium*-ID site hosted at the Pennsylvania State University (Fig. 5). To obtain the MLST, we used the most representative FVER sequences, which obtained the highest percentage of identity and the lowest E value in the sequence alignment using the BLAST tool, as seen in Table 3. The MLST schema shows that the isolate that previously identified *F. verticillioides* is clustered with similar types like *Fusarium verticillioides* (*Fusarium fujikuroi* species complex), excluding *Fusarium oxysporum* (*Fusarium oxysporum* species complex) that are grouped in a different phylogenetic group (Fig. 5).

Discussion

Symptomatic and morphological recognition

This study showed that FVER is a pathogen of the carnation, which can lead to a confused diagnosis with carnation plants infected with FOX. Here, we demonstrated that FVER attacks carnation plants, producing some symptoms like those of carnation plants attacked by other *Fusarium* species. The morphological description of the species FVER agreed with those of Leslie and Summerell (2006) and other authors (Deepa *et al*., 2018), with the presence of typical macroconidia, microconidia in a chain, and absence of chlamydospores.

In vitro tests showed resistance to FVER from cells of different hybrids and commercial varieties. This demonstrates that the plant can recognize FVER and elaborate a response to it. The pathogenicity test, the *in vitro* "dual" test is based on the chemical response, in which recognition of the pathogen activates the metabolism of defense in the plant cell wall (Bigeard *et al*., 2015). In this assay, the undifferentiated cells of the hybrid lines, which have some type of resistance towards the pathogen, induced smaller displacements on the fungus on the nutrient media, supporting the hypothesis that antifungal molecules are produced by callus into the media (Buiatti *et al*., 1987). This type of response has been already reported, characterizing lines of cells of tomato and evaluating them *in vitro* responses against FOX. In this case, a correlation between phytoalexin production and inhibition of fungal growth was reported (Storti *et al*., 1989). In our case, in some "dual" tests, changes were

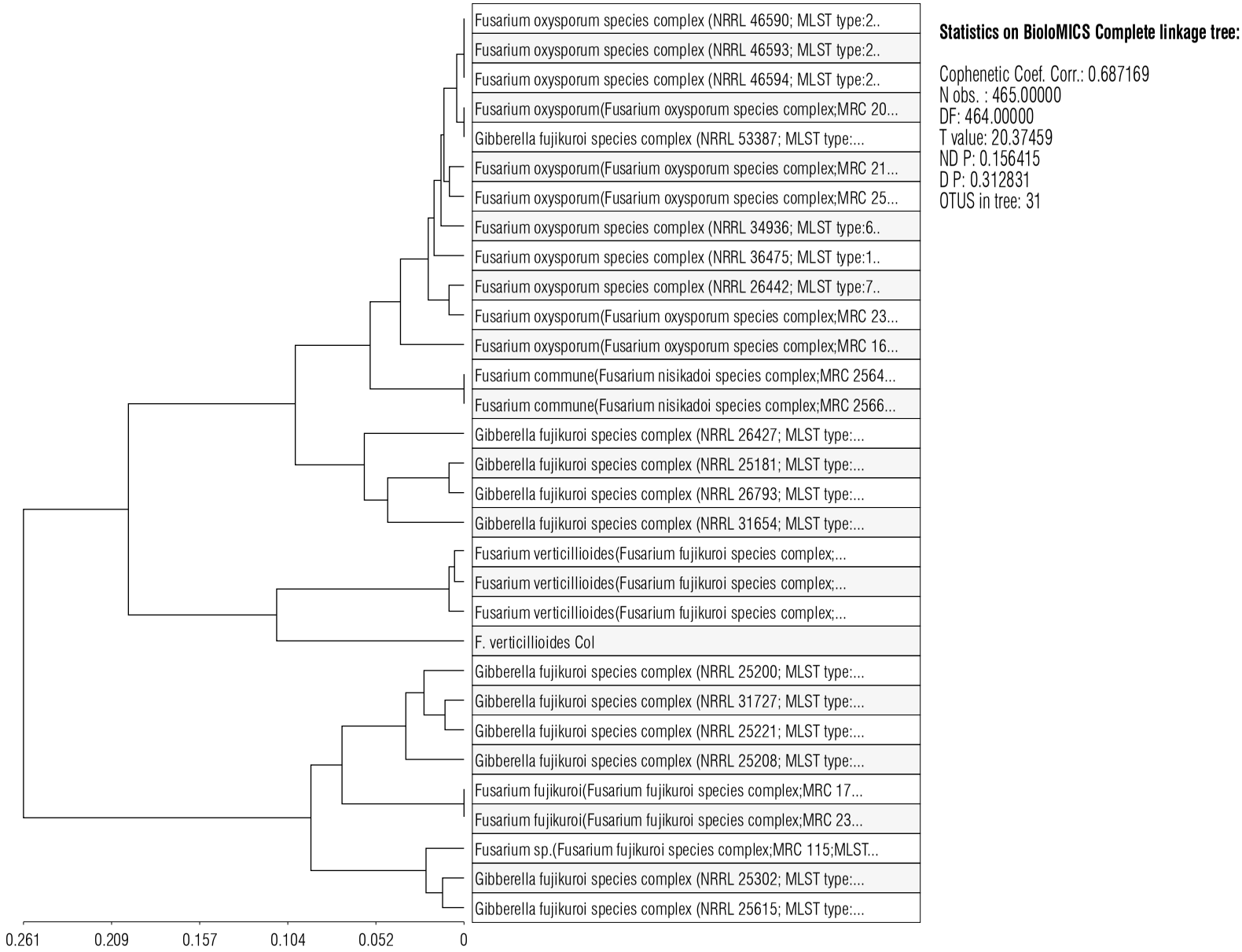

FIGURE 5. MLST scheme. FVER (*F. verticillioides* col) phylogeny inferred from an *EF1*, *Cmd*, *Bt1*, *VERT*, *VERTF*, *ITS1-2*, *ITS Fu*, and *NMS* dataset genes, in which six species complexes were resolved. The approximate number of phylogenetically distinct species within each species complex is based on an agglomerative clustering of complete linkage analysis.

presented in the form of the fungal growth front line. This clearly shows a response by the callus, which does not allow the fungi to continue its normal advance. Filgueira and Guardiola (1999) also reported this change in the growth direction of the fungus as a response to the resistance of the carnation callus to the pathogen presence. Several reports have shown similar results in their studies, such as the measured severity and disease incidence. They show correlations between the *in vitro* assays and those observed in the field (González *et al*., 2004).

In the carnation, FVER produces basal rot, which is characterized as dry rot in the base of the stem, as previously observed in maize by Leyva-Madrigal *et al*. (2015). Sometimes the rot can be presented in phases as Filgueira reported in 2007 in a study of the carnation, and when the infection is advanced, cottony mycelium can be seen in the cave produced by the medullar rot. Also, posterior expansion of the infection is possible toward the vascular bundles and other stem tissues, producing stem rot disease. Externally, the stem in the base presented chlorosis, cortical necrosis, dry rot, and yellowing foliage.

Symptoms of the disease caused by FVER expand in the plant from bottom to top, similar to wilting caused by FOX. These two pathologies can be differentiated doing cross-section cuts to determine if necrosis is present in the medullary zone of the vessel bundles. In addition, it should be kept in mind that FVER does not produce an asymmetric yellowish color on the leaves. On the contrary, the leaves lose hydration, and the wilting shows a strong pink color in the stem. The FVER sporulation on the outer side of the stem is remarkably similar and consistent with rot caused by FVER in corn, where the rot is accompanied by sporulation (Rossi *et al*., 2008).

In carnation plants, rot in the medullar zone can also be observed when attacked by species of *Fusarium* other than FOX, such as FVER, as reported by Filgueira *et al.* (2007). This suggests that the presence or proportion of these pathogenic species depends on the individual incidence of each pathogen. The greatest incidence of double infections was present with FOX and FVER together. These species colonizing the same plant evidenced low or non-existent ecological competition among the species. This result evidences a possible synergy between some of the species and stem colonization. Additionally, the presence of *Fusarium* in symptomatic plants was found in some carnation varieties.

Molecular identification

In all the cases analyzed using ITS primer, all the corresponding to the *Fusarium* genus were demonstrated in other cases by Glass and Donaldson (1995) and Tan and Niessen (2003). Furthermore, the amplicon obtained with the primers ITS 1/2 consistently showed that the species most related with the analyzed sequences was FVER. In general, the obtained size of the amplicons of this study did not differ significantly from the expected size, except for the case of β-tubulin which showed an appreciable difference (Tab. 1); the difference was not a problem when using the sequence as a tool to identify the related species in the GenBank. Most of the amplicons obtained with the different primers of the genes used in this study were identified as the species most related to FVER. This is the case of the β-tubulin, the Bt1a/Bt1b and Bt2a/Bt2b amplicons, the cytochrome C amplicon, histone-3, calmodulin, and mitochondrion small subunit amplicon, which presented identities above 97% and e-values in all cases near zero.

The product obtained by the primers VERT1/VERT2 and VERTF2/VERTF specific to identify FVER produced bands of the expected sizes of 800 and 400 bp, respectively, as reported by Patiño *et al*. (2004) in the isolates of FVER from the symptomatic flowers of farms. This is indicative of the species with Fumonisin gene presence. In addition, Fumonisin is a mycotoxin produced by some *Fusarium* species like FVER (Scott, 2012; Rosa Junior *et al*., 2019).

On the other hand, the phylogeny analysis with the MLST schema using the genes of FVER *EF1*, *Cmd*, *Bt1*, *VERT*, *VERTF*, *ITS1-2*, *ITS Fu*, and *NMS* produced a tree with a clear aggregation of FOX isolates in a separate group of FVER species and with an important genetic distance (Fig. 5). All these results evidence that 1) FVER is present in carnation growing fields. This is notable because FVER is an important parasite of different crops of economic importance like cereals; 2) FVER has a type host-pathogen relationship with the carnation that had not been studied until now and produces notable symptoms in the plant leading to the die; 3) the disease known as *F. roseum* by carnation growers is a basal rot caused by FVER; 4) FVER and FOX can act synergistically to produce an infection of the plant that presents symptoms of both disease, vascular wilt, and basal rot; and 5) FVER is producing an emergent disease that could be related to climate change and must be acted upon.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

All the authors of this article contributed in many forms to the final document. JJFD is the leader of the scientific group and compiled and wrote the manuscript. CMRS worked on the molecular identification of the species. CYQ discovered the parasite *F. verticillioides* in the carnation crop. JCS did the isolation and preliminary characterization of the fungi. IEM collected the samples on the farms and studied the incidence in the crop. JJFD formulated the overarching research goal and aims. CYQ and IEM applied statistical methods. JJFD obtained financial support. JJFD conducted the research and investigation process. JJFD and CMRS developed and designed the methodology. JJFD verified the overall reproducibility of results and other research outputs. JJFD, CMRS, CGQ and IEM prepared and presented the published work and oversaw its presentation. JJFD wrote and translated the initial draft. JJF carried out the critical review of the manuscript. All authors reviewed the manuscript.

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Growth, development, and chlorophyll indexes of glyphosate and glufosinate-tolerant maize under herbicide application

Crecimiento, desarrollo e índices de clorofila de maíz tolerante a glifosato y glufosinato bajo aplicación de herbicidas

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ABSTRACT

Glyphosate, glufosinate, and atrazine do not affect the growth and development of glyphosate and glufosinate-tolerant (RR2/LL) maize; however, the results are less consolidated for these herbicides applied in mixtures. The aim of the present study was to evaluate the effects of post-emergent application of glyphosate, glufosinate, and atrazine, alone and in mixtures, on the growth and development of the RR2/LL maize. The treatments consisted of the application of glyphosate (1080 g of acid equivalent [ae] ha^{-1}), glufosinate (500 g of active ingredient [ai] ha^{-1}), atrazine (2000 g [ai] ha^{-1}), glyphosate + glufosinate, glyphosate + atrazine, glufosinate + atrazine, glyphosate + glufosinate + atrazine, and the control (without application). The experiment was carried out in two locations under greenhouse conditions with different maize hybrids. Crop injury and variables of the agronomic performance (height, stem diameter, chlorophyll indexes, fresh and dry weight of shoot, root dry weight, and total dry weight) were evaluated. Herbicides applied alone or in binary mixtures did not cause major damage. However, the application of the three associated herbicides in some situations might result in detrimental effects on the development of the RR2/LL maize.

Key words: atrazine, crop injury, herbicide mixture, selectivity, *Zea mays*.

RESUMEN

Glifosato, glufosinato y atrazina no afectan el crecimiento y desarrollo de las plantas de maíz tolerantes a glifosato y a glufosinato (RR2/LL); sin embargo, los resultados están menos consolidados para estos herbicidas aplicados en mezclas. El objetivo del presente estudio fue evaluar los efectos de la aplicación post-emergente de glifosato, glufosinato y atrazina, solos y en mezclas, sobre el crecimiento y desarrollo del maíz RR2/LL. Los tratamientos se formaron por aplicación de glifosato (1080 g de ácido equivalente [ae] ha^{-1}), glufosinato (500 g de ingrediente activo [ia] ha^{-1}), atrazina (2000 g [ai] ha^{-1}), glifosato + glufosinato, glifosato + atrazina, glufosinato + atrazina, glifosato + glufosinato + atrazina, además del testigo (sin aplicación). El experimento se llevó a cabo en dos localidades, en condiciones de invernadero, con diferentes híbridos de maíz. Se evaluó la fitotoxicidad al cultivo y variables relacionadas con el crecimiento del mismo (altura, diámetro del tallo, índices de clorofila, masa fresca y seca de la parte aérea, masa seca de las raíces, masa seca total). Los herbicidas aplicados solos o en mezclas binarias, no causaron daños importantes. Sin embargo, la aplicación de los tres herbicidas en asociación en algunas situaciones puede resultar en efectos perjudiciales sobre el desarrollo del maíz RR2/LL.

Palabras clave: atrazina, fitotoxicidad, mezcla de herbicidas, selectividad, *Zea mays*.

Introduction

In recent years, glyphosate-tolerant crops have promoted the repetitive and large-scale use of this herbicide. The occurrence of glyphosate-resistant weed biotypes is directly related to the development and expansion of glyphosate-tolerant crops because of the high selection pressure of these biotypes due to the continued use of this herbicide (Albrecht *et al*., 2014).

For effective control of weeds and prevention of the appearance of resistant weed biotypes, the use of two or more herbicides with different mechanisms of action is recommended. It is important to use new technologies that confer tolerance to different herbicides, thus, providing conditions for the rotation of mechanisms of action (Riar *et al*., 2013), as well as the association of herbicides as an important tool in weed management (Gemelli *et al*., 2013).

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The second generation of glyphosate-tolerant maize is represented by the event NK603 (Roundup Ready™ 2 - RR2), approved in the USA and Brazil in 2000 and 2008; and the event MON87427 (Roundup Ready™), first approved in the USA in 2013 and later in 2016 in Brazil (Albrecht *et al*., 2021; ISAAA, 2021). Glyphosate tolerance is conferred by the expression of the *cp4 epsps* gene derived from the *Agrobacterium tumefaciens* strain CP4; this gene encodes a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) insensitive to glyphosate (Nielsen *et al*., 2004).

T25 maize (Liberty Link® - LL) shows tolerance to the glufosinate herbicide. The tolerance is conferred by the gene *pat*, from the bacterium *Streptomyces viridochromogenes* (Matsuoka *et al*., 2001). In addition, maize with insect resistance (TC1507 maize) also shows tolerance to glufosinate, since the *pat* gene was used as a marker during its selection (Silva *et al*., 2017; Albrecht *et al*., 2021).

With the expansion of areas with glyphosate-tolerant and glufosinate-tolerant maize (RR2/LL), doubts have arisen regarding their association with other herbicides. In some situations, glyphosate is associated with other herbicides or other pesticides; and crop injury may occur (Albrecht *et al*., 2014). If the mixtures of some herbicides have undesirable effects even in crops with tolerance, as highlighted by Taiz and Zeiger (2010), negative effects on plant growth and development can occur. This is plausible when we consider the recent use of RR2/LL maize by Brazilian farmers.

For maize, as alternative herbicides or as a complement to glyphosate, atrazine (mechanism of action of PSII inhibitors) can be highlighted. It can be applied in the pre- or post-emergence of the crop, with an effect on eudicotyledonous weeds in pre-emergence or post-initial (Barnes *et al*., 2020; Langdon *et al*., 2021); and it is selective for maize (Giovanelli *et al.,* 2018).

Araújo *et al*. (2021) do not observe reductions in yield or 100-grain weight or changes in nutrient contents, even with the use of high rates of glyphosate and glufosinate in RR2/LL maize. Silva *et al*. (2017) observe injury >5% in maize, for glyphosate (1080 g acid equivalent [ae] ha^{-1}), glufosinate (500 g active ingredient [ai] ha^{-1}) and atrazine (2000 g ai ha^{-1}), applied alone and in mixtures. Other studies suggest possible undesirable effects of the herbicide application in maize, such as symptoms of injury, when glyphosate was applied in mixtures with other herbicides (Giovanelli *et al*., 2018; Soltani *et al*., 2018).

Glyphosate, glufosinate, and atrazine do not affect the growth and development of RR2/LL maize plants; however, the results are less certain for these herbicides in mixtures. The present study aimed to evaluate the effects of the post-emergence application of atrazine, glyphosate and glufosinate, alone and in mixtures, on the growth and development of glyphosate and glufosinate-tolerant maize.

Materials and methods

Experimental conditions

The first experiment was conducted from January to March of 2014 in Piracicaba, São Paulo (SP, Brazil) (experiment I). The experiment was then repeated from January to March of 2015 in Palotina, Paraná (PR, Brazil) (experiment II). The treatments consisted of the application of glyphosate (1080 g of acid equivalent [ae] ha^{-1}), glufosinate (500 g of active ingredient [ai] ha^{-1}), atrazine (2000 g ai ha^{-1}), glyphosate + glufosinate, glyphosate + atrazine, glufosinate + atrazine, glyphosate + glufosinate + atrazine, and the control (without application), thus, totaling eight treatments. Commercial products were used: Roundup Ready® (480 g ae of glyphosate L^{-1}, Monsanto), Finale® (200 g ai of glufosinate L^{-1}, Bayer), and Proof® (500 g ai of atrazine L^{-1}, Syngenta).

Simple hybrids 2B810PW and 30F53HR were used in the experiment I and II. Both show resistance to lepidopterans and tolerance to glyphosate and glufosinate. The hybrids were chosen because they were suitable for the localities and mainly because they represented representative cultivated areas in Brazil.

The experiments were conducted in a completely randomized design with four replicates, up to 28 d after application (DAA). The pots for plant growth had a capacity of 7 L, with one plant per pot, and they were kept in a greenhouse at 25°C, relative humidity 60% and 5 mm day^{-1} of irrigation.

Physical and chemical analysis of soil was carried out as recommended by Donagema *et al*. (2011). The soil used for the experiment I showed the following results: pH ($CaCl_2$) = 5.3; Al = 0.0 $cmol_c\ dm^{-3}$, H+Al = 2.50 $cmol_c\ dm^{-3}$, C = 10.27 g dm^{-3}; P (resin) = 7.0 mg dm^{-3}; K = 0.26 $cmol_c\ dm^{-3}$; Ca = 3.90 $cmol_c\ dm^{-3}$; Mg = 1.60 $cmol_c\ dm^{-3}$; sum of bases (SB) = 5.76 $cmol_c\ dm^{-3}$. The clay, sand, and silt contents were 40%, 54%, and 6%, respectively. For the experiment II, the physical and chemical analysis of the soil used showed the following results: pH ($CaCl_2$) = 5.5; Al = 0.0 $cmol_c\ dm^{-3}$, H+Al = 4.28 $cmol_c\ dm^{-3}$, C = 5.39 g dm^{-3}; P (Mehlich) = 8.93 mg dm^{-3}; K = 0.51 $cmol_c\ dm^{-3}$; Ca = 5.39 $cmol_c\ dm^{-3}$; Mg = 0.87 $cmol_c\ dm^{-3}$; SB = 6.77 $cmol_c\ dm^{-3}$. The clay, sand, and silt contents were 65.7%, 17.8%, and 16.5%, respectively. All pots were kept free from weed interference through manual control.

The applications of the treatments were carried out in the V_4 phenological stage (Ritchie *et al.,* 1993), recommended for application of the herbicides used in the experiment. A CO_2 pressurized backpack sprayer was used, with a constant pressure of 2 bars, equipped with a bar 2 m wide, containing four fan-like tips (XR 110.02, Teejet®) that, working at a height of 50 cm from the target and at a speed of 1 m sec^{-1}, provided a spray volume of 200 L ha^{-1}.

Data collection

Chlorophyll indexes were evaluated at 7, 14, 21, and 28 DAA. For the experiment I, a portable meter (SPAD-502, Konica Minolta, Inc., Japan) was used that evaluates the intensity of the green leaves and calculates the SPAD index that is highly correlated with the total chlorophyll content of leaves (Uddling *et al*., 2007). For the experiment II, the indexes of chlorophyll a, b, and total (a + b) were measured. For this, an electronic chlorophyll meter (clorofiLOG - CFL1030, Falker Automação Agrícola Ltda., Brazil) was employed that determined the Falker chlorophyll indexes (FCI) (Barbieri Junior *et al*., 2012). The measurement was always performed on the first fully developed leaf.

Also, at 7, 14, 21, and 28 DAA, symptoms of injury were evaluated. These assessments were carried out through visual analysis at each experimental unit considering significantly visible symptoms of the plants according to their development. Scores from 0% to 100% were assigned, where 0 represented the absence of symptoms and 100% the death of the plant (Velini *et al*., 1995). Treatment without application (without herbicide effect) was used as a reference for evaluations. On the same four dates, the height of the plants and stem diameter were evaluated (for the experiment I, stem diameter was assessed only at 28 DAA). Height measurements were made from the soil surface to the last fully open leaf with the ligule visible, and the stem diameter was measured 4 cm above the soil.

At 28 DAA, the fresh mass of shoots and the dry mass of roots, shoots and total (roots + shoot) were determined. The shoots were cut at ground level, with subsequent weighing to determine the fresh mass. The soil with the plant roots was removed from the pots, and the roots were separated from the soil with the aid of running water and sieves. For drying, a greenhouse with forced ventilation was used for 72 h at 65ºC. To measure the weights, an analytical balance with a precision of three decimal places was used.

Statistical analysis

The results were tested for normality (Shapiro-Wilk test) and homogeneity (Levene test) and then tested by analysis of variance by the F-test ($P<0.05$), according to Pimentel-Gomes and Garcia (2002). The means of the treatments were compared by the Tukey's test ($P<0.05$). For this purpose, the Sisvar 5.6 software was used (Ferreira, 2011).

Results and discussion

Experiment I

For plant height evaluations, a significant difference was found in the evaluation at 28 DAA; treatment 6 had shorter plants than treatments 2 and 3, while the SPAD index evaluated at 7, 14, 21, and 28 DAA did not significantly differ between the treatments (Tab. 1).

TABLE 1. Height and chlorophyll index of maize plants at 7, 14, 21, and 28 d after application (DAA) of herbicides alone and in mixtures. Experiment I, Piracicaba, SP, Brazil, 2014.

Treatments	Height (cm)				Chlorophyll index (SPAD index)			
	7	14	21	28	7	14	21	28
	DAA							
1. Control	27.3	36.8	49.8	66.3 ab	41.5	35.7	38.6	43.8
2. gly	27.5	37.8	51.5	68.3 a	38.1	37.9	41.0	43.5
3. glu	27.3	32.4	48.0	68.0 a	40.6	38.6	42.4	41.0
4. atr	27.3	37.8	50.8	64.3 ab	40.1	36.4	40.9	43.5
5. gly + glu	25.3	34.9	48.8	65.5 ab	41.4	35.9	44.0	39.2
6. gly + atr	26.0	32.5	45.0	57.3 b	38.9	37.9	42.4	41.8
7. glu + atr	28.0	35.5	46.5	65.5 ab	40.3	39.0	42.1	42.2
8. gly + glu + atr	26.0	32.0	44.0	60.0 ab	39.1	36.7	41.6	42.3
Mean	26.8^{ns}	34.9^{ns}	48.0^{ns}	64.4*	39.4^{ns}	37.3^{ns}	41.6^{ns}	42.2^{ns}
CV (%)	8.1	12.1	7.5	7.0	7.4	5.8	7.2	7.5

gly (glyphosate - 1080 g ae ha^{-1}), glu (glufosinate - 500 g ai ha^{-1}), atr (atrazine - 2000 g ai ha^{-1}). CV - coefficient of variation. * Significant at 5% by the F test. ^{ns} not significant ($P<0.05$). Means followed by the same letter in the column did not differ significantly ($P<0.05$) according to the Tukey's test.

The test of means for the fresh weight of the shoots showed that treatment 8 (triple association) had a lower mass than treatments 1, 2, and 5. In relation to the dry weight of the shoot, treatment 8 expressed a lower mass than treatments 1, 2, 5, and 7 (Tab. 2).

These results indicated a potential negative effect for the association of the three herbicides (treatment 8) applied on the RR2/LL maize. The absence of visual symptoms of injuries on the plants (0%) is worth mentioning, a fact confirmed by the lack of significant differences between treatments in the plant height and SPAD index.

Experiment II

For plant height evaluations (Tab. 3), a significant difference was observed at 7, 14, and 28 DAA, with emphasis on treatment 8 that was significantly lower than the treatments 4, 5, 6, 7 (7 DAA), 1, 3, 4, 5 (14 DAA), and 1, 3 (28 DAA). In the stem diameter evaluations, the analysis of the variance was not significant for all the evaluated periods.

Table 4 shows the chlorophyll indexes. For these, a significant difference was observed at 14 DAA in which treatment 5 had a lower significant index than treatment 2. For the chlorophyll b indexes, the significant difference in the

TABLE 2. Stem diameter (SD), fresh weight of shoot (FS), dry weight of shoot (DS), dry weight of roots (DR), and total dry weight (TD) of maize plants at 28 d after application of herbicides alone and in mixtures. Experiment I. Piracicaba, SP, Brazil, 2014.

Treatments	SD (mm)	FS	DS	DR	TD
		(g)			
1. Control	17.5	145.1 a	27.2 a	12.3	39.5
2. gly	18.0	142.7 a	27.3 a	12.3	39.6
3. glu	17.0	120.1 ab	24.3 ab	12.9	37.2
4. atr	15.5	112.4 ab	21.5 ab	10.9	32.3
5. gly + glu	16.5	141.4 a	26.5 a	11.9	38.5
6. gly + atr	14.5	113.7 ab	21.7 ab	10.3	32.0
7. glu + atr	16.0	122.0 ab	26.1 a	11.5	37.6
8. gly + glu + atr	15.8	96.8 b	17.3 b	11.4	28.7
Mean	16.3^{ns}	124.3*	24.0*	11.7^{ns}	35.7^{ns}
CV (%)	13.3	14.7	15.5	14.6	13.2

gly (glyphosate - 1080 g ae ha^{-1}), glu (glufosinate - 500 g ai ha^{-1}), atr (atrazine - 2000 g ai ha^{-1}). CV - coefficient of variation. * Significant at 5% by the F test. ^{ns} not significant ($P<0.05$). Means followed by the same letter in the column did not differ significantly ($P<0.05$) according to the Tukey's test.

TABLE 3. Height and stem diameter of maize plants at 7, 14, 21, and 28 d after application (DAA) of herbicides alone and in mixtures. Experiment II, Palotina, PR, Brazil, 2015.

Treatments	Height (cm)				Stem diameter (mm)			
	7	14	21	28	7	14	21	28
	DAA							
1. Control	30.8 abc	44.5 a	58.8	79.0 a	11.0	14.2	14.5	15.1
2. gly	28.8 bc	39.0 ab	55.0	71.0 ab	11.2	13.2	14.3	14.8
3. glu	30.8 abc	41.3 a	56.8	79.3 a	11.8	15.2	16.2	16.7
4. atr	35.0 a	43.3 a	54.5	74.8 ab	11.6	15.2	16.1	16.5
5. gly + glu	33.8 ab	45.3 a	59.0	74.5 ab	11.7	15.3	16.1	16.5
6. gly + atr	32.5 ab	39.5 ab	54.0	71.5 ab	11.3	14.1	16.0	16.4
7. glu + atr	32.5 ab	39.8 ab	58.3	71.0 ab	11.4	15.4	16.1	16.5
8. gly + glu + atr	26.0 c	31.3 b	52.8	63.3 b	11.4	13.9	14.8	14.9
Mean	31.3*	40.5*	56.1^{ns}	73.2*	11.4^{ns}	14.6^{ns}	15.5^{ns}	15.9^{ns}
CV (%)	7.9	9.4	9.1	7.1	9.0	8.0	6.8	6.6

gly (glyphosate - 1080 g ae ha^{-1}), glu (glufosinate - 500 g ai ha^{-1}), atr (atrazine - 2000 g ai ha^{-1}). CV - coefficient of variation. * Significant at 5% by the F test. ^{ns} not significant ($P<0.05$). Means followed by the same letter in the column did not differ significantly ($P<0.05$) according to the Tukey's test.

evaluation at 14 DAA occurred in treatment 7. A mean lower than treatment 2 and the means of treatments 4 and 5 were significantly lower when compared to treatments 1 and 2. In the total chlorophyll indexes at 14 DAA, the difference was significant for treatment 5 and the mean was significantly lower than that in treatments 1 and 2.

There are two predominant forms of chlorophylls a and b that differ slightly in structure. The main function of chlorophyll is to convert light energy into chemical energy, a process that occurs in the chloroplasts (Streit *et al.*, 2005). These variables are important in terms of understanding selectivity and connections with plant development. The application of glyphosate associated with glufosinate (treatment 5) resulted in a lower chlorophyll a, b, and total index at 14 DAA than only applying glyphosate (treatment 2). This indicates the possibility of a negative effect on the association of the two products applied to the RR2/LL maize. The reduction in the chlorophyll content can be an indicator for investigating an injury to the plants (Song *et al.*, 2007). This is an important aspect for study, considering the immense potential of using these transgenic technologies that confer tolerance to herbicides (RR2/LL) and, at the same time, the lack of research results in this sense.

For the fresh weight of shoots and total dry weight of plants, treatment 8 had a lower weight than treatment 5 (Tab. 5). Despite the use of a different hybrid in experiment II, the

TABLE 4. Chlorophyll a, b, and total in maize plants at 7, 14, 21, and 28 d after application (DAA) of herbicides alone and in mixtures. Experiment II, Palotina, PR, Brazil, 2015.

Treatments	Chlorophyll a (FCI)				Chlorophyll b (FCI)				Total chlorophyll			
	7	14	21	28	7	14	21	28	7	14	21	28
	DAA											
1. Control	38.2	30.4 ab	35.1	30.3	9.6	6.6 ab	9.4	5.6	47.7	37.0 a	44.5	35.9
2. gly	36.7	30.9 a	35.2	29.1	9.1	6.7 a	9.0	5.2	45.7	37.6 a	44.2	34.3
3. glu	35.9	29.8 ab	37.6	28.8	9.9	5.5 abc	9.8	5.4	45.8	35.2 ab	47.3	34.2
4. atr	37.0	27.1 ab	36.1	28.2	9.6	4.9 c	8.6	5.2	46.6	32.0 ab	44.7	33.4
5. gly + glu	36.3	25.4 b	37.8	31.3	8.7	4.6 c	9.2	6.1	44.9	29.9 b	46.9	37.4
6. gly + atr	36.2	30.3 ab	36.6	30.4	8.8	5.6 abc	9.4	5.4	45.0	35.9 ab	45.9	35.8
7. glu + atr	35.0	28.5 ab	32.8	27.1	8.3	5.2 bc	9.0	5.0	43.3	33.7 ab	41.8	32.1
8. gly + glu + atr	36.4	29.8 ab	34.9	29.7	8.8	5.6 abc	8.7	5.4	45.2	35.3 ab	43.6	35.1
Mean	36.4^{ns}	29.0*	35.8^{ns}	29.3^{ns}	9.1^{ns}	5.6^{ns}	9.1^{ns}	5.4^{ns}	45.5^{ns}	34.6^{ns}	44.9^{ns}	34.8^{ns}
CV (%)	4.7	8.0	6.7	8.8	9.2	11.0	9.2	13.2	5.0	8.0	6.8	9.3

FCI (Falker chlorophyll index), gly (glyphosate – 1080 g ae ha^{-1}), glu (glufosinate – 500 g ai ha^{-1}), atr (atrazine – 2000 g ai ha^{-1}). CV – coefficient of variation. * Significant at 5% by the F test. ^{ns}not significant ($P<0.05$). Means followed by the same letter in the column did not differ significantly ($P<0.05$) according to the Tukey's test.

TABLE 5. Fresh weight of shoot (FS), dry weight of shoot (DS), dry weight of roots (DR), and total dry weight (TD) of maize plants at 28 d after application of herbicides alone and in mixtures. Experiment II, Palotina, PR, Brazil, 2015.

Treatments	FS	DS	DR	TD
	(g)			
1. Control	125.5 ab	21.1	9.6	30.6 ab
2. gly	117.3 ab	21.4	9.7	31.0 ab
3. glu	113.3 ab	22.6	11.2	33.7 ab
4. atr	121.0 ab	23.1	11.6	34.6 ab
5. gly + glu	128.3 a	23.6	11.4	35.0 a
6. gly + atr	117.5 ab	21.5	10.8	33.3 ab
7. glu + atr	121.8 ab	22.8	11.0	32.8 ab
8. gly + glu + atr	106.5 b	19.9	9.8	29.6 b
Mean	118.9*	22.0^{ns}	10.6^{ns}	32.6*
CV (%)	7.2	7.9	9.6	6.8

gly (glyphosate - 1080 g ae ha^{-1}), glu (glufosinate - 500 g ai ha^{-1}), atr (atrazine - 2000 g ai ha^{-1}). CV - coefficient of variation. * Significant at 5% by the F test. ^{ns} not significant ($P<0.05$). Means followed by the same letter in the column do not differ significantly ($P<0.05$) according to the Tukey's test.

pattern of behavior observed in the first experiment was maintained. One of the main responses to emphasize is the potential for damage related to the application of glyphosate, glufosinate and atrazine (treatment 8) to the RR2/LL maize.

We emphasize that visual analysis of crop injury was performed in both experiments, but not including notes, as no visual symptoms of injury were perceived. This confirms the results for the chlorophyll indexes, for which reductions were observed only at 14 DAA for the application of glyphosate + glufosinate in experiment 2. At 21 and 28 DAA no further reductions were observed. These findings are in line with those reported by Chahal and Jhala (2018) and Giovanelli *et al*. (2018), in which the high selectivity is linked to the high expression levels of the *pat* gene.

As already mentioned, in both experiments, the main highlight was the significant reduction observed in the fresh and dry weight of shoots for the treatment with the mixture of three herbicides (treatment 8), thus, being an indication to avoid this practice. It should be noted that there are no results in the literature in this sense. So, this study must be widened and repeated.

The application of atrazine did not damage the maize, confirming information in the literature (Dan *et al*., 2010; Giovanelli *et al*., 2018; Giraldeli *et al*., 2019; Richburg *et al*., 2020) and for LL maize. In the latter case, atrazine was used in combination with glufosinate (Silva *et al*., 2017). In the relevant literature, there are no reports of glyphosate damage in RR2 maize and, when reported, usually, they have higher rates than the one that was considered in this study (Gemelli *et al*., 2013; Albrecht *et al*., 2014; Langdon *et al*., 2020). The same fact occurs with glufosinate, alone or in mixtures, in which no harmful effects were observed in LL maize (Silva *et al*., 2017; Krenchinski *et al*., 2019).

Another aspect to be highlighted is that TC1507 maize hybrids (insect resistant) may have different levels of tolerance to glufosinate. The *pat* gene, as already mentioned, was used as a marker in the selection process of this event. According to Krenchinski, Carbonari, *et al*. (2018), the expression of the *pat* gene may vary according to the trademark of the hybrid, thus, hybrids with greater expression of the *pat* gene show greater tolerance to glufosinate.

The selectivity of glufosinate, alone or in mixtures, is known for maize in other studies (Lindsey *et al*., 2012; Ganie & Jhala, 2017). In these studies, the maize hybrids were T14 or T25 events, that is, the LL technology (properly said) guarantees a good level of selectivity in the maize plants. Insect-resistant maize hybrids that also show tolerance to glufosinate have been marketed in Brazil for several years (Borém, 2015). In recent years, in Brazil, tolerance to glufosinate is increasingly used in the management of weeds in post-emergence of insect-resistant maize (Albrecht *et al*., 2021), with good levels of tolerance to glufosinate, as demonstrated in this study.

The herbicide mixtures, in the RR2/LL maize hybrids, are a practice that needs to be preserved. Shaner (2000) already emphasizes that the intensive use of glyphosate in RR crops could lead to problems, especially, in the selection of resistant biotypes. Therefore, it is important to use technologies that confer tolerance to different herbicides, thus, providing favorable conditions for the rotation of action mechanisms (Gage *et al*., 2019; Summers *et al*., 2021). Results showing the advantages of the use of glufosinate-tolerant maize become another option for the control of weeds in this productive system (Armel *et al*., 2008; Everman *et al*., 2009; Krenchinski, Albrecht, *et al*., 2018).

Conclusions

Herbicides applied alone or in binary mixtures did not cause major damage. However, the application of the three associated herbicides in some situations may result in detrimental effects on the development of the RR2/LL maize. These herbicides in mixtures were generally safe for maize and could be considered for weed management. These mixtures have a broad spectrum of action for the control of weeds and may be important in the management of weeds resistant to herbicides. However, caution is important when using triple mixtures in the field, given the possible deleterious effects.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

AJPA, LPA and RVF designed the experiments, AJPA, VGCP, FHK, KSW, BFG and AFMS carried out experiments, AJPA, LPA and FHK contributed to the data analysis, AJPA and AFMS wrote the article. All authors reviewed the manuscript.

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Bioherbicidal activity of seed extract of *Campomanesia lineatifolia* on the weed *Sonchus oleraceus* L.

Actividad bioherbicida del extracto de semillas de *Campomanesia lineatifolia* sobre la maleza *Sonchus oleraceus* L.

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ABSTRACT

Sonchus oleraceus L. is a persistent weed in horticultural crops that generates high economic losses and its management is carried out with various chemical molecules. The objective of this research was to evaluate the bioherbicidal activity effect of the extract of *Campomanesia lineatifolia* seeds on the germination and growth of *S. oleraceus* L. seedlings. The study was carried out in two phases. In the first phase, germination was evaluated with two experiments, the first consisted of the addition of the ethanolic extract of *C. lineatifolia* every third day, in the second experiment the extract was only added at planting. In both cases, four concentrations of the extract (0%, 3%, 6%, and 9%) were evaluated. In the second phase, the same extracts were applied to plant leaves. *C. lineatifolia* extract inhibited the germination of *S. oleraceus*, when applied only once at sowing. As there was no germination with the extracts of *C. lineatifolia*, no direct inhibitory effect on the growth of the seedlings could be seen on the number of leaves, length of roots, and stem. With the foliar application, an incidence of 100% with symptoms of chlorosis and necrosis was observed using any of the concentrations evaluated, but the applications did not cause the plant death. The ethanolic extract from the seeds of *C. lineatifolia* has bioherbicide activity on *S. oleraceus*.

Key words: allelopathy, germination, growth, injury.

RESUMEN

Sonchus oleraceus L. es una maleza persistente en cultivos hortícolas que genera altas pérdidas económicas y su manejo se realiza con diversas moléculas químicas. El objetivo de este estudio fue evaluar la actividad bioherbicida del extracto de semillas de *Campomanesia lineatifolia* sobre la germinación y crecimiento de plántulas de *S. oleraceus* L. El estudio se llevó a cabo en dos fases. En la primera fase se evaluó la germinación con dos experimentos, el primero consistió en la adición del extracto etanólico de *C. lineatifolia* cada tercer día, en el segundo experimento, el extracto solo se adicionó en la siembra. En los dos casos se evaluaron cuatro concentraciones del extracto (0%, 3%, 6% y 9%). En la segunda fase se hizo la aplicación de los mismos extractos vía foliar a las plantas. El extracto de semillas de *C. lineatifolia* inhibió la germinación de las semillas de *S. oleraceus*, tanto con aplicación continua del extracto como aplicado sólo una vez a la siembra. Como no hubo germinación con los extractos de *C. lineatifolia* no se pudo ver efecto inhibitorio directo sobre el crecimiento de las plántulas a través del número de hojas, longitud de raíz y tallo. Con la aplicación foliar se observó incidencia del 100% con cualquiera de las concentraciones evaluadas con síntomas de clorosis y necrosis, pero no se causó la muerte de las plantas. El extracto etanólico de las semillas de *C. lineatifolia* presenta efecto como bioherbicida sobre *S. oleraceus*.

Palabras clave: alelopatía, germinación, crecimiento, daño.

Introduction

Sonchus oleraceus L. belongs to the Asteraceae family and has an annual life cycle, growing up to 1.5 m in height (Elkamali *et al*., 2011). The plant is characterized by a whitish latex and has alternate simple leaves and inflorescences with yellow terminal capitulum (Fuentes *et al*., 2011; Gámez *et al*., 2018). This species has different common names in Spanish, among them is *cerraja*, *lechosa* or *lechuguilla*. In English, it is known as dandelion sow thistle and hare´s thistle.

S. oleraceus is widely distributed throughout the world and, in Colombia, it is present in the highlands of almost the entire territory (Fuentes *et al*., 2011) and is considered invasive (Peerzada *et al*., 2019). As a weed, it has been reported in corn, grasslands, oats, vegetables, asparagus, garlic, feijoa, pea, quinoa, apple, leek (Gámez *et al*., 2018), among others.

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In peach orchard under tropical conditions, it was reported as the species in fifth place with the highest importance value index (Moreno-Preciado & Balaguera-López, 2021). *S. oleraceus* is of great interest to cause a detrimental impact on agriculture through crop competition and allelopathic interference (Chauhan *et al*., 2006; Elkhayat, 2009). It is a limiting species for crops because it has a great competitive capacity and produces a high number of most viable seeds (Peerzada *et al*., 2019). It is also considered the host of different pests and diseases, such as aphids, fungi, nematodes, besides other organisms that affect crops of economic interest (Gámez *et al*., 2018). Genetic diversity, efficient seed dispersal, resistance to some herbicides (groups M and B) are some of the factors that contribute to the successful distribution of *S. oleraceus* in the agroecosystems. In Australia, it is currently considered the second most important broadleaf weed (Peerzada *et al*., 2019).

Herbicide use and herbicide resistance hinder weed management (Guglielmini *et al*., 2007; Vencill *et al*., 2012) and cause a high negative impact on crop yields (Menalled, 2010), environment, and human health (Böcker *et al*., 2019). Therefore, new management alternatives must be found, within which biological management can be included (Peerzada *et al*., 2019) based on the use of allelochemicals (Jabran, 2017). Allelopathy is a tool that allows for the sustainable management of weeds (Arafat & Ali, 2015), allowing the maintenance of a balance in the ecosystem by reducing the use of chemical herbicides (Nawaz *et al*., 2014; Kaab *et al*., 2020). Allelochemicals (including phenolic compounds, terpenoids, and alkaloids) can be formulated into bioherbicides, but many aspects that should be studied are still unknown, such as an understanding of their mode of action (Jabran, 2017).

The champa (in Spanish) or perfume guava (in English) (*Campomanesia lineatifolia* R. & P.) is a fruit tree of the Myrtaceae family. Its fruits are edible berries with a pleasant taste (Balaguera-López *et al*., 2012; Porras *et al*., 2020; González *et al*., 2021), containing 6 to 8 seeds that represent an important percentage of the fruit volume (Balaguera-López *et al*., 2012). The seeds appear to have a high and diverse content of secondary metabolites. Bonilla *et al*. (2005) found that the seed of *C. lineatifolia* has ß-tricetone-like components, which are characterized by the presence of several methyl groups, a flavonoid ring or chalcone, making it a relatively rare class of secondary metabolites. These compounds are known to have antimicrobial activity (Bonilla *et al*., 2005; Osorio *et al*., 2006). Muñoz *et al*. (2015) report in this species the existence mainly of diphenols and polyphenols, Madalosso *et al*. (2012) mention the presence of flavonoids (catechin and quercitrin) and tannins, while Raphaelli *et al*. (2021) report that the *C. xanthocarpa* seeds contain phenolic compounds with different biological activities. The same authors also indicated that the species of the Myrtaceae family are recognized for having high levels of the bioactive compounds, mainly phenolics and carotenoids.

Some plants have biologically active compounds, whose crude, methanolic, and other allelopathic extracts inhibit the germination of weed seeds and affect plant growth causing necrosis or chlorosis (Kaab *et al*., 2020). Several studies with allelopathic extracts have been carried out on Myrtaceae. Verdeguer *et al*. (2009) mentioned that the essential oil of *Eucalyptus camaldulensis* inhibits the germination and growth of *Amaranthus hybridus* L. and *Portulaca oleracea* L. Puig *et al*. (2018) observed that the aqueous extract of *Eucalyptus globulus* presents phytotoxic effects in *Amaranthus retroflexus* L. and *Echinochloa crus-galli* L. Recently, an allelopathic effect of *C. lineatifolia* seed extract was found on the germination and physiology of *Taraxacum officinale* L. (Cabeza-Cepeda *et al*., 2021).

Therefore, the objective of this study was to evaluate the allelopathic activity of *C. lineatifolia* seed extract on the germination, seedling growth, and injury of *S. oleraceus* L. under laboratory conditions.

Materials and methods

The experiment was conducted in Tunja (Boyacá, Colombia), under laboratory conditions, with a mean temperature of 19°C and relative air humidity of 60.8%. *S. oleraceus* L. seeds were obtained from farms in the municipalities of Tunja and Paipa (Boyacá). *C. lineatifolia* seeds were collected from mature fruits in the Miraflores municipality (Boyacá) in "La playa" farm located at 1432 m a.s.l., 5°11'49'' N and 73°08'47'' W with a mean temperature of 24°C (González *et al*., 2021).

The ethanolic extracts of the *C. lineatifolia* seeds were obtained using the following procedure. Initially, the seeds were dried at room temperature (18°C) for 24 h, then this material was grounded, and weighed to obtain the extract. A 400 g sample of the grounded material was immersed in 1 L of ethanol (96%), the mixture was shaken and then left to stand in a glass bottle and covered with aluminum foil for a period of 48 h. The bottle was shaken regularly to homogenize the sample. Subsequently, the liquid was separated from the solid part through a filtering process (Whatman 1 filter paper), then it was subjected to

a distillation process with a rotary evaporator to obtain the extract. From the concentrated extract, the different evaluated concentrations were prepared (% w/v) using distilled water as the final solvent. The study was carried out in two phases.

In the first phase, which corresponded to application on pre-emergent plants, germination was evaluated with two experiments. The first experiment consisted of the application of *C. lineatifolia* extract every third day. In the second experiment, the extract was only added at the time of sowing. In both experiments, a completely randomized design was used with four treatments corresponding to concentrations of *C. lineatifolia* seed extract (0%, 3%, 6%, and 9%), concentrations evaluated previously by Cabeza-Cepeda *et al.* (2021). Each treatment had four replicates and 16 experimental units; each unit consisted of a Petri dish with 100 *S. oleraceus* seeds.

In the second phase or the post-emergent application, the extracts were applied over the leaves of the plants. A completely randomized design with the same four concentrations of *C. lineatifolia* seed extract was used. Each of the 16 experimental units was composed of a pot with five *S. oleraceus* plants.

The seeds were sown in Petri dishes, on the base of which there was absorbent paper. Periodically the seeds were hydrated with the allelopathic extracts, except for the control that was hydrated with distilled water. In the second experiment, periodic hydration was done with distilled water. The Petri dishes were left at room temperature (19°C) and relative humidity (60.8%) with a 12 h photoperiod. Germination readings were carried out every third day from the moment the first seed germinated. It was considered as germination criterion that the radicle measured a minimum of 2 mm. From these data and with the formulas used by Carranza *et al.* (2016) and proposed by Ranal and Santana (2006) the percentage of germination and the mean germination speed (germinated seeds/d) were calculated. At the end of the experiment, the height (cm) and the length of the longest root (cm) of each seedling were determined by measurement with a Vernier caliper.

For the second phase, ten seeds of *S. oleraceus* were sown in pots with a volume of 500 ml, and peat moss was used as substrate. When the seeds germinated, 5 seedlings of similar size were chosen, and the rest were eliminated. Irrigation was carried out periodically, and fertilizer was applied weekly to avoid nutritional deficiencies. The fertilizer mixture was prepared by taking 20 ml of commercial fertilizer 12-4-8 (Follaje®, Davalia Fertilizantes, Colombia) and dissolving it in 2 L of water. Of this solution, 40 ml per bottle was applied. When the plants had between 4 and 5 true leaves, the extracts were applied uniformly to the leaves of the entire foliage using a 10 ml atomizer. It was necessary to add an adjuvant (Cosmoflux® 411F, Cosmoagro, Colombia) to the extracts to achieve uniform coverage on the leaves.

At 2, 5, and 8 d after the application of the extracts, the following variables were determined: incidence of damage (%), defined as the number of plants with damage by the herbicide/total number of plants x 100. The severity of the damage (%) was determined with the scale used by Chaves Neto *et al.* (2020), where 0-20% indicates no injury or effect, 20-40% slight injury and/ or growth reduction with rapid recovery, 40-60% injury and/or growth reduction with slow or definitive recovery, 60-80% severe injury and/or reduction of non-recoverable growth and/or reduction of the stand, and 80-100% destruction or just a few live plants.

Statistical analysis

An analysis of variance was carried out with the data obtained to determine statistical differences. Where differences were observed, the Tukey´s test ($P<0.05$) was used. Variables that did not meet the assumptions of normality of the residuals (Shapiro-Wilk test) and homogeneity of variances (Levene test) were subjected to arcsine$\sqrt{x + 1}$ transformation and $\sqrt{x}$ transformation. The analyses were carried out with the statistical program SPSS IBM v. 19.

Results and discussion

Phase 1. Pre-emergence treatments

Germination percentage: there were significant differences between the concentrations of the extract and the control (without the extract) in the two experiments. Germination of *S. oleraceus* seeds with *C. lineatifolia* extract was found to be inhibited (Fig. 1). In agreement with these results, this extract also inhibited germination in *T. officinale* (Cabeza-Cepeda *et al.*, 2021).

The total inhibition of germination obtained with *C. lineatifolia* extracts may be due to the presence of secondary metabolites that directly affect the cells and cause the death of the seeds. *C. lineatifolia* seeds contain several compounds, such as catechins, phenolic acids, chlorogenic acid, flavonoids and anthocyanins, mainly (data not shown). This composition is according with that reported in other studies previously. According to Catunda *et al.* (2002), germination can be inhibited by the secondary metabolites (phenols, terpenoids, saponins, etc.) by

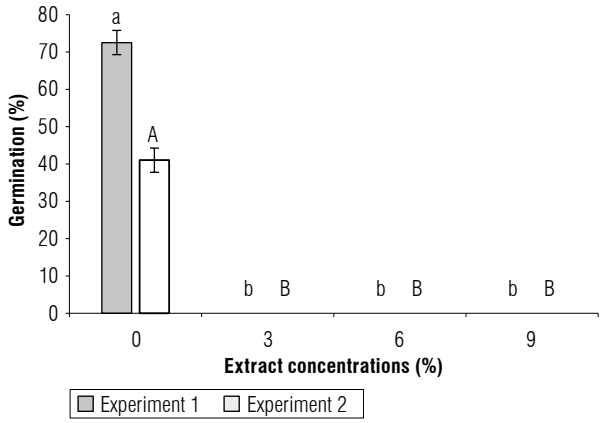

FIGURE 1. Bioherbicidal effect of the allelopathic extract of *C. lineatifolia* seeds on the germination percentage of *Sonchus oleraceus* seeds. Experiment 1: Continuous application of the extracts. Experiment 2: application of the extracts only at the sowing of the seeds. Means followed by different letters in each experiment indicate significant differences according to the Tukey test ($P<0.05$). Vertical bars for each mean indicate the standard error (n = 4).

affecting several fundamental processes such as respiration, cell division, synthesis of gibberellins, permeability of the seed coat, and other effects. Carvalho *et al*. (2019) reported that allelochemicals affected germination by inhibition of cell cycle in meristematic cells. To clarify this situation, it is recommended to perform a seed viability test with triphenyl tetrazolium chloride on seeds that did not germinate; if the test is positive, germination would be inhibited but the embryo is still alive; if the test is negative, this would indicate that the extract of *C. lineatifolia* caused the death of the seeds.

Imatomi (2010) evaluated the allelopathic activity of aqueous extracts of leaves of 15 species belonging to the Myrtaceae family on the germination and growth of *Lactuca sativa*, *Solanum lycopersicum,* and *Allium cepa*, and found that the species *Myrcia multiflora, Myrcia splendens* and *Eugenia punicifolia* exhibited the highest inhibition of seed germination in bioassays, followed by the species *Myrcia bella*, *Psidium laruotteanum*, *Campomanesia pubescens*, *Psidium cenereum*, *Eugenia myrcianthes*, *Myrcia lingua*, and *Psidium rufum*. This supports the results obtained in this study where *C. lineatifolia* of Myrtaceae has an allelopathic effect that inhibits germination. However, knowing with certainty how the applied extract works is very difficult since the extract can have a significant number of different secondary metabolites that can have allelopathic action. According to Jabran (2017), the chemical inhibitors contained in plant extracts produced by allelopathic agents correspond to secondary metabolites and belong to various classes of compounds such as phenols, aldehydes, glucosides, terpenes, and organic cyanogenic compounds.

Gil *et al*. (2010) reported that the application of the extracts of *Swinglea glutinosa* and *Lantana camara* inhibited the germination of *Senna obtusifolia*, *Amaranthus dubius*, *Rumex crispus*, *Brassica rapa,* and *Poligonum segetum*. In the case of *Swinglea,* the extracts inhibit germination with the concentration 0.5% and *Lantana* inhibits germination with an extract of 2.0% (Gil *et al*., 2010). Seabra *et al*. (2017) evaluated allelopathic concentrations of *Canavalia ensiformis* on the seed germination of *Carthamus tinctorius*, where the inhibition was obtained with an extract concentration of 75% indicating that the extract of *C. lineatifolia* is very efficient because it needed only 3% to inhibit germination.

For mean germination speed (MGS), in the two experiments, statistical differences were found between *C. lineatifolia* extracts. Any concentration inhibited the germination of *S. oleraceus*; and, therefore, there was no germination speed. With the control (0 % of the extract) the seeds had a MGS of 5 germinated seeds/d for experiment 1 and 3 germinated seeds/d for experiment 2 (Fig. 2).

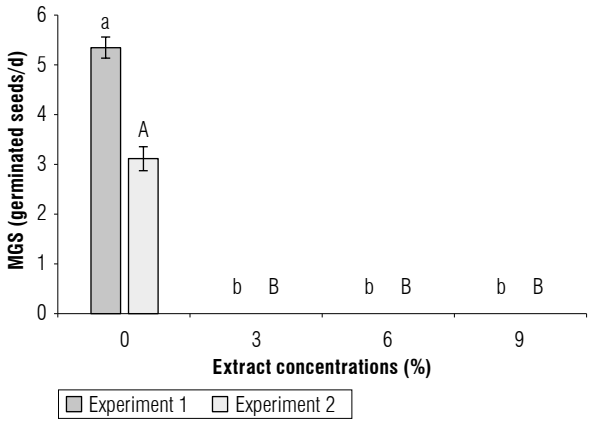

FIGURE 2. Bioherbicidal effect of the allelopathic extract of *C. lineatifolia* seeds on the mean germination speed (MGS) of *Sonchus oleraceus* seeds. Experiment 1: Continuous application of the extracts. Experiment 2: application of the extracts only at the sowing of the seeds. Averages followed by different letters in each experiment indicate significant differences according to the Tukey test ($P<0.05$). Vertical bars in each mean indicate the standard error (n=4).

This shows that the extract of *C. lineatifolia* caused phytotoxicity in the seeds of *S. oleareaceus*, which (as explained above) could be due to compounds derived from secondary metabolism such as phenolic compounds. That affect the growth and development of plants (Jabran, 2017), which, in this case, are the seeds.

Seabra *et al*. (2017) indicate that the allelopathic effects of plants can cause inhibition of the percentage and the germination speed and can reduce initial growth. This agrees with this study since the evaluated extract negatively affected germination. For their part, Gindri *et al*. (2020) found that the aqueous extract of *Lantana camara* causes inhibition of the germination speed index in *Bidens pilosa* seeds.

Length of roots and stems: since the extract of *C. lineatifolia* inhibited the germination of *S. oleraceus*, growth in seedlings that could only be measured in the control treatment, significant differences were observed concerning the other concentrations of the extract. On average, the seedlings had 2 leaves (Fig. 3A), root length was 1.98 cm and stem length were 0.18 cm (Fig. 3B). The number of leaves was 2 because only the cotyledonal leaves were present at the time of the experiment. The stem was very short at that time, the longest organ was the root. In addition, the stem had a reddish-green color and the leaves were completely green. The allelopathic effect of the extract on seedling growth cannot be evaluated because the effect was 100% inhibition in the germination process. In another study, the phenolic compounds were responsible for inhibiting the growth of the *Echinochloa crus-galli* root (Chon & Kim, 2004), while Gindri *et al*. (2020) indicate that the triterpenes lantadene A and B from the aqueous extract of *Lantana camara* causes growth inhibition in epicotyl and root of *Bidens pilosa*.

Gil *et al*. (2012) found that with *Swinglea* extracts there is complete root inhibition in the five weeds that they evaluated, which is consistent with the negative effect on germination. If there is no germination, there is no radicle protrusion and growth, as occurred in the present study.

Phase 2. Post-emergence treatments

Incidence (%): from 2 d on it was evident that all the plants showed symptoms of damage from the extract of *C. lineatifolia,* which is why 100% of incidence was evident. At 5 and 8 d, 100% incidence of damage continued to be observed (Fig. 4). The same values were found in *T. officinale* with applications of *C. lineatifolia* extract (Cabeza-Cepeda *et al*., 2021). As seen in Figure 5, the symptoms were localized yellowing and necrotic spots, showing that *C. lineatifolia* has potential as a bioherbicide. In this regard, Khan and Khan (2015) found that the extracts of several plants decrease the density of weeds to one-third about control in a wheat crop with the application of *Ammi visnaga* and *Convolvulus arvensis*.

For severity (%), significant differences were found throughout the study and the damage was greater as the concentration of the extract increased. At 2 d after application (first reading) the damage with the extracts was over 40.0 ± 0.4%; at 5 d (second reading) with 9% of the extract, 63.0 ± 2.0% damage was reached, and at 8 d (third reading) the damage with this treatment was 74.5 ± 1.7%. The other extracts produced lower effects but showed representative damage (Fig. 6). Although the plants did not die and some even tended to start flowering, perhaps due to the stress produced by the extract, this shows that *C. lineatifolia* exerts an allelopathic effect on *S. oleraceus*, apparently, due to the presence of phenolic compounds, therefore, it can be considered as a bioherbicide with great potential.

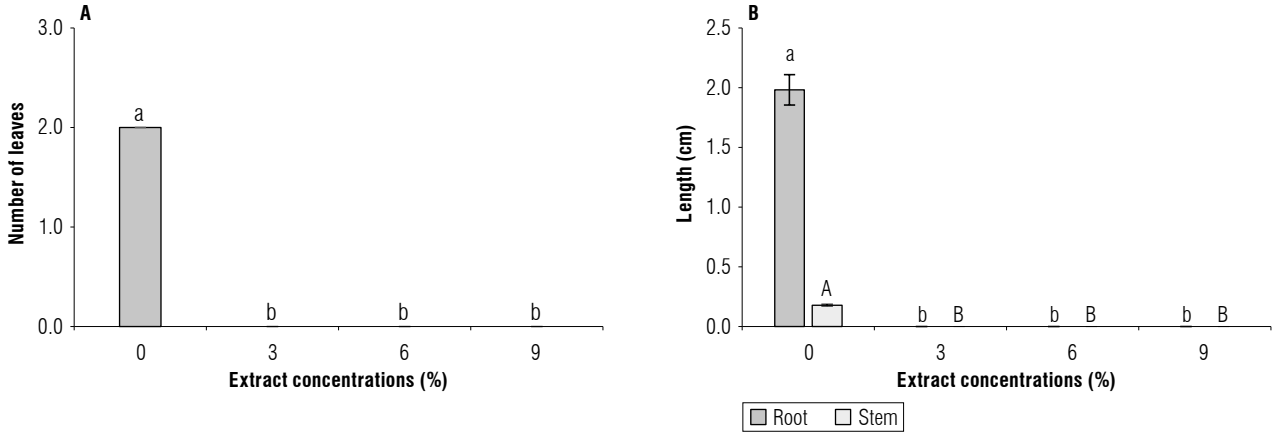

FIGURE 3. Bioherbicidal effect of the allelopathic extract of *C. lineatifolia* seeds on the number of leaves (A) and the stem and root length (B) of *Sonchus oleraceus* seedlings. Means followed by different letters indicate significant differences according to the Tukey test ($P<0.05$). Vertical bars for each mean indicate the standard error (n=4).

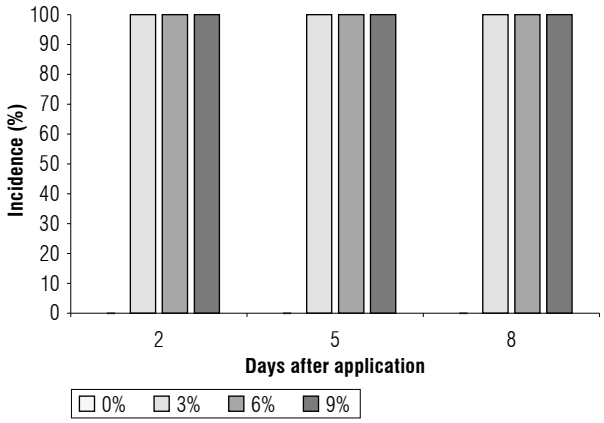

FIGURE 4. Bioherbicidal effect of the allelopathic extract of *C. lineatifolia* seeds on the incidence of damage in *Sonchus oleraceus* plants.

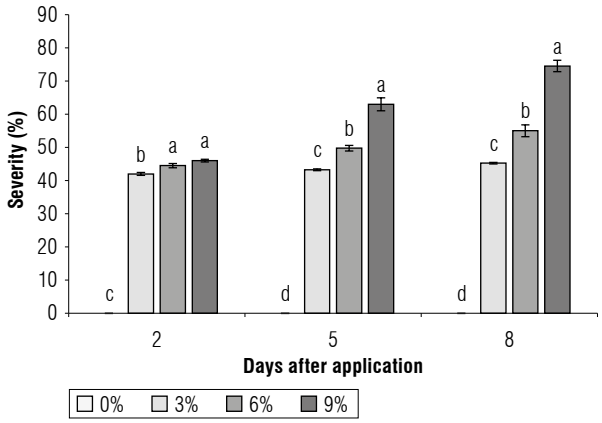

FIGURE 6. Bioherbicidal effect of the allelopathic extract of *C. lineatifolia* seeds on the severity of damage in *Sonchus oleraceus* plants. Means followed by different letters on each evaluation day indicate significant differences according to the Tukey test ($P<0.05$). Vertical bars of each mean indicate the standard error (n=4).

FIGURE 5. Symptoms of damage in *S. oleraceus* leaves caused by application of *C. lineatifolia* ethanolic extract.

In agreement with these findings, Gil *et al*. (2010) report from slightly visible toxic symptoms up to death for different weeds (*Senna obtusifolia*, *Amaranthus dubius*, *Rumex crispus*, *Brassica rapa*, and *Polygonum segetum*) with the application of *Swinglea glutinosa* and *Lantana camara* extracts. It is important to highlight that, in the study by Gil *et al*. (2010), the extracts were at the maximum of 5% concentration. Blanco (2006) mentions various mechanisms of action of allelopathic agents, for example, by stimulating stomatal closure, inhibiting electron transport in photosystem II and mitochondria, decreasing ATP synthesis, affecting cell membranes, and degrading auxins, among other effects. In *C. lineatifolia*, the effects are unknown. It is not known how the application affects the plant. Possibly, the application directly affects chlorophylls because it generates yellowing or chlorosis. It could also greatly affect cell membranes because the effects it generates appear very fast with typical symptoms such as necrosis. Among the chemical herbicides, those that cause these symptoms, include membrane disruptors and photosynthesis inhibitors (Zimdahl, 2018). In addition, it is still not known if the effects are selective, but perhaps it is contact because the damage was localized (Fig. 5). In *T. officinale*, the extract

of *C. lineatifolia* affected parameters of chlorophyll fluorescence (Cabeza-Cepeda *et al*., 2021) indicating that this bioherbicide can affect the photosynthetic process. In turn, the allelochemicals can be more biodegradable than traditional herbicides, but they can also have harmful effects on crops. For this reason, it is necessary to carry out more studies before adopting commercial use (Singh *et al*., 2003; Ma *et al*., 2006).

The results obtained are consistent with Stefanello (2016), in which allelopathic substances have been studied to minimize the application of agrochemicals, such as herbicides, that in the long term can minimize the negative effects on the environment and human health. Thus, Oliveros-Bastida (2008) mentioned a great diversity of substances with allelopathic potential. For phytotoxic evaluation, interest can be focused on the base molecules for the development of herbicides having around 100 molecules from each of the following groups or substances: terpenes, coumarin, benzoquinones, and alkaloids. This same author writes that the development of the use of herbicides from natural sources in the agrochemical industry has been very limited. With *C. lineatifolia* the first step is being taken, and other subsequent studies are necessary to make it viable as a bioherbicide on a commercial level.

Conclusions

The ethanolic extract of *Campomanesia lineatifolia* seeds inhibited the germination of *Sonchus oleraceus* seeds. With the foliar application, an incidence of 100% control was observed with and any of the concentrations evaluated showed symptoms of chlorosis and necrosis. The severity of damage was observed starting from d 2 after application, indicating that the bioherbicidal effect is rapid. At any concentration of the extract, symptoms of damage were observed in the *Sonchus oleraceus* plants, and, although it was not possible to cause the death of the plants, the ethanolic extract from the seeds of *C. lineatifolia* has bioherbicide activity on *S. oleraceus*.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

CAMC and JAF designed the experiments, CAMC carried out experiments, CAMC, HEB and JAF contributed to the data analysis, and CAMC, HEB wrote the article. All authors reviewed the manuscript.

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Postharvest application of acibenzolar-S-methyl and plant extracts affect physicochemical properties of blueberry (*Vaccinium corymbosum* L.) fruits

La aplicación poscosecha de acibenzolar-S-metil y extractos vegetales afecta las propiedades fisicoquímicas de los frutos de arándano (*Vaccinium corymbosum* L.)

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ABSTRACT

The demand for fruits with high anthocyanin content, such as blueberries, has increased in recent years due to their health benefits. However, few studies are known on the postharvest behavior of blueberry fruits subjected to the application of plant extracts and acibenzolar-*S*-methyl (ASM). The objective of this research was to investigate the effect of the application of ASM and vegetable extracts (mint and coriander) on the organoleptic characteristics of blueberry fruits stored at 16°C during postharvest through a completely randomized design with five treatments. The contents of total anthocyanins in fruits (TA) did not differ between treatments, with values that ranged between 74.1 mg and 83.9 mg 100 g^{-1} of fresh weight. The TA tended to increase during storage and then to decrease during senescence. The firmness of blueberry fruits increased during storage, but hardness did not increase, since the fruits are softer and show wrinkling over time, indicating that the epidermis of the fruits has great elasticity. The fruits with the application of vegetable extracts showed the highest total soluble solids and total titratable acidity. The values of red/green and yellow/blue ratio increased, so the blueberry fruits slightly lost their blue hue during postharvest. Fruits with ASM application reached a postharvest life of 15 d after harvest (dah), while the other treatments only maintained quality for 13 dah.

Key words: anthocyanins, coriander extract, mint extract, fruit firmness, respiratory rate.

RESUMEN

La demanda de frutos con alto contenido de antocianinas como el arándano ha aumentado en los últimos años debido a los beneficios para la salud. Sin embargo, se conocen pocos estudios sobre el comportamiento poscosecha de los frutos de arándano sometidos a la aplicación de extractos vegetales y acibenzolar-*S*-metil (ASM). El objetivo de este trabajo fue investigar el efecto de la aplicación de ASM y extractos de menta y cilantro en las características organolépticas de frutos de arándano almacenados a temperatura ambiente durante la poscosecha, mediante un diseño completamente al azar con cinco tratamientos. Las antocianinas totales (AnT) no presentaron diferencias entre tratamientos con valores que oscilaron entre 74.1 y 83.9 mg 100 g^{-1} de masa fresca. Las AnT presentaron una tendencia a aumentar durante el almacenamiento para después descender durante la senescencia. La firmeza de los frutos de arándano aumenta durante el almacenamiento, no así su dureza, ya que los frutos son más blandos y presentan arrugamiento a través del tiempo, lo que indica que la epidermis de los frutos tiene gran elasticidad. Los frutos con aplicación de extractos vegetales presentaron los valores más altos de sólidos solubles totales y acidez total titulable. Los valores de la relación rojo/verde y amarillo/azul aumentan, por lo que los frutos de arándano pierden ligeramente la tonalidad azul durante la poscosecha. Los frutos con aplicación de ASM alcanzaron una vida poscosecha de 15 d después de cosecha (ddc), mientras que los demás tratamientos sólo mantuvieron la calidad durante 13 ddc.

Palabras clave: antocianinas, extracto de cilantro, extracto de menta, firmeza de frutos, tasa respiratoria.

Introduction

The blueberry grows both in the wild and as a commercial crop. It has doubled its commercial production from the year 2010 to 2019 from 439,000 t to 1.0 million t, of which the main producing countries are the United States, Canada, Chile, and Peru (USDA, 2021). The production of blueberries in Colombia ranks sixth among South American countries, with 304 ha cultivated in 2018, located mainly in the departments of Cundinamarca and Boyacá and harvested to satisfy local consumption (Agronet, 2022). Blueberries are fruits with a low sugar content, a high antioxidant capacity and a high content of flavonoids, among which anthocyanins stand out. Their consumption

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provides many benefits for human health (Salgado *et al*., 2018), among which the improvement of vision and antidiabetic properties are highlighted (Yang *et al*., 2022).

Currently, there are many varieties of blueberry on the market, including the 'Biloxi' variety that could easily adapt to different altitudes (Araujo *et al*., 2015). The fruits of blueberry crops could suffer from physical damage and attacks by pathogens and diseases during storage, resulting in economic losses. Different natural extracts and compounds, such as acibenzolar-*S*-methyl (ASM), have been tested, with positive effects on the conservation of fruits of mango (*Mangifera indica* L.) and cabbage (*Brassica oleracea* L.) (Ishiga *et al*., 2021); thus, increasing the postharvest life of blueberry fruits is possible.

ASM is a synthetic compound and chemical activator of plant resistance to diseases through the activation of specific genes (Colombo *et al*., 2018). It acts as a functional analog of salicylic acid, inducing systemic acquired resistance (SAR) (Jepersen *et al*., 2017). ASM maintains fruit quality by regulating enzymes that degrade the cell wall during storage in apples (*Malus domestica* Borkh) (Li *et al*., 2020).

The effect of different essential oils, including the extract of red thyme and oregano, proved to be very effective against mold in storage, and extracts of thyme, mint, and limonene had the most effective preservative action on stored strawberries during 14 d of postharvest (Saxena *et al.,* 2020).

Applications of these products have been effective in the management of phytopathogens; however, the effect they have on the physical, chemical, and quality characteristics of blueberry fruits must be tested (Saxena *et al.,* 2020). Therefore, the application of ASM and vegetable extracts (mint and coriander) was evaluated on the organoleptic characteristics and respiration rate of blueberry fruits stored at room temperature (16°C) during postharvest.

Materials and methods

Location

This research was developed in the Plant Physiology Laboratory of the Universidad Pedagógica y Tecnológica de Colombia (Boyacá, Colombia), Tunja, at 5°33'16" N and 73°21'09" W, with an average annual temperature of 16°C and average relative humidity of 65%.

Materials

The fruits were harvested from an organic commercial crop two years of age, located on the 'La Nutria' farm, El Carmen village in the municipality of Combita, at an altitude of 2697 m a.s.l., 5°41'54" N and 73°16'11" W, with an average annual temperature of 12.5°C and relative humidity ranging from 70% to 80%.

Twenty kg of 'Biloxi' variety blueberry fruits was collected with average weights of 1.3 to 1.5 g per fresh fruit, between 6:00 and 8:00 a.m. These were transported in 10 kg plastic baskets from the farm to the laboratory, under ambient conditions in a time of 35 min. Ripe fruits were selected according to the Colombian technical standard for blueberries NTC-6373 (Icontec, 2019), free from mechanical damage and with a uniform dark purple color and a size greater than 8 mm in diameter, using a grading sieve in order to homogenize the sample.

To obtain coriander (*Coriandrum sativum* L.) and mint (*Mentha piperita* L.) extracts, 69.92 g of seed and 385.8 g of mint leaves were used, washed, dried at a temperature of 30°C at 35°C, respectively, macerated and immersed in 240 ml and 500 ml of 35% (v/v) ethyl alcohol, respectively. The extracts were stored at 4°C in glass jars in the refrigerator and in the dark for 15 d, then filtered for later application (Morais *et al*., 2015).

Experimental design

Extracts were applied to the blueberry fruits with a completely randomized design with five treatments as follows: T1) coriander extract (*Coriandrum sativum* L.), T2) mint extract (*Mentha piperita* L.), T3) 50 mg L^{-1} of acibenzolar-*S*-methyl (ASM), T4) 100 mg L^{-1} of ASM, and T5) fruits immersed in distilled water. Each treatment had four replicates, for a total of 20 experimental units (EU). Each EU had about 500 g of blueberry fruits, stored at 16°C in commercial plastic boxes KIT 250-H53 (Proplantas S.A.®, Colombia), after being subjected to immersion in the different treatments for 5 min.

Physicochemical properties

The following variables were evaluated daily: 1) fruit firmness using a GY-4 penetrometer (Yueqing Handpi Instruments Co., Ltd, China) with a 3.5 mm tip, a pressure depth of 10 mm, a 0.01 N precision, and 3 fruits with uniform size for each EU; 2) contents of total soluble solids (TSS) using a Hanna HI 96803 refractometer with a scale from 0% to 85% (Hanna Instruments, Woonsocket, RI, USA), and measuring the Brix degrees in juice extracted from 12 fruits; 3) total titratable acidity (TTA) quantified with the methodology used by Álvarez-Herrera *et al.* (2015) through calculations with data on the volume of sodium hydroxide (NaOH) incorporated in 5 ml of fruit juice brought to 50 ml

with distilled water, adding three drops of phenolphthalein as an indicator of color change and using equation (1).

$$TTA(\%) = \frac{(A * B * C) * 100}{D} \quad (1)$$

Where: A = volume of NaOH (ml); B = normality of NaOH (0.097); C = equivalent weight expressed in g of predominant acid in the fruit (citric acid 0.064 g meq^{-1}); D = mass in grams of the sample used (5 g).

a*: chromaticity from green to red, b*: chromaticity from blue to yellow, and L*: luminosity were determined. The hue angle and chroma were calculated according to the equations (2) and (3).

$$Chroma = (a^{*2} + b^{*2})^{½} \quad (2)$$

$$Hue = \left(\frac{tan^{-1}\left(\frac{b^*}{a^*}\right)}{2 * \pi * 360} \right) + 180 \quad (3)$$

The respiratory rate (RR) was determined with a 2 L SEE BC-2000 (Vernier Software & Technology, OR, USA) hermetic breathing chamber connected to a VER CO_2-BTA infrared sensor (Vernier Software & Technology, OR, USA) and Labquest2 interface (Vernier Software & Technology, OR, USA) for 10 min, expressed in mg of CO_2 kg^{-1} h^{-1}. The total anthocyanins (TA) in fruits were quantified with a 0.25 g blueberry fruit sample, homogenized in 1 ml of 0.1% citric acid solution, and 10 ml of 80% ethanol; 4 ml of the sample were centrifuged in a Unico C858 Model PowerSpin LX (Unico Scientific, Hong Kong) at 4000 rpm for 20 min. Absorbances were measured at 532 nm with a HumanCorp UV/Visible X-ma 1200V spectrophotometer (Human Corporation, Seoul, Korea). The TA content (mg 100 g^{-1} of fresh fruit weight (FW)) was calculated and reported as cyanidin-3-glucoside equivalents (Cy-3-glucoside) using equation (4) (Zapata *et al.*, 2014).

$$TA = \frac{abs * MM * DF * 1000}{e} \quad (4)$$

Where abs is absorbance measured in the spectrophotometer, MM is molecular mass of cyanidin-3-glucoside, 449.2 g mol^{-1}, DF is the dilution factor (10), *e* is the molar extinction coefficient for cyanidin-3-glucoside (26900), and 1000 is the conversion factor from g to mg.

Statistical analysis

The data were subjected to normality tests to eliminate erroneous and atypical values. Once the assumptions of normality had been verified, a transversal and longitudinal analysis of variance (ANOVA) was carried out to determine significant differences between the treatments and measurement times. The average of the variables was subjected to the Tukey's means comparison tests ($P \leq 0.05$); the analyses were performed with SAS v.9.2e (SAS Institute Inc., Cary, NC).

Results and discussion

Accumulated mass loss in fruits

There were significant differences for accumulated mass loss (ML) during the first three measurements at 1, 2, and 3 d after harvest (dah). Afterwards, it homogenized and was constant for all treatments during the postharvest phase of the blueberry fruits. The absence of significant differences between treatments agrees with Li *et al.* (2020) who found that apple fruits treated with ASM did not have ML that was significantly affected with respect to the control treatment.

At 13 dah, the treatment without applications had the highest ML, while the lowest ML values were seen in the fruits with applications of the mint extract. The ML of the blueberry fruits at 15 dah was 19.6% on average, higher than reported at 15 dah by Jaramillo-Sánchez *et al.* (2019) of 1.8% and 3% for the control and ozone treatment, respectively, and higher than reported by Paniagua *et al.* (2013) in the third postharvest week for chilled blueberries, which ranged between 1% and 15%.

At 4 dah, there were no significant differences in ML because ML in fleshy fruits is due to water loss, which is directly proportional to the vapor pressure deficit (VPD) between the fruit and the environment; therefore, at longer storage times there would be greater water losses that can be diminished by the initial protective effect of treatments. When a berry loses around 6% water, it loses commercial quality (Díaz-Pérez, 2019). So, the blueberry fruits reached 13 d with an acceptable consumption quality, which is longer than the commercial grade (4 dah).

Fruit firmness

Firmness only had significant differences at 6 and 13 dah (Fig. 1). The treatment with 50 mg L^{-1} of ASM reached the highest firmness at 13 dah. The firmness of the equatorial part of blueberry fruits increased throughout the postharvest phase with an average for all treatments of 5.06 N at harvest and 6.41 N at the end of the storage period. And the fruits that received applications of coriander extract at the beginning of the postharvest extract stood out, and achieved an increase in firmness of 50% (Fig. 1), from 4.5 N to 6.8 N. This was probably due to an increase in the

extensibility of the fruit epidermis, that means that, even though the fruits were softer to the touch due to the action of enzymes such as polygalacturonase (Yahia *et al*., 2019), they had greater resistance to breakage by the penetrometer.

Jaramillo-Sánchez *et al*. (2019) find firmness values in blueberry fruits ranging between 2.2 and 2.6 N with maximum displacement of the epidermis before rupture of 4-5 mm. Cortés-Rojas *et al*. (2016) find that 'Biloxi' blueberries have firmness averages of 2.1 N, similar to the softening data of Xu and Liu (2017), 3 N to 1.5 N throughout storage. These authors find lower firmness values in blueberry fruits than those in the present study, probably, because the latter were harvested at commercial maturity and brought directly to the laboratory the same day to start the postharvest measurements.

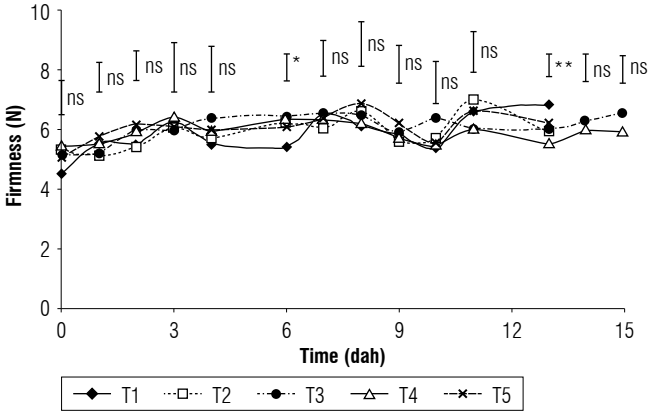

FIGURE 1. Firmness of blueberry fruits subjected to different preservative treatments during 15 d after harvest (dah): T1) coriander extract; T2) peppermint extract; T3) acibenzolar-*S*-methyl 50 mg L^{-1}; T4) acibenzolar-*S*-methyl 100 mg L^{-1}; T5) fruits immersed in distilled water. ns) no significant, * and ** indicate significant effect according to the ANOVA ($P<0.05$ and $P<0.01$, respectively) between treatments. Vertical bars indicate the Tukey range test ($P<0.05$).

Contents of total soluble solids

Although there were significant statistical differences in many of the measurements recorded over time, they did not occur at all sampling times (Tab. 1). However, the fruits that received applications of coriander or mint extracts at all times had higher average values of total soluble solids (TSS) (12.82 and 12.39 °Brix, respectively), while the treatments with applications of 50 and 100 mg L^{-1} of ASM and the control had lower average values (11.88, 11.95, and 11.94 °Brix). Similar results are reported by Saito *et al*. (2020) for the cultivars 'Draper' and 'Duke', which range between 11.4 and 13.2 °Brix, but higher results are obtained for the variety 'Biloxi' by Cortés-Rojas *et al*. (2016), whose averages oscillate around 13.6 °Brix.

All treatments significantly increased the TSS during postharvest storage, between 3% and 17%, except the fruits that received applications of 100 mg L^{-1} of ASM that had a reduction in TSS of around 4% (Tab. 1). Once the fruits were harvested, a greater breakdown of reserve carbohydrates to simpler sugars was generated. This degradation is normally faster in climacteric fruits after they reach physiological maturity that generates an increase in TSS. This results from an increase in the activity of enzymes such as α-amylase, β-amylase, and starch phosphorylase that hydrolyze starch (Yahia *et al*., 2019). The most abundant sugar in blueberry fruits is glucose, followed by fructose and galactose (Fotiric-Akšic *et al*., 2019). This TSS increase in blueberry fruits as they ripen and during storage is also reported by Gibson *et al*. (2013) but is contrary to that reported by Xu and Liu (2017), who obtain a decrease from 9 to 5 °Brix at 8 dah in fruits stored at 4°C. It is likely that the coating (quinoa protein-chitosan-sunflower oil edible film) has a positive effect on the accumulation of TSS during postharvest, as reported by Abugoch *et al*. (2015).

Total titratable acidity

There were significant differences between the treatments in total titratable acidity (TTA) only in the samplings carried out at 0, 3, 4, 9, and 13 dah (Tab. 1). The fruits subjected to applications of mint or coriander presented higher average TTA values (0.85% and 0.80%, respectively), while the fruits subjected to 50, 100 mg L^{-1} of ASM, and the control had values of 0.70%, 0.66%, and 0.75%, respectively. This highlights the importance of applying coatings to fruits during postharvest since it is clear that coatings delay the loss of TTA and conserve the TTA longer (Gol *et al*., 2013).

The average TTA for all treatments tended to decrease significantly during the storage period, like that reported by Xu and Liu (2017). This decrease is attributed to the fact that many organic acids in fruits are used as a substrate in enzymatic reactions of respiration, such as occurs in berries and tomatoes, where the amount and concentration of malate and citrate decrease over time. Likewise, in strawberries and mangos, the amount of citrate and malate also decreases during storage (Vallarino & Osorio, 2019). Once citrate has been produced in the tricarboxylic acid cycle (during the onset of maturation), it can be degraded to cytosol through the synthetic pathway of γ-aminobutyric acid or converted to oxaloacetate and acetyl-CoA (Lee *et al*., 2021) as respiration intensifies and postharvest begins. This explains the decrease in TTA during fruit storage.

TABLE 1. Contents of total soluble solids (TSS) and total titratable acidity (TTA) evaluated in blueberry fruits subjected to different preservative treatments 15 d after harvest (dah).

Variable	dah	Treatments				
		T1	T2	T3	T4	T5
TSS (°Brix)	0	11.65 ± 0.81^{A,a}	12.95 ± 0.41^{A,abc}	12.30 ± 1.13^{A,abc}	13.58 ± 0.91^{A,ab}	11.05 ± 1.06^{A,ab}
	1	13.03 ± 0.66^{A,a}	9.50 ± 0.25^{B,d}	10.03 ± 0.80^{B,c}	10.30 ± 1.04^{AB,de}	11.85 ± 0.32^{AB,ab}
	2	12.80 ± 0.62^{A,a}	11.18 ± 0.64^{A,bcd}	11.65 ± 0.59^{A,bc}	11.68 ± 0.33^{A,bcde}	12.20 ± 0.51^{A,ab}
	3	13.05 ± 0.44^{A,a}	12.38 ± 0.60^{A,abc}	11.33 ± 0.62^{A,bc}	11.83 ± 0.30^{A,abcde}	11.10 ± 0.42^{A,ab}
	4	12.30 ± 0.50^{AB,a}	13.63 ± 0.45^{A,a}	12.08 ± 0.30^{AB,abc}	11.35 ± 0.12^{B,bcde}	11.63 ± 0.35^{B,ab}
	6	12.55 ± 0.50^{A,a}	13.58 ± 0.21^{A,a}	12.10 ± 0.67^{A,abc}	12.30 ± 0.45^{A,abcde}	13.68 ± 0.65^{A,a}
	7	13.88 ± 0.38^{A,a}	13.20 ± 0.16^{A,abc}	11.28 ± 0.11^{A,bc}	11.15 ± 0.50^{A,bcde}	10.14 ± 0.07^{A,b}
	8	12.83 ± 0.35^{A,a}	11.03 ± 0.30^{AB,cd}	12.38 ± 0.31^{A,abc}	10.23 ± 0.38^{B,e}	12.08 ± 0.67^{A,ab}
	9	13.15 ± 0.64^{A,a}	12.55 ± 0.66^{A,abc}	11.78 ± 0.18^{A,abc}	12.75 ± 0.18^{A,abcde}	13.73 ± 0.66^{A,a}
	10	13.00 ± 0.07^{A,a}	11.30 ± 0.49^{BC,bcd}	10.50 ± 0.11^{C,bc}	10.55 ± 0.53^{C,cde}	12.58 ± 0.18^{AB,ab}
	11	12.03 ± 0.66^{AB,a}	14.13 ± 0.43^{A,a}	11.70 ± 0.48^{B,bc}	11.55 ± 0.38^{B,bcde}	11.08 ± 0.54^{B,ab}
	13	13.65 ± 0.99^{A,a}	13.33 ± 0.38^{A,ab}	13.00 ± 0.42^{A,ab}	12.78 ± 0.38^{A,abcd}	12.30 ± 0.62^{A,ab}
	14	---	---	12.88 ±0.53^{A,ab}	14.23 ±0.46^{A,a}	---
	15	---	---	14.48 ± 0.32^{A,a}	13.08 ± 0.19^{B,abc}	---
TTA (%)	0	1.14 ± 0.06^{A,a}	0.90 ± 0.09^{AB,abc}	0.71 ± 0.03^{B,abcd}	0.82 ± 0.07^{B,ab}	0.77 ± 0.03^{B,ab}
	1	0.74 ± 0.02^{A,bcd}	0.66 ± 0.08^{A,bc}	0.68 ± 0.08^{A,abcd}	0.69 ± 0.03^{A,abcdf}	0.75 ± 0.07^{A,ab}
	2	0.79 ± 0.02^{A,bcd}	0.89 ± 0.12^{A,abc}	0.74 ± 0.04^{A,abcd}	0.72 ± 0.02^{A,abcde}	0.83 ± 0.06^{A,ab}
	3	0.89 ± 0.15^{AB,abcc}	1.05 ± 0.10^{A,ab}	0.67 ± 0.06^{AB,abcd}	0.60 ± 0.03^{B,cdf}	0.72 ± 0.09^{AB,ab}
	4	0.96 ± 0.04^{A,abc}	0.96 ± 0.08^{A,abc}	0.88 ± 0.12^{A,ab}	0.65 ± 0,02^{A,bcdef}	0.79 ± 0.07^{A,ab}
	6	0.70 ± 0.05^{A,ab}	0.78 ± 0.05^{A,abc}	0.71 ± 0.07^{A,abcd}	0.76 ± 0.05^{A,abcd}	0.71 ± 0.04^{A,ab}
	7	0.61 ± 0.04^{A,bcd}	0.73 ± 0.06^{A,bc}	0.64 ± 0.01^{A,abcd}	0.62 ± 0.06^{A,cdef}	0.62 ± 0.06^{A,b}
	8	0.65 ± 0.04^{A,d}	0.74 ± 0.05^{A,bc}	0.62 ± 0.03^{A,bcd}	0.67 ± 0.05^{A,bcdef}	0.67 ± 0.05^{A,ab}
	9	0.65 ± 0.01^{A,cd}	0.62 ± 0.01^{A,c}	0.53 ± 0.01^{A,d}	0.52 ± 0.04^{A,ef}	0.66 ± 0.06^{A,ab}
	10	0.85 ± 0.04^{A,cd}	0.91 ± 0.03^{A,abc}	0.95 ± 0.07^{A,a}	0.79 ± 0.05^{A,abc}	0.92 ± 0.05^{A,a}
	11	0.95 ± 0.05^{A,ab}	1.14 ± 0.07^{A,a}	0.87 ± 0.09^{A,abc}	0.89 ± 0.05^{A,a}	0.93 ± 0.06^{A,a}
	13	0.74 ± 0.02^{AB,bcd}	0.89 ± 0.14^{A,abc}	0.67 ± 0.06^{AB,abcd}	0.53 ± 0.02^{B,ef}	0.65 ± 0.02^{AB,ab}
	14	---	---	0.56 ± 0.02^{A,cd}	0.55 ± 0.01^{A,def}	---
	15	---	---	0.62 ±0.05^{A,bcd}	0.49 ± 0.02^{A,f}	---

Means of 8 replicates ± standard error. Treatments correspond to blueberry fruits with applications of: T1) coriander extract; T2) peppermint extract; T3) acibenzolar-*S*-methyl 50 mg L^{-1}; T4) acibenzolar-*S*-methyl 100 mg L^{-1}; T5) fruits immersed in distilled water. Values followed by different uppercase letters in the same row indicate significant statistical differences between treatments ($P<0.05$). Values followed by different lowercase letters in the same column indicate significant statistical differences in postharvest time ($P<0.05$). (---) Loss of fruit quality of consumption.

Contents of total anthocyanins

There were no significant differences between the treatments of total anthocyanin (TA) contents in fruits for any of the measurements over time (Fig. 2); however, the fruits that received applications of mint or coriander extract had higher TA values on average (83.9 and 74.1 mg 100 g^{-1} FW), similar to those found by Gibson *et al.* (2013) for Cy-3-glucoside (76 mg 100 g^{-1} FW of ripe blueberries, and within the range reported by Xu and Liu, 2017), 50 to 300 mg 100 g^{-1} FW of blueberries subjected to 1-methyl-cyclopropene doses and refrigerated at 4°C.

When analyzing the TA over time, a significant tendency to increase the TA during storage up to 13 dah (83.9 mg 100 g^{-1} of fruit FW) was observed. TA then decreased (66.54 mg 100 g^{-1} of fruit FW), similar to that reported by Gibson *et al.* (2013), who states that, once the fruits reached the optimum state of maturity, they were overripe, and the anthocyanin value began to decrease, especially Pet-3-glucoside, Pet-3-galactoside, Cy-3-glucoside, Cy-3-galactoside, Del-3-glucoside, and Del-3-galactoside, the main anthocyanins in blueberry fruits. Pet-3-glucoside is one of the most abundant anthocyanins in ripe blueberry

fruits that changes from 0.4 mg 100 g^{-1} of dry mass in green fruits to 244 mg 100 g^{-1} of dry mass in ripe fruits (Gibson *et al*., 2013).

The increase in TA could be caused by an increase in the activity of enzymes, such as phenylalanine ammonium lyase (PAL) and chalcone synthase (CHA), whose product is chalcone, which degrades by chalcone isomerase to produce naringenin that then transforms into anthocyanin by a hydroxylation reaction and subsequent dehydration (Liu *et al*., 2018). This increase in the synthesis of anthocyanins may be due to abiotic stress from the preservative applied to fruits, which affects the production of secondary metabolites (Chiabrando & Giacalone, 2017).

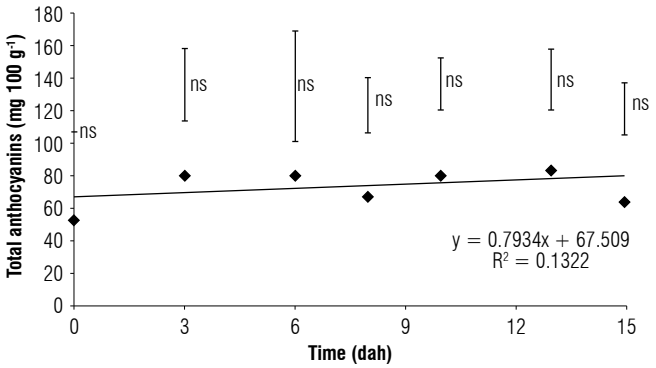

FIGURE 2. Total anthocyanin contents in blueberry fruits subjected to different preservative treatments during 15 d after harvest (dah). ns; no significant, * and ** indicate significant effect according to the ANOVA ($P<0.05$ and $P<0.01$) between treatments. Vertical bars indicate the Tukey range test ($P<0.05$).

Light/dark ratio

There were no significant differences between the treatments at any of the sampling points (Tab. 2); however, the control treatment reached, on average, the highest light/dark ratio (L*) value (33.2) throughout the postharvest phase, while the fruits that received applications of preservatives maintained lower L* values (31.5) because of the coating. These results agree with those of Chiabrando and Giacalone (2017), who report average values of L* 33.75 for all postharvest treatments applied at the beginning of storage and of L* 29.09 at the end of refrigerated postharvest (45 dah), higher than the average for the fruit treatment with chitosan of L* 17.51. It is likely that coatings with a preservative result in lower L* values because more layers are added to cover the fruit epidermis.

The L* values decreased slightly during storage, from 33.3 to 31.0 on average; they did not show significant differences over time, except for the fruits that received applications of 50 mg L^{-1} of ASM that decreased from the beginning of postharvest (33.2) until the end of storage at 15 dah (30.68). These results are also reported by Chiabrando and Giacalone (2017) and are similar to those of Xu and Liu (2017), who, during 8 d of postharvest, find that L* values in blueberry fruits vary from 28.78 to 25.01. This indicates that, during the shelf-life, the fruits had a generalized loss of luminosity that accords with a darkening of the fruits because of oxidation, water loss, and internal browning attributed to inherent degradation in ripening and senescence (Cogo *et al*., 2011).

Red/green ratio

The ANOVA for red/green ratio (a*) did not show significant differences for the treatments in the measurements, except at 8 dah. The control treatment had the lowest a* values (-6.59), followed by the treatments with coatings of coriander or mint that showed higher average values (-5.84 and -6.11) (Tab. 2). Even though no significant differences were observed over time, an increase in the a* values were observed from -6.32 to -5.82, probably caused by the slight increase in darkening that occurs in blueberry fruits during storage. Xu and Liu (2017) make a similar report, where a* increased from the beginning of postharvest (-0.01) to the end of storage (0.29). Likewise, Zou and Hou (2017) obtain average values of 1.02 for a*, indicating that postharvest blueberry fruits lose green color and gain red color.

Yellow/blue ratio

For the yellow/blue ratio (b*), there were significant differences on the 1 d, 7 d, 11 d, and 13 d of measurement between the treatments (Tab. 2). The control treatment had the lowest b* values (-1.64), followed by the treatments with ASM applications that had values of -1.36 and -1.43 for 50 and 100 mg L^{-1}, while the blueberries treated with the coriander or mint extracts had higher values (-0.92 and -1.20, respectively). These results contradict those of Zou and Hou (2017), who report b* values of 1.87 that are positive; however, for the color space, similar tones are close to zero.

Chroma

The chromaticity of the blueberries showed significant differences between treatments at 0, 7, 8, and 14 dah, with an average value of 6.3 ± 0.23 (Tab. 3) that was higher than the values of 3.84 and 2.86 reported by Eum *et al*. (2013) for the cultivars 'Bluetta' and 'Duke'. Likewise, Abugoch *et al*. (2015) report that blueberry fruits have an average chroma of 4.8, lower than in this study. In addition, the chromaticity did not show significant differences over time and had a downward trend from 6.48 to 5.98 during postharvest.

TABLE 2. Light/dark (L*), red/green (a*), and yellow/blue (b*) ratios evaluated in blueberry fruits subjected to different preservative treatments during 15 d after harvest (dah).

Variable	dah	Treatments				
		T1	T2	T3	T4	T5
L*	0	31.93 ± 0.82^{A,a}	33.85 ±1.24^{A,a}	33.28 ± 0.68^{A,a}	32.39 ± 0.14^{A,a}	35.12 ± 1.60^{A,a}
	1	31.59 ± 0.84^{A,a}	33.92 ± 1.14^{A,a}	32.76 ± 0.98^{A,ab}	32.42 ± 0.40^{A,a}	33.63 ± 1.23^{A,a}
	2	31.57 ± 0.83^{A,a}	31.73 ± 1.19^{A,a}	32.29 ± 0.56^{A,ab}	31.94 ± 0.03^{A,a}	33.34 ± 0.98^{A,a}
	3	31.42 ± 1.05^{A,a}	31.44 ± 1.06^{A,a}	32.37 ± 1.06^{A,ab}	31.87 ± 0.43^{A,a}	33.08 ± 0.98^{A,a}
	4	30.91 ± 1.11^{A,a}	32.42 ± 0.99^{A,a}	32.15 ± 0.40^{A,ab}	32.29 ± 0.27^{A,a}	33.42 ± 1.21^{A,a}
	6	30.36 ± 0.76^{A,a}	32.18 ± 1.29^{A,a}	31.82 ± 0.54^{A,ab}	31.75 ± 0.41^{A,a}	33.06 ± 1.03^{A,a}
	7	30.35 ± 0.77^{B,a}	31.69 ± 1.00^{AB,a}	31.56 ± 0.57^{AB,ab}	31.72 ± 0.53^{AB,a}	33.38 ± 1.28^{A,a}
	8	29.22 ± 1.10^{A,a}	32.35 ± 1.24^{A,a}	31.84 ± 0.53^{A,ab}	32.09 ± 0.10^{A,a}	33.02 ± 0.71^{A,a}
	9	29.90 ± 0.94^{A,a}	31.97 ± 1.30^{A,a}	32.36 ± 0.68^{A,ab}	32.20 ± 0.94^{A,a}	33.28 ± 1.06^{A,a}
	10	30.18 ± 0.79^{A,a}	31.90 ± 1.07^{A,a}	31.48 ± 0.92^{A,ab}	31.68 ± 0.09^{A,a}	32.23 ± 1.10^{A,a}
	11	29.71 ± 1.37^{A,a}	30.81 ± 0.99^{A,a}	30.91 ± 0.57^{A,ab}	31.49 ± 0.45^{A,a}	32.28 ± 0.92^{A,a}
	13	29.45 ± 1.12^{A,a}	30.58 ± 1.00^{A,a}	30.67 ± 0.68^{A,ab}	31.29 ± 0.24^{A,a}	32.78 ± 1.13^{A,a}
	14	---	---	29.44 ±0.47^{A,b}	30.66 ± 0.47^{A,a}	---
	15	---	---	30.69 ± 0.27^{A,ab}	31.37 ± 0.23^{A,a}	---
a*	0	-5.92 ± 0.11^{A,a}	-6.33 ± 0.34^{A,a}	-6.43 ± 0.14^{A,a}	-6.24 ± 0.6^{A,ab}	-6.71 ± 0.17^{A,a}
	1	-5.89 ± 0.22^{A,a}	-6.40 ± 0.28^{A,a}	-6.32 ± 0.28^{A,a}	-6.20 ± 0.17^{A,ab}	-6.49 ± 0.20^{A,a}
	2	-6.03 ± 0.20^{A,a}	-6.05 ± 0.30^{A,a}	-6.33 ± 0.13^{A,a}	-6.22 ± 0.02^{A,ab}	-6.60 ± 0.12^{A,a}
	3	-6.02 ± 0.28^{A,a}	-6.05 ± 0.35^{A,a}	-6.55 ± 0.30^{A,a}	-6.35 ± 0.15^{A,ab}	-6.50 ± 0.18^{A,a}
	4	-6.04 ± 0.33^{A,a}	-6.06 ± 0.38^{A,a}	-6.34 ± 0.10^{A,a}	-6.44 ± 0.11^{A,b}	-6.73 ± 0.19^{A,a}
	6	-5.94 ± 0.20^{A,a}	-6.30 ± 0.32^{A,a}	-6.45 ± 0.13^{A,a}	-6.43 ± 0.11^{A,b}	-6.76 ± 0.19^{A,a}
	7	-5.74 ± 0.16^{A,a}	-6.32 ± 0.29^{AB,a}	-6.27 ± 0.19^{AB,a}	-6.33 ± 0.12^{AB,ab}	-6.78 ± 0.28^{B,a}
	8	-5.43 ± 0.32^{A,a}	-6.08 ± 0.24^{AB,a}	-6.15 ± 0.29^{AB,a}	-6.45 ± 0.06^{AB,b}	-6.55 ± 0.16^{B,a}
	9	-5.82 ± 0.24^{A,a}	-5.95 ± 0.47^{A,a}	-6.36 ± 0.25^{A,a}	-6.50 ± 0.25^{A,b}	-6.91 ± 0.30^{A,a}
	10	-6.14 ± 0.23^{A,a}	-6.22 ± 0.24^{A,a}	-6.15 ± 0.23^{A,a}	-6.38 ± 0.07^{A,b}	-6.57 ± 0.25^{A,a}
	11	-5.80 ± 0.43^{A,a}	-5.80 ± 0.30^{A,a}	-5.79 ± 0.19^{A,a}	-6.19 ± 0.15^{A,ab}	-6.25 ± 0.30^{A,a}
	13	-5.42 ± 0.20^{A,a}	-5.76 ± 0.34^{A,a}	-5.74 ± 0.32^{A,a}	-6.16 ± 0.12^{A,ab}	-6.35 ± 0.38^{A,a}
	14	---	---	-5.47± 0.08^{A,a}	-5.76 ± 0.07^{A,a}	---
	15	---	---	-5.69 ± 0.21^{A,a}	-5.96 ± 0.10^{A,ab}	---
b*	0	-1.10 ± 0.12^{A,a}	-1.7 ± 0.11^{A,a}	-1.46 ± 0.16^{A,a}	-1.36 ± 0.08^{A,a}	-1.78 ± 0.10^{A,a}
	1	-1.07 ± 0.15^{A,a}	-1.6 ± 0.09^{A,a}	-1.50 ± 0.21^{AB,a}	-1.50 ± 0.13^{AB,a}	-1.58 ± 0.12^{B,a}
	2	-1.00 ± 0.19^{A,a}	-0.96 ± 0.12^{A,a}	-1.26 ± 0.19^{A,a}	-1.48 ± 0.07^{A,a}	-1.61 ± 0.07^{A,a}
	3	-1.06 ± 0.18^{A,a}	-1.14 ± 0.21^{A,a}	-1.46 ± 0.27^{A,a}	-1.36 ± 0.18^{A,a}	-1.78 ± 0.12^{A,a}
	4	-0.98 ± 0.22^{A,a}	-1.35 ± 0.11^{A,a}	-1.52 ± 0.10^{A,a}	-1.57 ± 0.10^{A,a}	-1.63 ± 0.23^{A,a}
	6	-0.93 ± 0.18^{A,a}	-1.37 ± 0.19^{A,a}	-1.37 ± 0.17^{A,a}	-1.41 ± 0.08^{A,a}	-1.62 ± 0.16^{A,a}
	7	-0.90 ± 0.21^{A,a}	-1.29 ± 0.13^{AB,a}	-1.30 ± 0.22^{AB,a}	-1.45 ± 0.16^{AB,a}	-1.77 ± 0.25^{B,a}
	8	-0.85 ± 0.22^{A,a}	-1.01 ± 0.12^{A,a}	-1.26 ± 0.25^{A,a}	-1.22 ± 0.16^{A,a}	-1.57 ± 0.21^{A,a}
	9	-0.81 ± 0.18^{A,a}	-1.28 ± 0.28^{A,a}	-1.37 ± 0.27^{A,a}	-1.44 ± 0.14^{A,a}	-1.71 ± 0.20^{A,a}
	10	-0.84 ± 0.21^{A,a}	-1.12 ± 0.26^{A,a}	-1.31 ± 0.37^{A,a}	-1.34 ± 0.05^{A,a}	-1.45 ± 0.19^{A,a}
	11	-0.89 ± 0.12^{A,a}	-1.17 ± 0.27^{AB,a}	-1.27 ± 0.13^{AB,a}	-1.36 ± 0.07^{AB,a}	-1.82 ± 0.26^{B,,a}
	13	-0.69 ± 0.16^{A,a}	-1.40 ± 0.08^{B,a}	-1.29 ± 0.20^{B,a}	-1.50 ± 0.08^{B,a}	-1.43 ± 0.10^{B,a}
	14	---	---	-1.47 ± 0.06^{A,a}	-1.57 ± 0.10^{A,a}	---
	15	---	---	-1.28 ± 0.18^{A,a}	-1.47 ± 0.10^{A,a}	---

Means of 8 replicates ± standard error. Treatments correspond to blueberries with applications of the following: T1) coriander extract; T2) peppermint extract; T3) acibenzolar-*S*-methyl 50 mg L^{-1}; T4) acibenzolar-*S*-methyl 100 mg L^{-1}; T5) fruits immersed in distilled water. L*: luminosity; a*) chromaticity from green to red; b*) chromaticity from yellow to blue. Values followed by different uppercase letters in the same row indicate significant statistical differences between treatments ($P<0.05$). Values followed by different lowercase letters in the same column indicate significant statistical differences in postharvest time ($P<0.05$). (---) Loss of fruit quality of consumption.

Hue

The blueberries had significant differences at 0, 11, and 13 dah in terms of tonality (Tab. 3), with an average value for all treatments of 282 ± 1.1, within the range of 282 to 284 obtained by Abugoch *et al*. (2015) and less than the 290 and 302 obtained by Eum *et al*. (2013) for the cultivars 'Bluetta' and 'Duke'. The treatment with the highest tonality was the control (283.9), while the coated treatments had a lower tonality on average (281.3) because these fruits did not have applications on the epidermis that decreased the intensity of the color, according to Abugoch *et al*. (2015), who confirms that the coated fruits had lower tonality values.

Respiratory rate

There were only significant statistical differences in fruit respiration rate (RR) at d 1, 10, and 11 after harvest. Figure 3 shows that the ASM treatments slightly decreased the RR during most of the measurements throughout the postharvest, according to the report by Li *et al*. (2020) for apple fruits where the ASM applications inhibited RR. The blueberry fruits have non-climacteric behavior, similar to that established by Saltveit (2019). The average values at the beginning of storage were around 30.93 ± 2.65 mg of CO_2 kg^{-1} h^{-1}, within the range reported by Brackmann *et al*. (2010), but higher than that found by Xu and Liu (2017),

TABLE 3. Chroma and hue evaluated in blueberries subjected to different preservative treatments during 15 d after harvest (dah).

Variable	dah	Treatments				
		T1	T2	T3	T4	T5
Chroma	0	6.03 ± 0.13^{B,a}	6.46±0.36^{AB,a}	6.60± 0.16^{AB,a}	6.39± 0.05^{AB,a}	6.94± 0.19^{A,a}
	1	5.99 ± 0.24^{A,a}	6.49± 0.28^{A,a}	6.50± 0.32^{A,a}	6.39± 0.19^{A,a}	6.68± 0.23^{A,a}
	2	6.11 ± 0.23^{A,a}	6.13± 0.31^{A,a}	6.47± 0.562^{A,a}	6.40±0.02^{A,a}	6.79± 0.13^{A,a}
	3	6.12 ± 0.30^{A,a}	6.16± 0.38^{A,a}	6.72± 1.060^{A,a}	6.50± 0.18^{A,a}	6.74± 0.20^{A,a}
	4	6.12 ± 0.35^{A,a}	6.21± 0.39^{A,a}	6.52± 0.398^{A,a}	6.63± 0.12^{A,a}	6.93± 0.23^{A,a}
	6	6.01 ± 0.22^{A,a}	6.45± 0.35^{A,a}	6.60± 0.544^{A,a}	6.58± 0.12^{A,a}	6.95 ± 0.22^{A,a}
	7	5.81 ± 0.19^{B,a}	6.45± 0.31^{AB,a}	6.42± 0.568^{AB,a}	6.50± 0.14^{AB,a}	7.01 ± 0.33^{A,a}
	8	5.51 ± 0.34^{B,a}	6.17± 0.25^{AB,a}	6.28± 0.529^{AB,a}	6.57± 0.05^{AB,a}	6.74 ± 0.20^{A,a}
	9	5.88 ± 0.25^{A,a}	6.10± 0.51^{A,a}	6.51± 0.684^{A,a}	6.66± 0.27^{A,a}	7.12 ± 0.33^{A,a}
	10	6.20 ± 0.25^{A,a}	6.34± 0.26^{A,a}	6.31± 0.918^{A,a}	6.52± 0.08^{A,a}	6.74 ± 0.28^{A,a}
	11	5.87 ± 0.43^{A,a}	5.92± 0.34^{A,a}	5.93± 0.566^{A,a}	6.34± 0.16^{A,a}	6.51 ± 0.36^{A,a}
	13	5.47 ± 0.19^{A,a}	5.93± 0.35^{A,a}	5.89± 0.683^{A,a}	6.34± 0.14^{A,a}	6.51 ± 0.381^{A,a}
	14	---	---	5.67 ± 0.07^{B,a}	5.97± 0.09^{A,a}	---
	15	---	---	5.84 ± 0.23^{A,a}	6.14±0.12^{A,a}	---
Hue	0	280.49 ± 0.89^{B,a}	281.27± 0.44^{AB,a}	282.69± 1.29^{AB,a}	282.30± 0.77^{AB,a}	284.85±0.56^{A,a}
	1	280.13 ± 1.07^{A,a}	279.37 ± 0.62^{A,a}	283.17± 1.51^{A,a}	283.58± 0.97^{A,a}	283.64± 0.61^{A,a}
	2	279.26 ± 1.44^{A,a}	279.01 ± 0.96^{A,a}	281.16± 1.55^{A,a}	283.37± 0.63^{A,a}	283.68± 0.42^{A,a}
	3	279.85 ± 1.14^{A,a}	280.40 ± 1.36^{A,a}	282.31± 1.74^{A,a}	281.97± 1.31^{A,a}	285.27± 0.60^{A,a}
	4	279.04 ± 1.66^{A,a}	282.53 ± 0.29^{A,a}	283.42± 0.72^{A,a}	283.65± 0.68^{A,a}	283.48± 1.57^{A,a}
	6	278.75 ± 1.46^{A,a}	282.18 ± 1.08^{A,a}	281.97± 1.36^{A,a}	282.37± 0.48^{A,a}	283.44± 1.02^{A,a}
	7	278.78 ± 1.79^{A,a}	281.48 ± 0.72^{A,a}	281.56± 1.73^{A,a}	282.89± 1.20^{A,a}	284.41± 1.45^{A,a}
	8	278.75± 1.83^{A,a}	279.37 ± 0.80^{A,a}	281.40± 1.73^{A,a}	280.70± 1.40^{A,a}	283.38± 1.49^{A,a}
	9	277.87 ± 1.70^{A,a}	281.99 ± 1.83^{A,a}	281.96± 1.98^{A,a}	282.45± 0.70^{A,a}	283.74± 1.08^{A,a}
	10	277.57 ± 1.59^{A,a}	280.10 ± 2.10^{A,a}	281.70± 3.13^{A,a}	281.85± 0.33^{A,a}	282.34± 1.15^{A,a}
	11	278.64 ± 0.80^{B,a}	281.11± 2.08^{AB,a}	282.33± 1.30^{AB,a}	282.36± 0.41^{AB,a}	285.96± 1.52^{A,a}
	13	277.33 ± 1.87^{B,a}	283.72 ± 0.50^{A,a}	282.58± 1.56^{A,a}	283.64± 0.47^{A,a}	282.72± 0.52^{A,a}
	14	---	---	285.10± 0.77^{A,a}	285.22±0.87^{A,a}	---
	15	---	---	282.52± 1.44^{A,a}	283.84± 0.76^{A,a}	---

Means of 8 replicates ± standard error. Treatments correspond to blueberry fruits with applications of T1) coriander extract; T2) peppermint extract; T3) acibenzolar-*S*-methyl 50 mg L^{-1}; T4) acibenzolar-*S*-methyl 100 mg L^{-1}; T5) fruits immersed in distilled water. Values followed by different uppercase letters in the same row indicate significant statistical differences between treatments ($P<0.05$). Values followed by different lowercase letters in the same column indicate significant statistical differences in postharvest time ($P<0.05$). (---) Loss of quality of consumption.

who reported variations in the RR of 1.6 to 2.6 mg of CO_2 kg^{-1} h^{-1} for blueberries refrigerated at 4°C. RR values in blueberries are higher when fruits are not refrigerated.

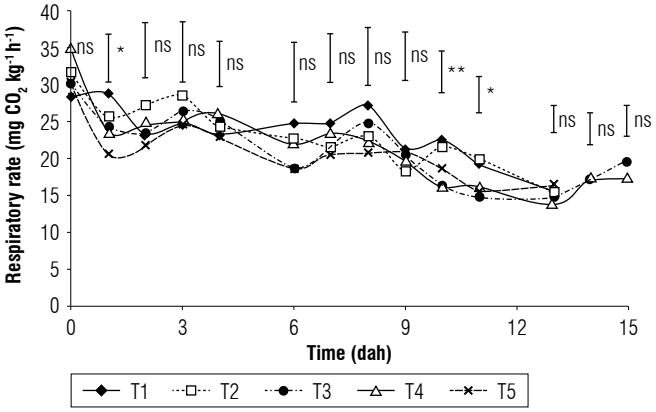

FIGURE 3. Respiratory rate in blueberry fruits subjected to different preservative treatments during 15 d after harvest (dah). T1) coriander extract; T2) peppermint extract; T3) acibenzolar-*S*-methyl 50 mg L^{-1}; T4) acibenzolar-*S*-methyl 100 mg L^{-1}; T5) fruits immersed in distilled water. ns: no significant, * and ** indicate significant effect according to the ANOVA ($P<0.05$ and $P<0.01$, respectively) between treatments. Vertical bars indicate the Tukey range test ($P<0.05$).

There were significant differences over time because the RR of the blueberries decreased throughout the postharvest period until reaching an average of 15.11 mg of CO_2 kg^{-1} h^{-1} at 13 dah, similar to the findings of Brackmann *et al*. (2010). This decrease of the RR in the blueberries was also obtained by Chiabrando and Giacalone (2017) and is attributed to a decrease in metabolism that is reflected in an increase in the concentration of TSS because they are used less in respiration (Guadarrama & Peña, 2013), as seen in the present study. In contrast, Xu and Liu (2017) find that the RR increased during storage, up to 8 dah in fruits refrigerated at 4°C that could have been due to the fact that refrigeration decreases the respiratory processes throughout the postharvest, while the fruits of this study had high RR at the beginning because they were stored at 16°C.

Conclusions

The application of mint extract decreased the loss of the accumulated mass in the blueberries. The firmness of the blueberries increased during storage, but the hardness did not. The fruits were softer and had wrinkling over time, that indicated that the epidermis of the fruits had high elasticity. The total anthocyanin values tended to increase during the first postharvest d and decreased once the fruits begin to lose their commercial quality. The fruits with applications of extracts of mint or coriander maintained higher values of total soluble solids and total titratable acidity. The red/green and yellow/blue ratio values increased. The blueberries had a slight loss of the blue hue during the postharvest, while the luminosity decreased. The respiration rate decreased throughout the storage period, while the total soluble solids increased. The application of acibenzolar-*S*-methyl extended the postharvest life of the blueberries, up to 15 dah (days after harvest), as compared to the other treatments (13 dah).

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contribution

MJG conceived the experiments. MJG and HDRB conducted the experiments. JGAH supervised the work and performed the data analysis. JGAH and HDRB wrote and edited the manuscript. All authors have reviewed the manuscript.

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Quality of *Butia capitata* fruits harvested at different maturity stages

Calidad de frutos de *Butia capitata* cosechados en diferentes estados de madurez

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ABSTRACT

Butia capitata (Mart.) Becc. or "coquinho azedo" is a native palm species of the Brazilian savannah, bears fruits which are sold fresh or as frozen pulp. This study examined postharvest changes in "coquinho azedo" harvested at a commercially immature stage and later evaluated the quality of these fruits by comparing them with those harvested fully ripe. Fruits purchased in the 2020 harvest in Santo Antônio do Retiro, MG (Brazil), were harvested at different degrees of maturity, namely, commercially immature and ripe, according to the point of harvest adopted in the region. For 7 d, weight loss, skin color and respiratory activity were evaluated in the commercially immature-harvested fruits. At 7 d postharvest, physical and chemical evaluations of the pulp were performed. The ripe-harvested fruits were subjected to the same evaluations, but only at 1 d postharvest. The skin color of commercially immature-harvested fruits tended to yellow over the days. Respiration postharvest increased in the immature-harvested fruits. Fresh weight loss exceeded 10% but without compromising appearance. The commercially immature-harvested fruits had 55% more total phenols, whereas the ripe-harvested fruits had higher soluble solids and ascorbic acid contents. Pulp color, soluble solids/titratable acidity ratio and total carotenoid contents were similar regardless of the degree of maturity at harvest. During the days after the harvest of the commercially immature "coquinho azedo", changes take place which cause them to resemble the fruit harvested ripe. Based on the evaluated traits, the early harvest did not compromise the ripening of the fruits.

Key words: coquinho azedo, postharvest, quality, ripening.

RESUMEN

Butia capitata (Mart.) Becc. o "coquinho azedo" es una especie de palma nativa de la flora brasileña, produce frutos que se venden frescos o como pulpa congelada. Este estudio examinó los cambios poscosecha en los frutos de "coquinho azedo" recolectados en su etapa comercialmente inmaduros y evaluó su calidad comparándola con los cosechados completamente maduros. Los frutos adquiridos en la cosecha de 2020 en Santo Antônio do Retiro, MG (Brasil), fueron cosechados en diferentes grados de madurez: inmaduros o maduros según el punto de cosecha adoptado en la región. Durante 7 d, se evaluó la pérdida de peso, el color de la epidermis y la actividad respiratoria en los frutos inmaduros recolectados. A los 7 d poscosecha, se realizaron evaluaciones físicas y químicas de la pulpa. Los frutos cosechados maduros fueron sometidos a las mismas evaluaciones, pero 1 d después de recolectados. El color de la epidermis de los frutos inmaduros tendió a amarillearse con el paso de los días. La respiración poscosecha aumentó en los frutos inmaduros. La pérdida de peso fresco superó el 10%, pero sin comprometer la apariencia. Los frutos cosechados inmaduros tuvieron un 55% más de fenoles totales, mientras que los maduros tuvieron mayores niveles de sólidos solubles y ácido ascórbico. El color de la pulpa, la relación solidos solubles/acidez titulable y el contenido de carotenoides totales fueron similares independiente del estado de madurez en la cosecha. Durante los días posteriores a la cosecha del "coquinho azedo" comercialmente inmaduro, ocurren cambios que los hacen similares a los cosechados maduros. Con base en las características evaluadas, la anticipación de la cosecha no comprometió la maduración de los frutos.

Palabras clave: coquinho azedo, poscosecha, calidad, maduración.

Introduction

Butia capitata, commonly known as "coquinho azedo" in Portuguese, is a Brazilian endemic palm tree that grows in the Cerrado regions of the states of Bahia, Minas Gerais, and Goiás (CNCFlora, 2020). The "coquinho azedo" fruits constitute an important source of income for producers during the harvest period, which runs from October to January (Lima *et al*., 2010). The fruit is sold fresh and as frozen pulp for use also during the off-season. According to Castricini *et al*. (2020), "coquinho azedo" pulp frozen for eight months exhibited variations in instrumental color (lightness, chroma and °*hue*), pH, titratable acidity (TA), contents of soluble solids (SS), and ascorbic acid content,

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without prejudice to the minimum standards of identity and quality.

The "coquinho azedo" pulp is a source of vitamin C, β-carotene and phenolic compounds (Barbosa *et al*., 2021). According to these authors, the consumption of the fruit should be encouraged due to the antioxidant action of these compounds on growth, development, and protection against diseases.

Optimum postharvest fruit quality is influenced by the maturity stage at the time of harvest, with fruits harvested immature or overripe having inferior quality and a shorter shelf life (Braman *et al*., 2015). In addition, Chitarra and Chitarra (2005) stated that early harvested (immature) fruits will have poor sensory attributes, even if they reach maturity some time later. This was demonstrated in fruits of *Ziziphus jujube*, cv. Dongzao harvested with a light green color (80 d after full flowering), which showed lower sensory acceptance up to the sixth day of shelf life, compared with fruits harvested with 50% of the skin red (110 d after full flowering) (Zhao *et al*., 2021). Finally, Lobos *et al*. (2018) mentioned that *Vaccinium corymbosum* L. fruits harvested at a higher degree of maturity exhibited higher soluble solids contents and soluble solids/titratable acidity ratio at 30 and 40 d of cold storage.

Lima *et al.* (2010) suggested that the best time for harvesting "coquinho azedo" is when most of the fruits in the bunch are yellowish-green (color-turning stage), since, if harvested green, not all of them will ripen and become good for consumption. However, if they are harvested ripe, the percentage of fruit losses due to decay can be high, reducing marketing time.

Therefore, this study was undertaken to examine the postharvest changes (physical, chemical, and physiological) of "coquinho-azedo" harvested at a commercially immature stage of maturity and evaluate the characteristics of these fruits by comparing them with those harvested fully ripe.

Material and methods

Fruit collection

The "coquinho azedo" fruits were acquired from the 2020 harvest period in the municipality of Santo Antônio do Retiro - MG, Brazil. The fruits were harvested by the producer at different degrees of maturity, namely, ripe and commercially unripe, according to the point of harvest adopted in the region.

B. capitata is a native species of the Brazilian flora and, for this reason, the fruit collection activity was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under no. A8E6425.

Experimental design

The study was divided into two stages, according to the degree of maturity at harvest: ripe and commercially immature. A completely randomized experimental design with six replicates was used, with each replicate consisting of three fruits.

The ripe-harvested fruits had completely yellow skin (lightness=64.07, chroma=63.33, and °*hue*=72.02), whereas the skin of those harvested when commercially immature was green with yellow spots (lightness=57.39, chroma=37.19, and °*hue*=103.99).

Evaluations of fruits picked ripe and commercially immature

After harvesting, the fruits were sent to the laboratory, and on the following day (1 d postharvest), respiratory activity was evaluated for 1 d in the ripe and for 7 d in the commercially immature-harvested fruits. The average temperature and relative humidity of the environment in the experimental period were 26.66ºC and 79.43%, respectively. Respiratory activity was measured simultaneously by two methods, to confirm the respiratory trends of the commercially immature fruits.

The evaluation by titrimetry followed the methodology of Crispim *et al*. (1994), adapted by Deliza *et al*. (2008), with results expressed in mg CO_2 kg h^{-1} on each evaluation day. Instrumental evaluation consisted of measuring the percentage of carbon dioxide (CO_2) accumulated inside the container holding the fruits, which was determined by direct measurement with a CO_2 Analyzer (MOCON, Ametek®) throughout the experimental period. In both methodologies, each replicate was placed in a covered 5.2-L PET bottle protected by a PVC film to ensure better sealing, preventing and/or reducing gas exchange with the external environment.

Parallel to measurement of respiratory activity, the fruits harvested at the commercially immature stage were weighed daily on a digital scale to determine weight loss. Losses were calculated each day relative to the initial weight of the fruits, with the result expressed in percentage terms.

Skin color in the immature fruits was also measured daily (before packaging), using a colorimeter (CR 400 Chroma meter, Minolta®) operating in the LCH system (lightness, chroma and °*hue*). Three readings were performed in the mid-region of each fruit.

On the last day of evaluation (7 d postharvest), these fruits were crushed without the skin and the pulp subjected to the following assessments:

— instrumental color, following the methodology described for whole fruits;

— pH, by potentiometry;

— titratable acidity, by titration with 0.1 M NaOH, with results expressed in g citric acid 100 g^{-1};

— soluble solids (°Brix), determined by digital refractometry, according to the Adolfo Lutz Institute – IAL (2008);

— ascorbic acid (mg 100 g^{-1}), measured by the reduction of the indicator 2,6-dichloroindophenol (DCIP) by ascorbic acid (Brasil, 2013);

— total carotenoid contents (mg 100 g^{-1}), by spectrophotometry, as proposed by Lichtenthaler (1987); extraction took place in ethyl alcohol (95% ethanol), in a dark and refrigerated environment, for 24 h. The filtered extract was read in a spectrophotometer, with the absorbance (A) of chlorophyll "a" determined at 664 nm, chlorophyll "b" at 648 nm, and total carotenoids at 470 nm;

— total contents of phenolic compounds, determined according to Singleton and Rossi (1965) and Georgé *et al.* (2005) with modifications. The extraction took place in 70% acetone for 20 min in an ultrasound device and centrifuging at 4,000 rpm for 20 min. The mixture was filtered through rapid-filtration filter paper and the extraction (successive extraction) was repeated. The 10% Folin-Ciocalteu and 7.5% calcium carbonate reagents were added to the extract. Total phenolic compounds were quantified using a gallic acid calibration curve, with values expressed in mg gallic acid 100 g^{-1}.

The ripe-harvested fruits were subjected to these same assessments, 1 d post-harvest, following the methodology described for immature fruits.

Data analysis

The data were subjected to the Shapiro-Wilk and Bartlett tests to check for the normality of errors and homogeneity of variances, respectively. Because they did not meet the assumptions, the skin color data of "coquinho azedo" collected at the commercially immature stage were subjected to non-parametric statistics, with the color change over the days of storage analyzed by the Kruskal-Wallis test and, subsequently, Dunn's test.

The other characteristics met the assumptions. The fresh weight loss data of the immature-harvested fruits were subjected to analysis of variance, in which losses over the storage days were analyzed by regression.

Data on the physical and chemical characteristics of the pulp (destructive evaluations) of fruits harvested at the ripe stage (1 d postharvest) and commercially immature (7 d postharvest) were compared by the t-test, to observe whether the commercially immature-harvested fruits would complete their ripening and resemble the ripe produce.

The respiratory activity of commercially immature-harvested fruits (by both methods used) during ripening was evaluated by descriptive statistics. All analyses were performed at the 5% significance level, using Sisvar statistical software (Ferreira, 2007).

Results and discussion

Skin color, weight loss, and respiratory activity of fruits picked commercially immature

The skin color of the immature-harvested fruits (Tab. 1) changed over the days. Between the first and fifth days postharvest, there was a 27.51% decrease in °*hue*, with the fruit skin shifting from green (°*hue* values between 90 and 180) to a color tending to yellow (°*hue* values between 0 and 90), which remained until the end of the experimental period. Chroma was 56.71% higher at 5 d postharvest, indicating that the skin was a more intense (or "purer") yellow. Because of yellowing or degreening, the skin was lighter, with higher lightness values (closer to 100) seen after the 4 d postharvest. The change in the color of the commercially immature-harvested "coquinho azedo" fruits was due to their ripening over the 7 d of study. This was mentioned by Lima *et al.* (2010), who stated that the yellow color is an indication of ripeness in "coquinho azedo". Additionally, according to Spoto *et al.* (2020), changes in color allow the consumer to identify the maturity of fruits.

Fruit ripening results in color changes due to biosynthesis, degradation and appearance of pigments, events catalyzed by enzymes activated at this stage of fruit development (Kapoor *et al.*, 2022).

Carotenoids are pigments present in green leaves and fruits, red, yellow and orange flowers, roots, and seeds, whose biosynthesis begins with the production of phytoene by the condensation of two molecules of geranylgeranyl diphosphate by the enzyme phytoene synthase (PSY) (Gonzalez-Jorge *et al*., 2013). Saini *et al.* (2015) mentioned that, after their biosynthesis, the main carotenoids accumulate in specialized plastids, chromoplasts, chloroplasts or leucoplasts, and the main carotenoids present in yellow-orange fruits are β-carotene and α-carotene. Additionally, Gonzalez-Jorge *et al.* (2013) argued that the concentration of carotenoids can also be regulated by enzymatic degradation (carotenoid cleavage dioxygenases) in plastids.

Fresh weight loss was significantly influenced by the days postharvest, fitting a quadratic model and reaching a maximum value of 13.42% at 7 d postharvest (Fig. 1). Although most fruits have their quality compromised when they lose 5 to 10% of moisture (Chitarra & Chitarra, 2005), our results suggest that the final percentage of fresh weight loss in the fruits did not cause them to wilt.

TABLE 1. Instrumental color of "coquinho azedo" (*B. capitata*) fruits harvested at a "commercially immature" stage and stored at an average temperature of 26.55ºC and 79.43% relative humidity.

Days after harvest	Lightness	Chroma	°*hue*
1	57.64 b	37.09 e	103.98 a
2	59.28 b	39.43 de	99.96 ab
3	60.05 b	42.31 edc	92.48 abc
4	64.83 a	51.71 cdb	81.54 bcd
5	65.03 a	55.97 cba	75.97 cde
6	65.31 a	58.25 ba	73.05 ed
7	64.88 a	58.91 a	71.94 e

Medians followed by the same lowercase letter do not differ from each other by Dunn's non-parametric test at 5% significance.

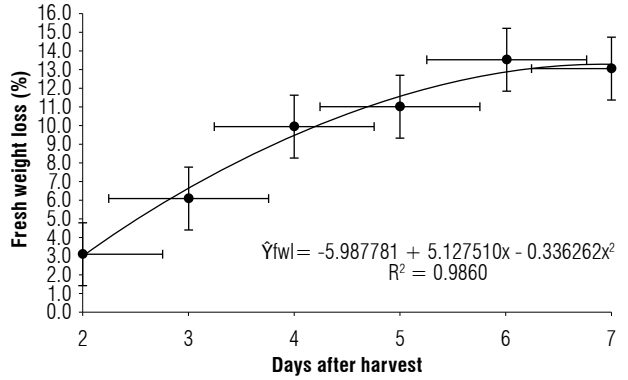

FIGURE 1. Fresh weight loss (%) in "coquinho azedo" (*B. capitata*) fruits harvested at the "commercially immature" stage and stored at an average temperature of 26.55ºC and 79.43% relative humidity. Significance of the quadratic model at $P<0.05$.

The transpiration caused by the difference in vapor pressure between the plant tissue and the surrounding atmosphere compromises fruit quality, inducing loss of fresh weight (Khaliq *et al*., 2015). Coatings on the fruit surface can reduce transpiration and the consequent fresh weight losses, as observed by the authors in mango. Similarly, fruits of *Physalis peruviana* L. (cape gooseberry) packed in a PET tray with cast PP film showed reduced weight loss (Garavito *et al*., 2022).

Respiratory rate decreased between the 1 and 2 d of storage, with a subsequent increase until the 5 d, followed by a decline until the last day, in both analyzed methods (Figs. 2A-B). This behavior suggests a climacteric pattern, since, after harvest, climacteric fruits exhibit a significant increase in respiratory activity and rapid ripening, with color changes, increases in sugar concentration and texture changes (Chitarra & Chitarra, 2005). In non-climacteric fruits, after harvest, there is a decrease in respiratory activity, regardless of the stage of development at which they were harvested (Spoto *et al.* 2020).

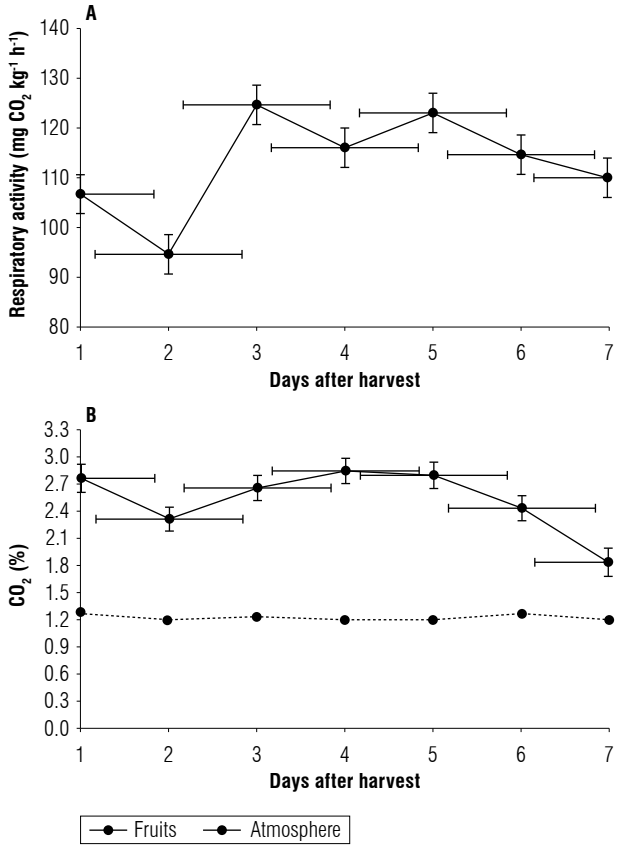

FIGURE 2. A) Respiratory activity and B) CO_2 production of "coquinho azedo" (*B. capitata*) fruits harvested at the "commercially immature" stage of maturity, during 7 d of storage at average temperature of 26.55ºC and 79.43% relative humidity.

Respiratory activity and percentage CO_2 of fruits picked commercially immature (7 d postharvest) and ripe (1 d postharvest)

There was no significant difference in respiratory activity and percentage CO_2 of the commercially immature-harvested fruits at 7 d postharvest and ripe-harvested fruits 1 d postharvest (Tab. 2). Considering that as the fruit ripens its respiratory rate generally decreases (Saltveit, 2016), it can be assumed that, in terms of these evaluations, the immature-harvested "coquinho azedo" is able to complete its ripening in a similar way to the fruits that ripened on the plant. Therefore, anticipating the harvest is not detrimental from the maturation standpoint.

TABLE 2. Respiratory activity (mg CO_2 kg^{-1} h^{-1}) and CO_2 production (%) of "coquinho azedo" (*B. capitata*) harvested "ripe" (1 d postharvest) and 7 d after harvesting the fruits "commercially immature".

Characteristics	Degrees of maturity at harvest		
	Commercially immature*	Ripe	CV (%)
Respiratory activity	110.18 ±7.65 a	123.64 ±3.49 a	10.40
CO_2 production	1.89 ±0.10 a	2.52 ±0.28 a	22.86

Means of six replicates ± standard error. Means followed by the same letter in the rows do not differ by the t-test at 5% significance. *Evaluation performed at 7 d postharvest.

Physical and chemical characteristics of pulp from fruits picked at different degrees of maturity

As for pulp color (Tab. 3), at 7 d postharvest, the commercially immature-harvested fruits had a higher lightness value than those that were harvested ripe. The chroma in the pulp of ripe-harvested fruits was higher, whereas the pulp color *hue* was similar between the fruits of both groups. The yellowish color of the fruit pulp indicates that both fruit groups were ripe, corroborating Lima *et al.* (2010). Nonetheless, due to the higher lightness (although this difference was not visually observable) and chroma values in the ripe-harvested "coquinho azedo", their pulp color was a lighter and more intense yellow shade.

During the ripening of "coquinho azedo" fruits, the change from greenish to yellowish color is due to the unmasking of preexisting pigments by the degradation of chlorophylls and synthesis of carotenoids (Maduwanthi & Marapana, 2019). In addition, according to Li and Yuan (2013), the yellowish color of the fruits is due to the synthesis and deposit of carotenoids in the chromoplasts, with β-carotene being the predominant carotenoid in "coquinho-azedo" (Faria *et al.*, 2011).

The pH of the immature-harvested fruits was significantly higher, with a value inversely proportional to titratable acidity. At 7 d postharvest, this fruit category exhibited a lower citric acid content. Following the same response, the ripe-harvested "coquinho azedo" had a higher soluble solids content. Soluble solids are mostly composed of sugars, which makes the sweetness of a fruit dependent on this trait (Cao *et al.*, 2015). During the ripening of fruits in a non-refrigerated environment, there is an increasing accumulation of organic acids and sugars within the vacuole (Ventura *et al.*, 2022), which will be used in respiration or in the conversion to sugars (Tosun *et al.*, 2008). In addition, during ripening, the partial degradation of the cell wall contributes to the increase in sugar levels in the fruits (Canton *et al.*, 2020).

TABLE 3. Lightness, chroma and ^{o}*hue* of "coquinho azedo" (*B. capitata*) fruits harvested "ripe" (1 d postharvest) and harvested "commercially immature" (7 d postharvest).

Characteristics	Degrees of maturity at harvest		
	Commercially immature**	Ripe	CV (%)
Lightness	65.10 a	64.06 b	1.18
Chroma	58.59 b	63.33 a	4.22
^{o}*hue*	72.07 a	72.02 a	2.53

Means followed by the same letter in the rows do not differ by the t-test at 5% significance. **Evaluation performed at 7 d postharvest.

In the commercially immature-harvested "coquinho azedo", the fruits, possibly, continued the expected respiratory process after harvest, with the use of organic acids and sugars, which possibly explains the lower acidity and soluble solids contents in the commercially immature-harvested fruits at 7 d postharvest.

The higher soluble solids content in the ripe-harvested "coquinho azedo" can be explained by the longer time the fruit remained on the plant, which favors a greater accumulation of sugars. This phenomenon was demonstrated in papaya, where the soluble solids content in the fruits remained practically constant after harvest despite the change in skin color, loss of firmness, among others (Gutierrez & Watanabe, 2017). The soluble solids content determines the degree of sweetness of fruits (Spoto *et al.*, 2020) due to the higher proportion of soluble sugars in their composition (Fernandes *et al.*, 2017). In the literature, average soluble solids levels of 7.70 and 9.60 °Brix were reported in mature coconuts (Souza, 2016; Souza *et al.*, 2018).

The SS/TA ratio was significantly the same in fruits harvested at different ripening degrees. According to Spoto *et al.* (2020), the SS/TA ratio is a criterion for the evaluation of flavor, which can express the degree of fruit ripeness.

Chitarra and Chitarra (2005) stated that the SS/TA ratio provides a good idea of the balance between the soluble solids content and titratable acidity, constituting a more representative measure than the evaluation of these traits in isolation. Therefore, it is likely that the "coquinho azedo" did not have their flavor altered 7 d after being harvested commercially immature.

Ascorbic acid, also known as vitamin C, is an important compound that has antioxidant and metabolic functions (Cruz-Rus *et al*., 2011), and its content was higher in the ripe-harvested fruits. In contrast, the total carotenoid content did not differ between the fruits harvested ripe or commercially immature.

After harvest, the concentration of organic acids in the fruits commonly declines due to their use as a respiratory substrate (Chitarra & Chitarra, 2005), which may explain the lower ascorbic acid content in the immature-harvested "coquinho-azedo". Greater synthesis of ascorbic acid in papaya fruit occurred in parallel with higher respiratory activity in the fruits, with a subsequent decrease during storage (Maringgal *et al*., 2021). This is because, as stated by Mellidou and Kanellis (2017), ascorbic acid participates in the synthesis of ethylene as a cofactor of 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase).

The similar total carotenoids contents in both studied fruit groups may be due to the continued biosynthesis of this phytochemical 7 d after the fruits were harvested commercially immature. This agrees with the findings of Rodriguez-Amaya (2001) that the carotenoids content intensifies with fruit ripening.

Phenolic contents were higher in the immature-harvested fruits, which showed approximately 55% more of these compounds. With respect to phenolic compounds, the literature reports that in citrus fruits, the concentration of flavonoids (a specific class of phenols in vegetables) decreases with increasing fruit size and maturity. This statement corroborates Mcharek and Hanchi (2017), who observed a reduction in the concentration of phenols throughout the ripening of lime. Kosar *et al*. (2004) did not observe significant differences in the flavonoid content of green and ripe strawberries. In the present study, the total phenolic content in the "coquinho azedo" was higher than that reported by Nascimento *et al*. (2020) in fully ripe fruits. The higher total phenolic content in this study may have been due to the two successive extraction steps used, which resulted in more compounds being extracted.

Phenolic compounds are aromatic organic compounds including secondary metabolites, which determine the color and flavor of fruits, in addition to actively participating in the mechanism of resistance to insects and diseases (Milind, 2010). In a study with guava, papaya and mango, Oliveira *et al*. (2011) stated that these metabolites are the antioxidant compounds that most contribute to antioxidant activity in these fruits. Therefore, the "coquinho azedo" can be a good source of natural oxidants for human consumption, especially if the fruits are harvested commercially immature and consumed 7 d postharvest.

TABLE 4. Means of pH, titratable acidity (TA-g citric acid 100 g^{-1}), soluble solids (SS-ºBrix), SS/TA ratio, ascorbic acid (mg 100 g^{-1}), total carotenoids (mg 100 g^{-1}) and total phenols in the pulp of "coquinho azedo" (*B. capitata*) fruits harvested at different degrees of maturity and evaluated at different times after harvest.

Characteristics	Degrees of maturity at harvest		
	Commercially immature**	Ripe	CV (%)
Respiratory activity	110.18 a	123.64 a	10.40
pH	3.80 a	3.25 b	0.94
Titratable acidity (TA)	1.39 b	2.32 a	7.00
Soluble solids (SS)	4.45 b	6.53 a	9.19
SS/TA ratio	3.21 a	2.84 a	10.36
Ascorbic acid	7.51 b	52.91 a	10.31
Total carotenoids	15.42 a	19.12 a	19.90
Total phenolic compounds	729.99 a	408.26 b	18.20

Means followed by the same letter in the rows do not differ by the t-test at 5 % significance.
**Evaluation performed at 7 d postharvest.

Conclusions

"Coquinho-azedo" fruits harvested commercially immature had an increase in respiratory activity after harvest, with changes in skin color and fresh weight losses of over 10% throughout storage. At the end of the ripening period after harvest, the color (º*hue*), respiratory activity, SS/TA ratio and total carotenoids in "coquinho azedo" resembled those of fruits that ripened on the plant. "Coquinho-azedo" fruits harvested fully ripe had higher titratable acidity, soluble solids and ascorbic acid contents than the fruits harvested commercially immature, when ripe. The total phenolic content of "coquinho-azedo" fruits harvested at the commercially immature stage, when ripe, was 55% higher than that of the ripe-harvested fruits.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

MM designed the experiments, carried out the laboratory experiments, performed the statistical analyses, and wrote this manuscript. AC contributed to the design of the experiments and the writing and review of this manuscript. JLS and LPS carried out the laboratory experiments and reviewed the manuscript. CMAM contributed to the data analysis and reviewed the manuscript. All authors have reviewed the manuscript.

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Plant growth and phosphorus uptake of coffee seedlings through mycorrhizal inoculation

Crecimiento y absorción de fósforo en plántulas de café a través de la inoculación micorrizal

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ABSTRACT

Soil phosphorus (P) availability is a limiting factor for coffee seedling growth. Usually, large amounts of P fertilizers are required, generating nutritional imbalance, increasing production costs, and raising environmental concerns in water pollution. The use of arbuscular mycorrhizal fungi (AMF) can enhance plant P uptake and growth and reduce the dose of P fertilizers. A greenhouse experiment was conducted in a substrate containing Paleudult soil and quartz sand, with low level of soluble P (1 mg kg^{-1}), to establish the effect of AMF inoculation with *Rhizoglomus fasciculatum* on coffee (*Coffea arabica* L. cv. Colombia) seedlings growth and P uptake under three levels of P in soil solution (0.002, 0.02, and 0.2 mg L^{-1}). AMF colonization was significantly reduced when contents of P in solution increased. Shoot dry weight and P foliar concentration were increased by the AMF inoculation when soil P in solution was 0.02 mg L^{-1}; these effects were lower at 0.2 mg L^{-1} and null at 0.002 mg L^{-1} P. Results showed that AMF inoculation can play an important role in the growth of coffee seedlings as long as the content P in soil solution maintains intermediate level. At the lowest P level, the response of coffee seedlings to AMF inoculation was ineffective, while at the highest level, AMF application was unnecessary for coffee growth.

Key words: *Coffea arabica*, *Rhizoglomus fasciculatum,* mycorrhizal colonization, shoot dry weight, soil testing.

RESUMEN

La disponibilidad de fósforo (P) es limitante para el crecimiento del café. Usualmente se recomiendan altas cantidades de fertilizantes fosfóricos, causando desbalances nutricionales, incrementos en los costos y contaminación. El uso de hongos arbusculares formadores de micorrizas (HFM) puede promover el crecimiento y la absorción de P del café, reduciendo las cantidades de fertilizantes fosfóricos. Se condujo un experimento en condiciones de invernadero, en un sustrato compuesto por un suelo Paleudult mezclado con arena cuarcítica y con bajo nivel de P soluble (1 mg kg^{-1}), con el objetivo de evaluar el efecto del HFM *Rhizoglomus fasciculatum* en el crecimiento y la absorción de P en plántulas de café (*Coffea arabica* L. cv. Colombia) a tres niveles de P en solución del suelo (0.002, 0.02 y 0.2 mg L^{-1}). La colonización de HFM se redujo significativamente con los incrementos en P del suelo. La masa seca total de las plantas y la absorción de P se incrementaron con el HFM a niveles de 0.02 mg L^{-1} de P en solución, fueron bajos a 0.2 mg L^{-1} de P y nulos a 0.002 mg L^{-1} de P. Los resultados mostraron que la inoculación con HFM juega un importante papel en el crecimiento de las plántulas de café, siempre y cuando el P de la solución del suelo se mantenga en un nivel intermedio. A niveles de P en solución muy bajos, la respuesta al HFM fue inefectiva, mientras que, a valores muy altos, la aplicación de HFM fue innecesaria para el crecimiento del café.

Palabras clave: *Coffea arabica*, *Rhizoglomus fasciculatum,* colonización micorrizal, masa seca aérea, análisis de suelo.

Introduction

Coffee represents one of the most important agricultural products of Colombia (Barjolle *et al.,* 2017). Currently, Colombia has 844,700 ha planted with coffee, 84% corresponding to Colombia, Castillo®, Cenicafé 1® and Tabi®, cultivars developed by Cenicafe, which are resistant to the coffee leaf rust disease (FNC, 2021). These coffee cultivars are highly productive and have an outstanding beverage quality (Echeverry-Giraldo *et al*., 2020). The major concern for coffee growers is to maintain high production levels at a low cost; coffee contributes significantly to the national productivity and gross domestic product and supports approximately 540,000 families, mostly small farm-holders (CCGF, 2020).

Coffee plants have a high demand for phosphorus (P) during nursery stage and vegetative growth. These requirements are commonly satisfied by a high dose of soluble P fertilizers (Ávila *et al*., 2007; Sadeghian & González, 2012), which increase production costs and increase environmental concerns (Ni & Wang, 2015; Tian *et al*., 2017).

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A solution to this problem is the biotechnological use of arbuscular mycorrhizal fungi (AMF), which can enhance plant P uptake (Cardoso *et al.,* 2017) and growth of coffee seedlings (Rivillas & Dodd, 1996; Osorio & Habte, 2014), due to its capability to explore higher soil volume through extra-radical hyphae (Andrade *et al*., 2009). These effects are also positive under drought conditions, where AMF colonization efficiently makes use of water conditioning the plant stomatal opening and the leaf turgor (Augé, 2004).

AMF has an important role in organic/biological coffee system production where the use of chemical synthesis P fertilizers is restricted (Chiputwa *et al.,* 2015; Sepúlveda *et al*., 2016). This bio-technology is environmentally friendly (Cardoso & Kuyper, 2006), cost-effective and contributes to reducing P fertilization (Jaramillo & Osorio, 2009; Rai *et al*., 2013), considering that the demand of phosphoric fertilizers has exceeded the supply and the prices have globally been increased (Williams, 2021), demonstrating, in a short time, high sensitivity on market (Alewell *et al*., 2020).

Several studies have been published about AMF colonization in different *Coffea arabica* varieties (Bolaños *et al*., 2000; Lebrón *et al*., 2012; Sewnet & Tuju, 2013; Franca *et al*., 2014; De Beenhouwer *et al*., 2015). In respect to the response of coffee plant growth during the nursery state to AMF applications, Orozco (1988) evaluated *Gigaspora margarita* (GM), *Entrophospora colombiana* (EC), *Glomus manihotis* (GMH), AMF native (N), and GM-EC-GMH-N combination, in a sterile soil (P-Bray II level: 151 mg kg^{-1}) and found best results with GM and combining GM-EC-GMH-N. Recently, Sadeghian and Ospina (2021), working with an AMF containing 50 spores/g inoculum according to technical sheet of product and composed by *Glomus sp., Entrophospora* sp. and *Scutellospora* sp. (20 g/plant) at two levels of P (1 and 2 g P_2O_5/plant), did not find an additive effect of AMF. Hernández-Acosta *et al.* (2020) carried out an experiment in coffee seedlings cultivars Garnica, Catimor, Caturra and Catuaí growing in a sterilized substrate (P-Bray II level: 33 mg kg^{-1}) and inoculated with different AMF (10 g/plant), with its quality previously verified (colonization between 57 and 65%). Plant height and dry biomass were positively affected by the joint application of *Glomus claroides, Rhizophagus diaphanus*, and *Paraglomus albidum*. Changes in the leaf P content varied among varieties, achieving highest levels when the AMF were applied in consortium (Hernández-Acosta *et al*., 2020).

Based on the last consideration, despite promising effects reported by using AMF, the response of coffee during the nursery stage to its application has been uncertain, and in some cases, inconsistent, due to variations in the experimental conditions, soil fertility status (sterile o non-sterile substrate, P level), species, formulations of AMF (single species application o joint application of various AMF species), dose per plant, as well as quality of AMF. In addition, few studies carried out verifications of AMF colonization at the end of the experimental goal to corroborate the real effect of AMF on plant growth under soil conditions.

Although more than 100 species of AMF establish association with coffee plants (Hernández-Acosta *et al*., 2021), in Colombia, there are few options based on AMF formulations as related to their quality for use in soil-coffee systems and to variability in soil fertility, particularly P availability in different soils.

Taking in account these issues, the hypothesis of this study was: plant P uptake and growth promotion of coffee seedlings due AMF inoculation depend on the soil P availability. Thus, our aim was to evaluate the response of coffee seedlings of *C. arabica* cv. Colombia to the inoculation of the AMF *Rhizoglomus fasciculatum* under different levels of P in the soil solution.

Materials and methods

The study was conducted under greenhouse conditions in the Universidad Nacional de Colombia in Medellín (6°15´ N, 75°35´ W, 1495 m a.s.l.). A sub-superficial (30-50 cm) soil sample from a Paleudult - Bt horizon (P-Bray II: 1 mg kg^{-1}) was air-dried, passed through a 4 mm sieve, mixed with quartz sand (soil:quartz sand ratio w/w of 2:1), and autoclaved twice at 120°C and 0.1 MPa for 1 h. Based on a soil test, the following fertilizers were applied to 1 kg of the soil-sand mixture: 2 g of calcium carbonate, 436 mg of ammonium sulfate, 1550 mg of calcium sulfate, 980 mg of magnesium sulfate, 5 mg of Fe-EDTA, 5 mg of Cu-EDTA, 5 mg Zn-EDTA, and 5 g Borax.

A soil P adsorption isotherm was conducted following the procedure proposed by Fox and Kamprath (1970) to determine the P requirement and to achieve three levels of P in soil solution: 0.002, 0.02, and 0.2 mg L^{-1}. Accordingly, KH_2PO_4 was applied at three doses: 0.950, and 2,800 mg kg^{-1}, respectively, and mixed thoroughly. To balance the level of potassium added, potassium sulfate was applied to the first two levels (1,533 and 1,066 mg kg^{-1}). The substrate was transferred into black plastic bags of 17x23 cm with capacity of 1.8 kg per bag, dry basis, the recommended conditions to grow coffee seedlings at the nursery stage. Then, the substrate was left uninoculated (control) or inoculated with 50 g of a crude mycorrhiza containing 250 spores of

R. fasciculatum per g and then mixed throughout. The mycorrhizal inoculum was previously multiplied in maize roots under controlled conditions in the Soil Microbiology Laboratory of the Universidad Nacional de Colombia (Habte & Osorio, 2001). The uninoculated bags received 20 ml of a 10% mycorrhizal suspension, which was previously filtered through a No. 42 Whatman filter paper.

Seeds of coffee *C. Arabica,* cv. Colombia, were germinated in sterile sand and grown for 60 d and then transplanted into the P-amended and inoculated/uninoculated substrate. A thin layer of fine quartz sand was applied on the surface of each pot to prevent cross contamination. The seedlings grew for 150 d under sunlight exposure. The substrate was watered with distilled water to maintain it at 50-60% of the maximum water holding capacity. To prevent plant nutrient deficiencies, 50 cm^3 of P-free Hoagland solution were supplied to each bag once a week.

The experimental design was completely randomized. Treatments had a factorial arrangement 2x3, two levels of mycorrhizal inoculation (inoculated and uninoculated) and three levels of soil solution P (0.002, 0.02, and 0.2 mg L^{-1}). Each treatment had five replicates. The foliar P content was monitored at 30, 50, 70, 92, 110, 130, and 150 d after transplanting; for this purpose, a leaf-disk of 6 mm in diameter was collected from the newest fully extended mature leaf of each plant, then oven-dried, weighed, and ashed in a muffle furnace (500°C, 3 h) (Aziz & Habte, 1987). The ashes were dissolved in 10 ml of distilled water and the P concentration was measured by the molybdenum blue method (Murphy & Riley, 1962). At the time of harvesting at 150th d, the mycorrhizal colonization was determined in fine root fragments, which were washed with water, cleared by immersion in 10% KOH for 24 h and then acidified with 10% HCl for 5 min. After that, the root samples were stained with 0.15% of fuchsine acid (Kormanik *et al*., 1980). The extent of root colonization was determined by the grid-line intersection method (Giovannetti & Mosse, 1980). The shoot dry weight (SDW) was determined after oven-dry the samples at 60°C for 96 h.

The data were subjected to ANOVA tests and to the Duncan multiple range test for mean separation. In both cases, a level of significance $P \leq 0.05$ was used. Both tests were performed with the statistical software Statgraphics.

Results and discussion

The variables under study were affected by the treatments. For instance, mycorrhizal colonization was only detected in those plants grown in the inoculated substrate, regardless of the soil solution P level (Fig. 1). Mycorrhizal colonization did not differ between 0.002 mg L^{-1} and 0.02 mg L^{-1} P levels (54% and 49%, respectively), but these two were statistically different ($P<0.05$) from that at 0.2 mg L^{-1} P level, which had the lowest value (9%). These results indicate that, in young coffee seedlings, mycorrhizal colonization depended on the P level in the soil solution.

The AMF colonization was between 1.4x and 3.6x the results in Hernández-Acosta *et al*. (2020) in *C. arabica* varieties Catuaí and Garnica using 10 g/plant of AMF in consortium and between 0.5x and 1.5x the results in Cuervo (2017) in *C. arabica* cultivars Tabi® and Castillo® planted in an Andisol (Bw horizon) and inoculated with 50 g/plant of *R. fasciculatum* containing 500 spores/g. Orozco (1988) reported AMF concentration around 60% in *C. arabica,* cv. Colombia. Differences among the results could be explained by variations between AMF species and formulations, such as the dose of inoculum and the concentration of spores, although these effects are likely controlled by the AMF or coffee specie (Jaitieng *et al*., 2021).

Phosphorus concentration in soil is a key factor to explain AMF colonization; while Hernández-Acosta *et al*. (2020) and Cuervo (2017) worked under P concentration in soil corresponding to 33.0 and 2.0 mg kg^{-1}, respectively, soil P concentration in our study was 1.0 mg kg^{-1}. According to Moreira *et al*. (2019), AMF colonization decreased significantly (around 50%) as P applications increased up to 0.74 g kg^{-1} of soil in coffee Catuaí Vermelho IAC 99 growing in substrate without sterilization. These findings demonstrate that AMF symbiosis is activated as the defense of plants against combined stress conditions as documented by Rashad *et al*. (2021).

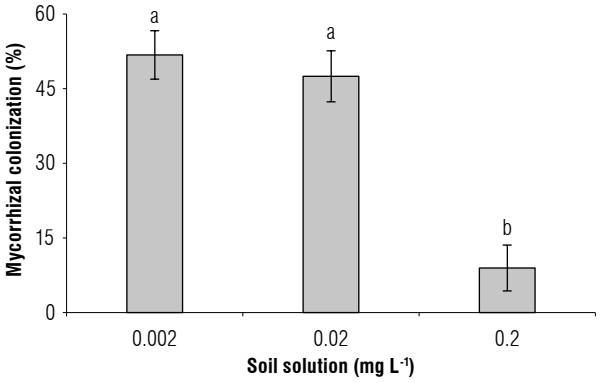

FIGURE 1. Mycorrhizal colonization in roots of *C. arabica* cv. Colombia, in a substrate inoculated with the mycorrhizal fungus *R. fasciculatum* at three levels of P in solution. Columns a are significantly different from b ($P<0.05$) according to the Duncan multiple range test. Bars represent the standard errors.

The shoot dry weight (SDW) was significantly affected by the soil solution P concentration, the AMF inoculation, and the interaction between both factors. At the 0.002 mg L^{-1} level of P, the AMF inoculation did not affect the SDW, which fluctuated between 0.39 and 0.52 g/plant (Fig. 2). By contrast, at the 0.02 mg L^{-1} level of P, the inoculation with *R. fasciculatum* significantly increased the SDW by 3.05x respect to the uninoculated control at 1.03 g/plant on average. Similarly, at 0.2 mg L^{-1} level, the SDW was significantly affected by the inoculation with *R. fasciculatum* (3.13 g/plant), while the uninoculated control was 2.07 g/plant.

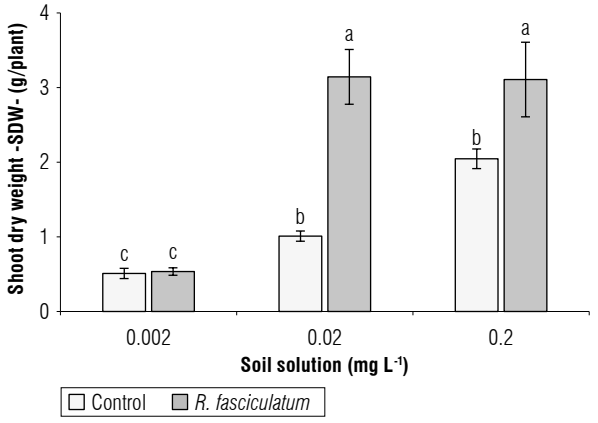

FIGURE 2. Shoot dry weight (SDW) of coffee cv. Colombia seedlings grown in a substrate either uninoculated (control) or inoculated with *R. fasciculatum* at three levels of P in solution. Different lowercase letters indicate significant differences according to the Duncan multiple test ($P<0.05$). Bars represent the standard errors.

The foliar P concentration was significantly affected by the interaction soil P level x AMF inoculation only at day 150. At the 0.002 mg L^{-1} level, the AMF inoculation did not increase the foliar P content at any time of evaluation; the values ranged from 0.07 to 0.15%. By contrast, at 0.02 mg L^{-1} the inoculation with *R. fasciculatum* significantly increased the foliar P concentration from 110 d after transplanting; the uninoculated plants had values between 0.10 and 0.13%, whereas the inoculated plants had between 0.17 to 0.19%, respectively. On the other hand, at 0.2 mg L^{-1} of P the AMF inoculation did not significantly affect the foliar P concentration (Figs. 3A-C).

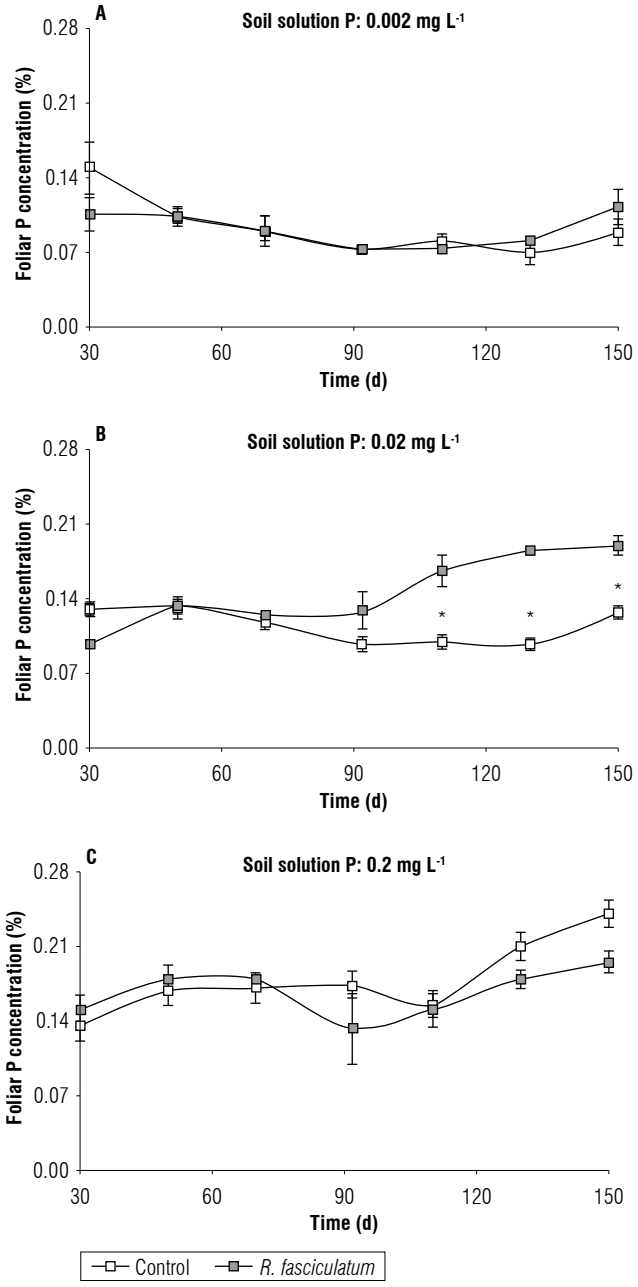

FIGURE 3. Foliar P concentration (%) in *C. arabica* cv. Colombia seedlings grown in a substrate either uninoculated or inoculated with *R. fasciculatum* and three levels of P in solution. The asterisks indicate significant ($P<0.05$) differences at the respective time between the respective means. Bars represent standard errors.

In addition, the P foliar content (µg/leaf disc) showed a similar behavior to the P concentration (%). At the 0.002 mg L^{-1} level, the AMF did not promote increase of foliar P content at any time, the values ranged from 0.93 to 1.19 µg/leaf disc. By contrast, at the 0.02 mg L^{-1}, the inoculation with *R. fasciculatum* significantly increased this nutrient in the leaf tissues, particularly after 110 d of growth. For example, uninoculated plants had values among 1.11, 1.08, and 1.22 µg/leaf disc at 110, 130, and 150 d, respectively, while the inoculated plants had 2.44, 2.38, and, 2.34 µg/leaf disc at the same sampling days. These results represent increases of foliar P content of 119%, 120%, and 92%, respectively (Figs. 4A-C).

The results suggest that the use of *R. fasciculatum* is adequate to promote plant growth and P uptake for coffee

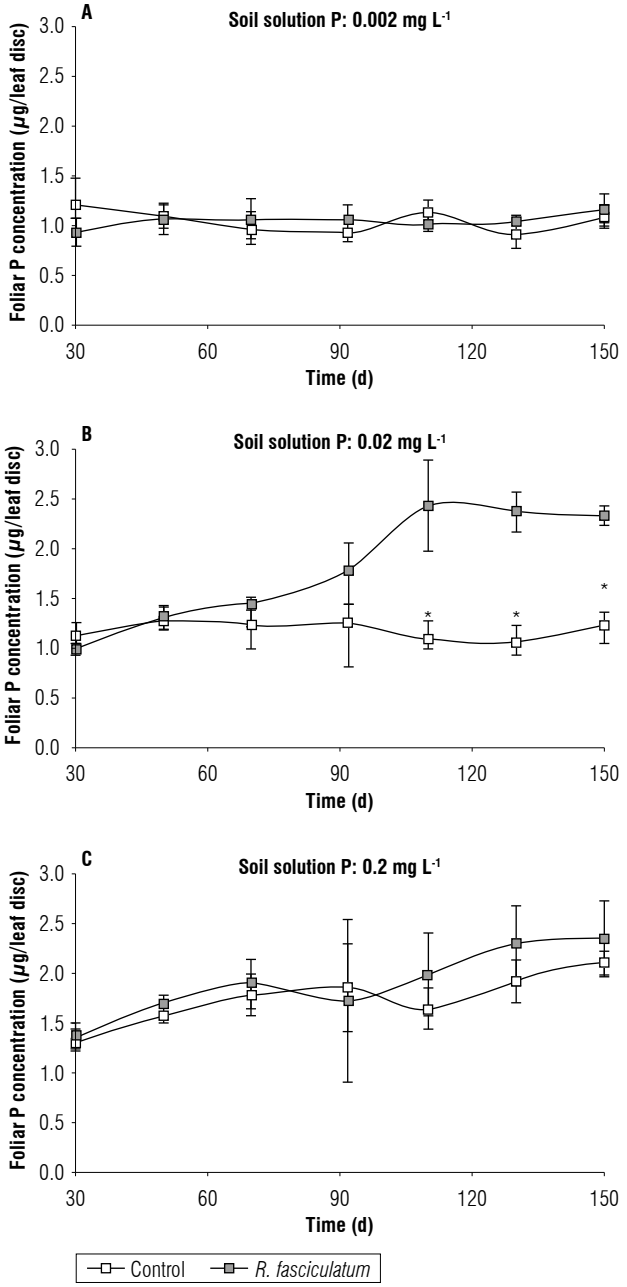

FIGURE 4. Foliar P content (μg/leaf disc) of coffee C. arabica cv. Colombia seedlings grown in a soil either uninoculated (control) or inoculated with the AMF *R. fasciculatum* at three levels of soil solution P. The asterisks indicate significant ($P<0.05$) difference at the respective time between the respective means. Bars represent standard errors.

seedlings cv. Colombia. The results agree with those of Jaramillo and Osorio (2009), which studied the mycorrhizal and effectiveness dependency of coffee seedlings of *C. arabica* cv. Colombia and Caturra at the same soil P levels used in this study. Cuervo (2017), working with a sterile Bw horizon (Andisol) and with the same AMF and soil P levels, found for the coffee cultivars Castillo® Naranjal, Castillo® Tambo, and Tabi® comparable results in SDW and foliar P uptake. In that case, the SDW increase with AMF inoculation was 10 times higher in respect to the uninoculated plants. Earlier works of Habte and Biitenbender (1999) in an Oxisol soil from Hawaii with coffee seedlings variety Typica showed positive response in plant growth when *Glomus aggregatum* was used.

The lack of response at the lowest level of soluble P is because this concentration is too low for uptake by both plant and AMF. In other words, the low soil P availability was a limiting factor to the mycorrhizal dependency (Osorio & Habte, 2014). Since the plant cannot grow properly under such condition, it cannot share carbon with the mycorrhizal fungus. Mycorrhizal plants invest nearly 20 to 30% of the fixed photosynthate carbon compounds in order to satisfy the nutritional requirements of the fungus (Eskandari *et al*., 2017), and specifically in coffee plants increasing photosynthetic rate (Cruz *et al*., 2020). This carbon allocates to the extra radical fungal hyphae, which operate as an extension of the plant root system (Lebrón *et al*., 2012) and acquire P from surrounding soil areas beyond the plant roots zone (López-Arredondo *et al*., 2014).

An opposite scenario occurred at the highest soil P availability level, where the AMF inoculation seems to be unnecessary, because there is enough P for plant growth and the magnitude of the response is lower (Harrison, 1999). In fact, several authors showed that AMF inoculation at that high level of soil P can produce negative effects due to an imbalance associated to an expensive metabolic cost required to support a micro-symbiont system with carbon, which does not improve the plant performance (Roth & Paszkowski, 2017; Wang *et al*., 2017). For instance, Jaramillo and Osorio (2009) indicated negative effects in coffee seedlings cultivars Caturra and Colombia using *Glomus fistulosum* inoculum when the soil P availability was 0.2 mg L^{-1}.

In order to contextualize the P values in the soil solution, González (2018) found that many soils in the Colombian coffee region according to the magnitude of P fixation in soils present values between $P_{0.1}$ and $P_{0.2}$ in soil solution, corresponding to P-Bray II levels between 10 and 30 mg kg^{-1}.

As shown by Sadeghian and Ospina (2021), coffee plants require between 1.0 and 2.0 g of P_2O_5/plant to satisfy the requirements during the nursery stage. This amount generates, according to the soil order, P levels in the soil from 200 to 490 mg kg^{-1} P-Bray II. In consequence, high doses of fertilizers generate soil salinity, with a negative impact

on the AMF colonization (Rashad *et al*., 2021) and, hence, a lower effect on plant growth.

It is also likely that at 0.2 mg L^{-1} the availability of other essential nutrients (*e.g*., Zn) can decrease (Bhattacharya & Bagyaraj, 2002; Ozdemir *et al.,* 2010; Sewnet & Tuju, 2013; Zhang *et al.,* 2017), thus, affecting the functioning, formation, and multiplication of AM fungi in the rhizosphere (Dutt *et al*., 2013; Sewnet & Tuju, 2014).

In summary, this study clearly demonstrated a positive effect of AMF *R. fasciculatum* inoculation on SDW and foliar phosphorus (P) content of coffee seedlings only if the P level in the soil solution is 0.02 mg L^{-1}. At 0.002 mg L^{-1} of P, the AMF inoculation proceeds only if P fertilizers were added to reach an optimal P level for the mycorrhizal association. On the other hand, if the P level in the soil solution is 0.02 mg L^{-1}, the AMF inoculation can be used without any P fertilization. By contrast, when P concentration in soil solution is as high as 0.2 mg L^{-1}, the AMF inoculation seems to be unnecessary.

Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

WO conceived the idea, designed the research, and was involved in planning and supervising the work. HG and WO performed statistical analysis of all data. HG, CEG and WO wrote the manuscript. SPJ conducted the laboratory experiments. HG, WO and CEG provided critical feedback, helped shape the research, and read and approved the manuscript.

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Control of $N\text{-}NH_4^+$ and K^+ leaching in potato using a carrageenan hydrogel

Control de la lixiviación de $N\text{-}NH_4^+$ y K^+ en papa mediante un hidrogel de carragenina

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ABSTRACT

Potato cultivation requires fertilizers to sustain crop yields, but a significant percentage of added nutrients is lost by leaching. The use of coating materials for fertilizers is currently being considered to reduce these losses. The objective of this study was to determine if a carrageenin based hydrogel (CBH), used to coat fertilizer, decreases NH_4^+ and K^+ leaching from a potato crop without affecting growth, specific gravity, and tuber yield. The CBH was tested in a diploid potato crop, cultivar Criolla Colombia (*Solanum tuberosum* L., Phureja Group) using a randomized full block design including the treatments noncoated fertilizer (T1), CBH coated fertilizer (T2), and no fertilizer (T3). Mineral nutrients in soil leachates together with dry biomass, foliar area, chlorophyll, tuber specific gravity, and yield were quantified. The nutrient content in leachates from T2 were below those from T1. No significant differences between treatments were observed for growth factors, yield, and tuber specific gravity. This study confirms the controlling effect of the CBH, ensuring the retention of the nutrients added in the fertilizer and preventing them from easily leaching. Future field studies are worthwhile to establish the amount of fertilizer this coating could save.

Key words: controlled release fertilizers, soil pollution, coating, environmental protection.

RESUMEN

El cultivo de papa requiere fertilizantes para mantener su rendimiento. Sin embargo, un porcentaje significativo de los nutrientes añadidos se lixivia. Actualmente el uso de materiales de recubrimiento para los fertilizantes se está considerando para disminuir estas pérdidas. El objetivo de este estudio fue determinar si un hidrogel a base de carragenina (CBH, por sus siglas en inglés), utilizado para recubrir el fertilizante aplicado, puede disminuir la lixiviación de NH_4^+ y K^+ en un cultivo de papa, sin afectar el crecimiento, gravedad específica y el rendimiento de tubérculo. El CBH se probó en un cultivo de papa diploide, cultivar Criolla Colombia (*Solanum tuberosum* L., Grupo Phureja) utilizando un diseño de bloques completos al azar. Se evaluaron los tratamientos: fertilizante sin recubrimiento (T1), fertilizante recubierto con CBH (T2) y sin fertilizante (T3). Se cuantificaron los nutrientes minerales en los lixiviados junto con biomasa seca, área foliar, clorofila, gravedad específica del tubérculo y rendimiento. Los contenidos de nutrientes en los lixiviados de T2 fueron inferiores a los de T1, y no se observaron diferencias entre estos tratamientos para factores de crecimiento, rendimiento y gravedad específica. Los resultados evidencian que el CBH tiene potencial como material de recubrimiento para fertilizantes en papa y, se debe complementar con otros ensayos para determinar la cantidad de fertilizante que este recubrimiento podría ahorrar.

Palabras clave: fertilizante de acción controlada, polución del suelo, revestimiento, protección del medio ambiente.

Introduction

The potato has been recognized as a key product for providing food security for the growing human population, particularly in developing countries (Devaux *et al*., 2020). This food crop stands out among others because it is an accessible source of nutrients (Wijesinha-Bettoni & Mouillé, 2019) that grows in a wide range of environmental conditions (International Potato Center, 2017), and its commercial value is resilient to price volatility at the global level due to its local production and distribution (Campos & Ortiz, 2019). Therefore, potato crops are expected to strengthen a sustainable capacity to supply sufficient human nutrition. Nevertheless, there are important challenges that must be overcome to ensure environmental sustainability. One of the biggest issues in potato production is to optimize the use of fertilizers to minimize the negative impacts of current fertilization practices on the environment (Tilman, 1999). Potato is a species with high nutritional demand per kg of produced dry mass. A commercial crop of diploid

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potato extracts, in a productive cycle, 124 kg ha^{-1} of nitrogen (N), 25.4 kg ha^{-1} of phosphorus (P) and 258.2 kg ha^{-1} of potassium (K) (Suarez &Torres, 2014). The demand for nutrients implies high fertilization doses to sustain high crop yields (Rajiv & Kawar, 2016). Consequently, potatoes are one of the crops with the highest application doses using 243 kg ha^{-1} of fertilizers (FAO, 2006) for a production of 370 million t over more than 17 million ha around the world (FAOSTAT, 2019). Locally in Colombia, applications doses in commercial diploid potato are around 833.3 kg ha^{-1}, and the nutrient doses applied of added fertilizers are around to 125 kg ha^{-1} N, 54.6 kg ha^{-1} P, and 104 kg ha^{-1} K (Alvarado & Ramírez, 2018), exceeding those reported by FAO (2006).

Despite the increase in yields per unit area achieved with the application of chemical fertilizers (FAO, 1981), only 30-35% of the N, 18-20% of the P and 35-40% of K present in the chemical mixtures are actually absorbed and used by the plants in agricultural crops (Subramanian *et al*., 2015), meaning that more than half of the applied fertilizers are quickly lost into the environment through runoff, leaching, and/or volatilization (Huang *et al*., 2017). The non-absorbed N and P are the main drivers of serious environmental problems such as water pollution, eutrophication-caused reductions in biodiversity (Whitters *et al*., 2014; Diatta *et al*., 2020), global warming (Bouwnman *et al*., 2002), and reductions in the ozone layer (Molina-Herrera *et al*., 2016). Unless there is a rise in fertilizer efficiency, a significant increase in NPK fertilizer application is expected by 2050 due to the increasing demand for food (Drescher *et al*., 2011) triggering a greater negative impact on the environment.

One of the strategies for facing this issue is to optimize the use of fertilizers in potato crops by implementing controlled release fertilizers. This technology maintains constant slow rates of nutrient release into the soil allowing synchronization between the onset of nutrient uptake by the plants and availability of nutrients (Naz & Sulaiman, 2016). Meanwhile, this technology reduces leaching by rain or irrigation water, mitigating eutrophication and the release of greenhouse gases into the atmosphere (Cong *et al*., 2010). To achieve controlled release, fertilizers are encapsulated in mineral and organic polymers known as coating agents (Azeem *et al*., 2014; Ali & Danafar, 2015; Guilherme *et al*., 2015). However, the materials used in the coating agents are often non-biodegradable, costly, toxic, and inconsistent in their release patterns and rates (Azeem *et al*., 2014; Naz *et al*., 2016). Recently, a carrageenin based hydrogel (CBH) has been proposed as a new encapsulating agent for fertilizer granules. This has extra advantages: its main component is carrageenan, a sulfated polysaccharide from the wall of red algae *Hypnea musciformis* (Wulfen) J.V. Lamouroux (Rozo *et al*., 2019); the alga is abundant in the coasts and widely distributed throughout the tropics and warm temperate seas in the eastern and western Atlantic. This alga is found in Southeast Asia (Ang *et al*., 2014), the Philippines (Lastimoso & Santiañez, 2021), Asia (Titlyanova *et al*., 2016), the southern China (Phang *et al*., 2016), and the Caribbean (Camacho & Montaña-Fernández, 2012). In addition, this natural hydrogel is relatively simple in structure and chemical composition, porous, semi-permeable, easy and inexpensive to synthetize, biodegradable and non-toxic (Blakemore, 2016; Hilliou, 2021; Guo *et al*., 2022). Fertilizers coated with urea and acrylamides have existed since the 1960s. However, since 1996 alginate and chitosan biopolymers were developed with a different synthesis technology than the one used for the CBH (Fertahi *et al*., 2021). Chitosan has shown good results in corn (Kumaraswamy *et al*., 2021). The use of kappa carrageenan as a coating material is still very new and there are still no studies evaluating this material as a coating in the field.

The CBH is a natural material that seems to have high potential as a coating to optimize fertilizers, ensuring the same yields while minimizing negative impacts in the environment; however, its efficiency at the field scale remains unexplored. Although it is known that the charges in its structure may have a natural potential to retain cations, it has not yet been explored whether, once the ions are retained, they are released from the hydrogel and absorbed by the plant. Knowledge of the integrity of the gel under the environmental conditions of the crop is absent but it is known that the encapsulating fertilizers with the CBH significantly reduced the $N\text{-}NH_4^+$ and K^+ in leachates from laboratory soil column experiments carried out with a soil from an Andean potato crop (Santamaría *et al*., 2019). The CBH did not have a major impact on the P leaching because in these soils this element is not leached. In general, potato crops in Colombia are in soils with high iron and aluminium content and a pH ranging between 4.5 and 6.0 (FAO, 2019), inducing the P in the fertilizer to absorb by the Fe/Al oxides (Hanyabui *et al*., 2020); therefore, P leaching is low. Furthermore, CBH encapsulation did not have a negative impact on the growth and quality of potato (*Solanum tuberosum* L., Phureja Group, cv. Criolla Colombia), since the encapsulated fertilizer was as effective as the non-encapsulated in green house experiments with plants cultivated in pots with soil (Santamaría *et al*., 2019). These results look promising; however, in order to propose the CBH as an environmentally friendly alternative to be implemented in potato crops, an evaluation of its efficacy in field experiments is necessary. The objective

of this study was to test whether the CBH coating around granulated fertilizer could reduce NH_4^+ and K^+ leaching without impacting plant growth, specific gravity, and yield of a commercial potato crop.

Materials and methods

Study area

This study was conducted in a *Solanum tuberosu*m L. crop at the San Isidro farm in the municipality of Sibaté (4°25'42"N; 74°17'58.4"W), located at 2720 m a.s.l. (Cundinamarca, Colombia) that supplies local markets of the Cundinamarca region. The production cycle took place between August and December of 2018, a period with an average temperature of 12.7°C and a maximum/minimum of 15.3/7.1°C. Collected soil samples were analyzed at the CIAT analytical laboratory for the physicochemical properties of the top (0 to 15 cm) soil layer (Tab. 1). Based on the levels of nutrient availability, the soil had a deficit in the amount of assimilable nitrogen (16.8 kg ha^{-1}), since 1 ha of the cv. Criolla Colombia potato extracts on average 124 kg of nitrogen (Suarez &Torres, 2014). Both phosphorus and potassium are present in optimal concentrations with respect to the concentration requirements of the crop (Suarez & Torres, 2014).

TABLE 1. Main soil properties at the San Isidro farm.

Property	Method	Value
pH	1:1 Soil water	4.92
Organic carbon	Walkley-Black	25.40%
Total N	Acid digestates-(sulfuric-salicylic)	5094 mg kg^{-1}
N-NH_4^+	1 M KCl-extraction	9.7 mg kg^{-1}
Extractable P	Bray II	31.6 mg kg^{-1}
Extractable K	Bray II	0.252 cmol kg^{-1}
Ca	1 M KCl-extraction	5.16 cmol kg^{-1}
Mg	1 M KCl-extraction	0.44 cmol kg^{-1}
Al	1 M KCl-extraction	1.245 cmol kg^{-1}
Fe	Dilute double acid extraction: 1 M HCl and 5 M H_2SO_4	6,62 cmol kg^{-1}
CECe	Sum of interchangeable bases plus Al	7.10 cmol kg^{-1}

Data source: soil samples collected at the study site and analyzed at CIAT analytical laboratory.

Field experiment

Experimental design

Three treatments were compared in a randomized block design with three replicates for a total of nine experimental plots of 15 m^2 each. Each plot was planted with two rows spaced 1 m apart and 25 plants per row with 0.3 m between plants in a row for a population of 33,333 plants ha^{-1}. The treatment of the plot T1 was fertilized with the nonencapsulated fertilizer and the treatment plots T2 were fertilized with the CBH hydrogel encapsulated fertilizer. A control treatment T3 was established with no fertilizer added to the plots. We used cv. Criolla Colombia (*S. tuberosum* L., Phureja Group), because this is the most cultivated diploid variety in Cundinamarca, with 120 d of cultivation cycle at 2600 m a.s.l (Ñústez & Rodríguez, 2020) and because of its excellent nutritional attributes (Thomas *et al*., 2021) and high required fertilizer applications (Alvarado & Ramírez, 2018), demand the design of innovative management strategies to reduce environmental impact. Also, the Phureja group is diploid and displays superior performance of agronomic traits, unlike *Solanum tuberosum* L. that is autotetraploid (Tai & Xiong, 2003) and has restrictions in the advancement of genetic improvement (Camadro & Mendiburu, 1988).

Fertilization treatments

Field preparation and management practices followed those used at the San Isidro farm. The land was dredge plowed and the furrows were constructed using a manual hoe. Fertilizer treatments with and without coating, consisted of 25 g of a granulated fertilizer (Vecol 15-15-15-11 with N 15%, P_20_5 15%, K_2O 15%, S 11% (Phosagro, Rusia) that contains only ammoniacal N) applied once per plant at the time of sowing. This amount corresponded to 833.3 kg ha^{-1}. In T2, the 25 g of fertilizer were distributed in 11 capsules of the CBH hydrogel. The capsules had a cylindrical shape of 15.4 cm^3 and contained 2.27 g of fertilizer (14% w/v). The encapsulated fertilizer was prepared as described in Santamaría *et al*. (2019). For both treatments, the 25 g of fertilizer were placed at 10 cm around the seed (Fig. 1). Finally, fertilizer and seed were covered with a layer of about 5 cm of soil. Selected seed tubers, about 5 cm in diameter, of the cv. Criolla Colombia were obtained from farmers of the region.

Rainfall was recorded daily using a conventional rain gauge. Precipitation registered a total of 481 mm during the production cycle. This precipitation corresponded to the second rainy peak at this locality. Supplemental irrigation was not performed.

Collected samples

Soil leachates

To collect leachates containing the nutrients not absorbed by the roots, three suction lysimeters were installed (SSAT

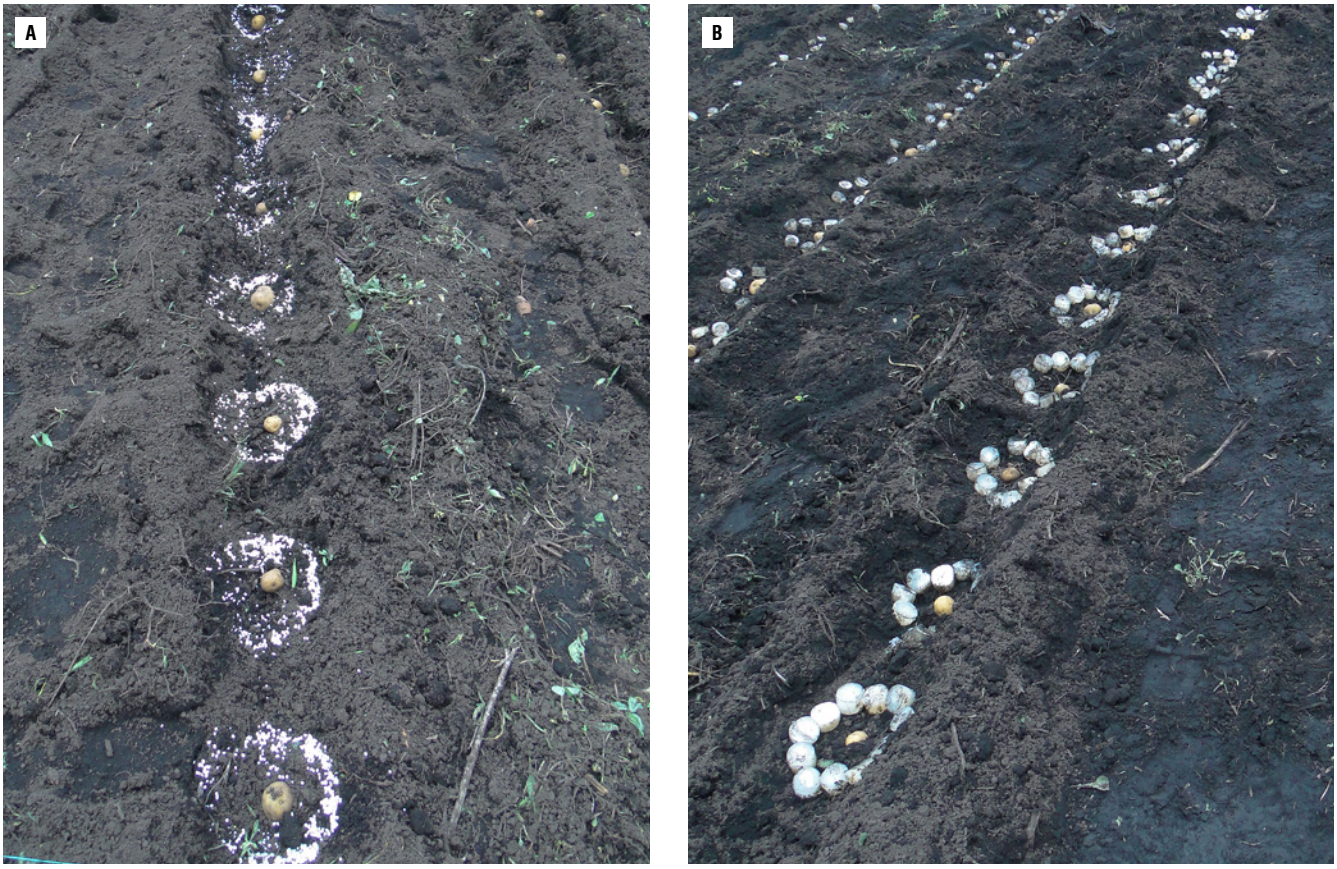

FIGURE 1. Fertilizer distribution around the potato seed. A) Treatment 1=noncoated fertilizer and B) treatment 2=CBH coated fertilizer.

Model, Irrometer Company, Inc., USA) the planting day in each experimental plot. Lysimeters were installed at 40 cm depth to ensure that the sampling of soil draining water not absorbed by the plants. At this depth, the leachates have moved beyond the reach of the roots that are typically 30 cm long in the cv. Criolla Colombia (C. Ñústez, personal communication, February 15, 2019). Lysimeters were placed next to the plants. Leachate volumes were sampled weekly up until 50 d after sowing (das). This time interval included the phenological growth stages of germination (BBCH 0), leaf development (1), formation of lateral shoots (2), longitudinal growth (BBCH 3), development of harvestable vegetative parts (BBCH 4), and appearance of floral organs (BBCH 5) based on the BBCH scale of phenological growth stages (Hack *et al*., 1993). Their content of NH_4^+ and K^+ was quantified in the Analytical Services Laboratory of the International Center for Tropical Agriculture (CIAT). The NH_4^+ was analyzed by the automated spectrophotometry method using a Skalar Sanplus Analyzer SA 3000/5000 Mode 5000-01 (Skalar Analytical B.V, Netherlands) and atomic absorption spectrophotometry (Unicam Solar 969 spectrophotometer, Unicam company, England) was used to quantify K^+.

Plant material samples and measured growth variables

To compare plant growth among the treatments, destructive samplings were conducted on three plants from each experimental plot fortnightly from 14 d after emergence (dae), until 8 d before harvest, which took place 126 d after planting. We quantified the total plant dry mass (PDM) (g), tuber dry mass (TDM) (g), foliar area (FA) (cm^2), and chlorophyll content (SPAD units). In all cases, the result was expressed as the average of the nine sampled plants.

The PDM was the sum of the dry weight of leaves, stems, flowers, and tubers on each plant. Dry weight of plant organs was determined after dehydration of the fresh samples in an oven at 70°C until they reached constant weight. Tuber dry mass content was determined by chopping all the tubers from the same plant and drying this material in an oven at 70°C until a constant weight was reached. Leaf area was quantified using a digital camera (Canon EOS-Rebel T3) and following the methodology of Campillo *et al*. (2008). The relative content of chlorophyll was measured with a portable chlorophyll meter (SPAD-502 plus, Konica Minolta, Inc., Japan) on the third or fourth fully expanding

leaf of the upper third in three plants per experimental plot. Three measurements per leaf were averaged.

At the time of harvest, we conducted destructive sampling to quantify tuber weight and size. Tubers from 30 different plants from each plot were classified according to the local commercial category bases on tuber diameters: > 4 cm, between 2 and 4 cm and < 2 cm. The tuber yield per plot was expressed in t ha^{-1}. The specific gravity, a commercial quality indicator, was determined for 10 tubers (> 4 cm diameter) randomly taken from each treatment after harvest, using both the weight in water and weight in air method (Bonierbale *et al*., 2010).

Data analysis

The assumptions of normality were verified by the Shapiro-Wilk and Kolmogorov-Smirnov tests. The homogeneity of variance was verified by the Levene test. If any variable did not meet the assumptions, a Box-Cox transformation was performed, and an analysis of variance was performed again.

Concentrations of N-NH_4^+ and K^+ in leachates did not meet the assumptions after transformation. Therefore, we tested if the treatment and the das explained the concentrations of N-NH_4^+ and K^+ in the leachates using generalized linear models (GLM, R core packages Stats version 3.6.2) followed by model selection. We conducted two independent models, one for each nutrient. The response variable was the concentration (mg L^{-1}), while the treatment and das were the fixed effects. We used a GLM from a Gaussian family, since a preliminary analysis, (Supplementary material 1 [S1]) showed our data deviated from the linear regression assumptions only slightly. To increase the robustness of our analysis, we used the corrected Akaike's information criterion (AICC) and dredge automated model selection tool (dredge, R package MuMIn; Bartón, 2020), instead of minimum squares to determine the significance of each predictor. We report the model averaged coefficients and 95% confidence intervals (considered significant if they did not overlap with 0) of the resulting models. Models with ΔAICC < 2 were not considered different, so the simpler model was preferred for that case. All statistical analyses were performed in R 3.6.3 (R Core Team, 2018).

Regarding plant growth and yield evaluation, a main effects analysis of variance (ANOVA) was applied to determine differences among treatments. The data from each sampling date were analyzed separately to determine the treatment effect on a phenological crop stage. Average values were compared by the Tukey's HSD post hoc analysis. These statistical analyzes were conducted with the software STATISTICA (version 7: StatSoft Inc., Tulsa, USA), with a significance level of α=0.05.

Results and discussion

Nutrient leaching

Overall, the greatest effect of the CBH on nutrient leaching occurred from the second week after sowing (Fig. 2 A, B). The highest leachate concentrations of N-NH_4^+ occurred in T1 (1.05±0.51 mg L^{-1}, mean ±standard deviation [SD]) at 15 das (Fig. 2A), a time at which the plants had not yet developed a root system to take the nutrients supplied through the fertilizer. The N-NH_4^+ in the leachates collected in T2 at 15 das (0.16±0.03 mg L^{-1}), was lower than in T1 and showed similar N-NH_4^+ values to those in leachates from the control treatment T3 (0.12±0.005 mg L^{-1}). Likewise, at 22 das the N-NH_4^+ in the leachates from T2 (0.12±0.01 mg L^{-1}) was lower than in T1 (0.423±0.145 mg L^{-1}), although the difference between T1 and T2 decreased. At 29 das, an important reduction in the N-NH_4^+ concentration was observed in leachates from T1 (0.28±0.13 mg L^{-1}) with closer but still higher values to those observed in T2 (0.13±0.01 mg L^{-1}) and T3 (0.10±0.002 mg L^{-1}). After 36 d, high N values were observed in the leachates from T1, but leachates from T2 and the control remained as in the previous sampling dates. Regarding the K^+ ion (Fig. 2B), its concentration in the leachates from T2 (4.18±0.75 mg L^{-1}) was lower than in T1 (6.39±1.09 mg L^{-1}) from 15 d.

In previous studies, the mathematical models that follow the ions leaving the gel as a function of time describe a gradual release (Rozo *et al*., 2019). Results for AIC model selection criteria (S2) showed that only the variable treatment had an effect on the concentration of N-NH_4^+ and K^+ in the soil. Results of the linear models are shown in Table 2. The estimate for T1 was different from the control, indicating that the application of uncoated fertilizer increased the ammonia and potassium ions in leachates. Conversely, T2 was no different from the control for any of the two nutrients, indicating that the coating reduced nutrient leaching to the same level as the control, reducing the environmental risk of using fertilizers.

It is important to highlight the large variation for N-NH_4^+ concentration in the leachates from plots under T1 (Fig. 2A). This may be the result of non-homogeneous fertilizer distribution in the crop soil plus the lateral flow of the water produced by the soil heterogeneity and the land slope that together may change the expected downward vertical transport of the infiltration water towards horizontal

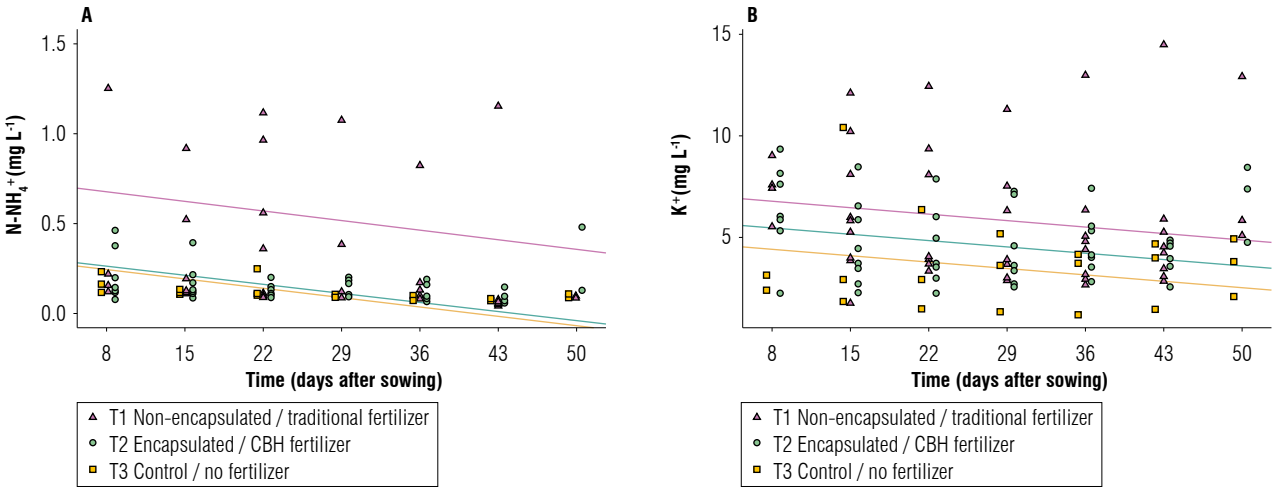

FIGURE 2. Nutrient concentration in soil leachates over time. The first seven weeks of the crop cycle in the San Isidro Farm are shown. A) Generalized linear models-GLM for $N\text{-}NH_4^+$ and B). generalized linear models-GLM for K^+. Number of analyzed leachate samples per treatment 1 and 2 in each sampling date=9.

TABLE 2. Results of the generalized linear models for the concentrations of the $N\text{-}NH_4^+$ and K^+.

	R^2 (%)	Parameter	Estimate	95% C.I.
$N\text{-}NH_4^+$ (mg L^{-1})	10.15	Intercept	0.305	
		Time	-0.007	-0.0148; 0.0017
		T1 (Uncoated fertilizer)	0.384	**0.0758; 0.6992**
		T2 (Coated fertilizer)	0.015	-0.2874; 0.3179
K^+ (mg L^{-1})	12.38	Intercept	4.169	
		Time	-0.019	-0.0556; 0.0172
		T1 (Uncoated fertilizer)	2.469	**1.1169; 3.8220**
		T2 (Coated fertilizer)	1.151	-0.1946; 2.4971

The estimates for each parameter are shown. The intercept corresponds to ion concentration in the control (no fertilizer) at the beginning of the experiment (d=0). The parameter "Time" indicates the change in concentration according to the d, which is constant for all treatments (no interactions). The percentage of variance explained is shown as the R^2 in %. The effect of each treatment (coated or uncoated) was determined as the difference between their intercept and the control given by a 95% confidence interval (C.I.) around the estimate. The intervals that indicate a significant difference from the control (those that do not overlap with 0) are highlighted in bold.

transport of the water (Hardie *et al*., 2012; Kim & Mohanty, 2016). Ideally, diffused nutrients from the fertilizer placed around the tuber seed would be expected to be mobilized primarily vertically into deeper layers of the soil profile by water infiltration. As a result, the fraction of the volume of the water infiltration sucked by the lysimeters placed in T1 plots would be expected to contain a high concentration of nutrients in all leachate samples. Nevertheless, the redistribution of the infiltration that resulted from the preferential water flow could avoid the infiltration water to move vertically into deeper layers of the soil profile towards the lysimeter (Bundt *et al.,* 2001; Starr & Timlin, 2001; Zhang *et al*., 2018). Therefore, some lysimeters in the T1 plots probably collected infiltrated water coming from soil areas not directly influenced by the fertilizer, yielding lower contents of $N\text{-}NH_4^+$ for some of the leachate samples. On the other hand, the variation in nutrient content of leachates collected from T2 was small and similar to that of the control, indicating that plots under T2 had a homogeneous low $N\text{-}NH_4^+$ concentration across the soil surface as a result of the control that CBH exerts on the exit rate of $N\text{-}NH_4^+$ from the fertilizer.

The CBH is more efficient regulating the $N\text{-}NH_4^+$ than the K^+ leaching. When the mean ion concentration in T2 is subtracted from T1, the difference is higher for K^+ than for $N\text{-}NH_4^+$, suggesting a greater effect of the CBH on K retention. However, there was a wider range of K^+ concentration values in leachates sampled in T2 (from 22 d until the last sampling date) than the one registered for $N\text{-}NH_4^+$ in T2 (Fig. 2B), pointing out that the CBH exerts less control over the diffusion of this ion from the fertilizer granule and reiterating previous laboratory results. This occurs because the presence in the hydrogel network of carboxyl

and sulfate negative charges had a greater influence on the retention of the ammonium ion as a result of a greater hydration radius in NH_4^+ than in K^+ (Guilherme *et al.,* 2015; Xu, 2019). Anyway, less variation was observed for K^+ in T2 than in T1 due to the presence of the CBH. Greater retention of potassium in the CBH could be achieved by using carrageenan with a higher content of sulfates.

Subsequent field trials with an experimental design including a greater number of replicates and plots are necessary. This is essential to measure the amount of fertilizer that can be saved using the CBH encapsulated fertilizer.

Crop growth and yield

From the beginning of tuberization detected at 42 d after emerging (dae), the PDM, FA, and TDM tended to have lower values in the plots treated with the CBH encapsulated fertilizer, but no significant differences were found among T1 and T2 for most sampling dates (Fig. 3).

No significant differences were observed among treatments for chlorophyll content (Fig. 3D) and specific gravity that showed an average value of 1.081 (±0.003) for T1, 1.078 (±0.010 SD) for T2 and 1.106 (±0.025) for T3 (Fig. 4A). Potatoes in T2 had lower mean yields than T1 in all categories of tuber diameter, although there were no significant differences between treatments (Fig. 4B). The lowest yields were recorded in T3.

The lower PDM and TDM trend in plants under T2 warns of the possibility that the current CBH formulation might decrease the crop yield below that achieved by using the non-CBH encapsulated fertilizer. The CBH could be retaining nutrients around the fertilizer, harming the development of the leaf area, and diminishing the interception of photosynthetically active radiation, hence affecting the total biomass production (Allen & Scott, 1980; Santos Castellanos *et al*., 2010; Gómez *et al*., 2017). Nonetheless, more field trials are needed to confirm a negative effect of the

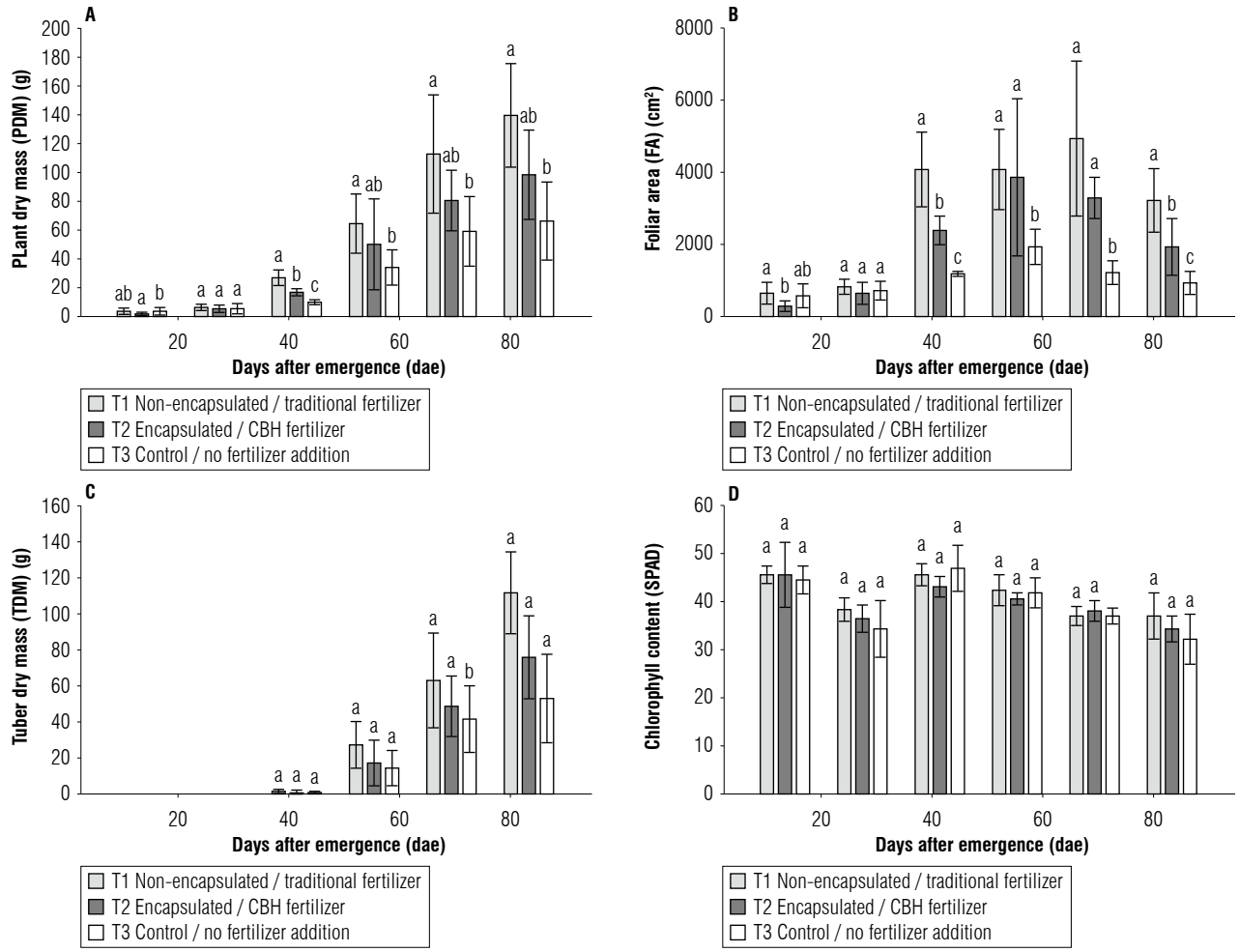

FIGURE 3. Plant dry mass A), foliar area B), tuber dry mass C) and chlorophyll content D) (mean ± standard deviation [SD]); n=9. Different letters above the bars indicate differences between treatments at $P<0.05$ (Tukey HSD *P*-values). Bars=SD.

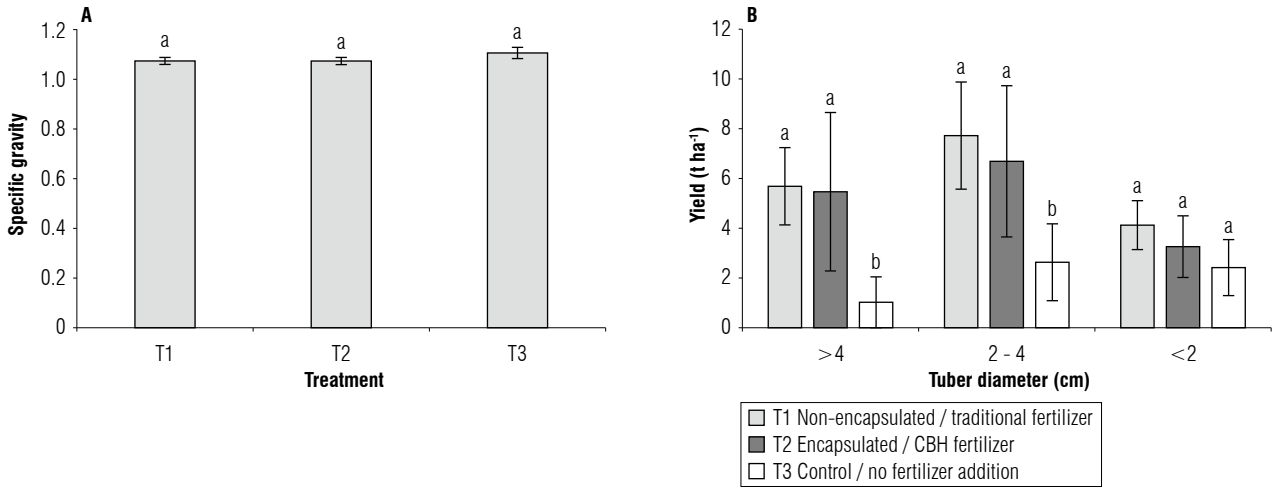

FIGURE 4. Specific gravity A) and yield B) (mean ± standard deviation [SD]); n per treatment for the yield data=3 blocks (30 plants per block) and n for the specific gravity=10 plants per treatment. The same letters above the bars indicate no differences between treatments (Tukey HSD, $P>0.05$). Bars=SD.

CBH on biomass production. Despite this trend towards a lower FA and PDM in T2, the chlorophyll content and the specific gravity were not affected. The chlorophyll ranged between 34.47 and 46.9 SPAD units between the 14 and 84 dae, coinciding with values previously reported by Ariza *et al.* (2020) for the cv. Criolla Colombia (42 SPAD at 70 das) under optimal irrigation and fertilization conditions. Therefore, in this study, normal chlorophyll levels were maintained with all treatments so nitrogen may not be a limiting factor in any of the treatments for chlorophyll synthesis. The specific gravity, an important quality indicator for the industrial processing of potato and directly correlated with the dry matter and starch content (Lulai & Orr, 1979), showed values (T1=1.081±0.003 and T2=1.078 ± 0.01) recommended for most processed products (Kirkman, 2007) like those reported for the cv. Criolla Colombia in previous studies (Rozo & Ñústez, 2011). The total tuber yield reported for cv. Criolla Colombia ranged between 11.15 and 20.48 t ha^{-1} in six localities in Antioquia department (Rodriguez *et al.*, 2009). The tuber yield result in this experiment is within this reported range, except for T3, a result that was expected due to lack of fertilizer.

Even so, contrary to the expected, a slight trend towards a lower yield was observed when CBH is used. This is not desirable because this would translate into a lower profit at the time of harvest. It is necessary to verify whether this effect is a consequence of the CBH nutrient retention that would make it necessary to modify the hydrogel formula to ensure the right flow of nutrients at the beginning of tuber development. It might also be an effect of the field trial size and the number of samples collected for yield estimation. Therefore, new trials should include larger crops and larger samplings.

Conclusions

This study confirmed the controlling effect of the CBH on the exit of nutrients from the soil solution at the field scale and during a rainy season, ensuring the retention of the nutrients added in the fertilizer on the soil surface and preventing them from easily leaching, especially at the beginning of the productive cycle when the seed potato tubers have not emerged and do not have roots to absorb the nutrients. At the same time, it was confirmed that the CBH is less efficient controlling the exit of K^+. The encapsulated CBH fertilizer allowed the same yield for crops at a field scale as the traditional fertilizers, with the additional advantage of reducing the negative environmental impact of leachates.

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Conflict of interest statement

The author´s declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

JSV and GRT formulated the overarching research goals and aims, JSV and GRT obtained the financial support

for the project leading to this publication. JSV and CEÑL developed or designed the methodology. NPM and JSV conducted the research. JSV, NPM, GRT and CEÑL contributed to the data analysis. JSV, NPM and GRT wrote the article. All authors reviewed the manuscript.

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S.1. Linear models

The effect of our predictors over the concentration of ammonia N-NH_4^+ (mg L^{-1}) and potassium K^+ (mg L^{-1}) was tested.

The model was set as: Concentration ~ Treatment + Days. The interaction between treatment and d was not considered, since there was not a powerful reason to believe that the effect of the treatments on the leachates would vary over time. This is because this is the first study of the performance of our carragean-based gels as coating for fertilizers at a field scale. In addition, it was done to prevent overfitting in our model.

The equation of the model was $Y = \beta 0 + \beta 1\ T1 + \beta 2\ T2 + \beta 3\ T3 + \alpha\ Days$, where Y represents the concentration, $\beta 0$ is the overall intercept, the value when all predictors are 0 (no biological meaning). $\beta 1$, $\beta 2$, $\beta 3$ are the values of the effect of each of the three treatments, uncoated fertilizer, coated and control, respectively. Finally, α represents the slope, *i. e.,* the effect of time over the concentration in the leachates.

The distribution of the residuals of the model was analyzed in order to determine if the suppositions of a linear regression are met. We evaluated if residuals ~ N (0, θ). Results are shown in Figure S1 for ammonium and S2 for potassium. In both cases, the general pattern seems to be the same: the residuals are distributed regardless of the treatments and

TABLE S1. Results for the linear models. The units of the Estimate and standard error are mg L^{-1}. The percentage of variance explained is shown as the R^2 in %, and the *P*-values indicating significant effects are highlighted in bold.

	R^2 (%)	Parameter	Estimate	Std error	*P*-value
N-NH_4^+ (mg L^{-1})	10.15	Intercept	0.305	0.1774	0.088
		Time	-0.007	0.0041	0.116
		T1*	0.384	0.1556	**0.015**
		T2**	0.015	0.1529	0.921
K^+ (mg L^{-1})	12.38	Intercept	4.169	0.7954	<0.001
		Time	-0.119	0.0183	0.298
		T1*	2.469	0.6827	**0.0004**
		T2**	1.151	0.6793	0-092

*T1 = Uncoated fertilizer, **T2 = Coated fertilizer.

time (Figs. S1A and S2A). Residuals are distributed with means approximately 0. Slight deviations from homoscedasticity and normality are observed, but mostly driven by the outliers. Overall, the analysis of residuals suggests the assumptions for linear regressions are met with only subtle deviations.

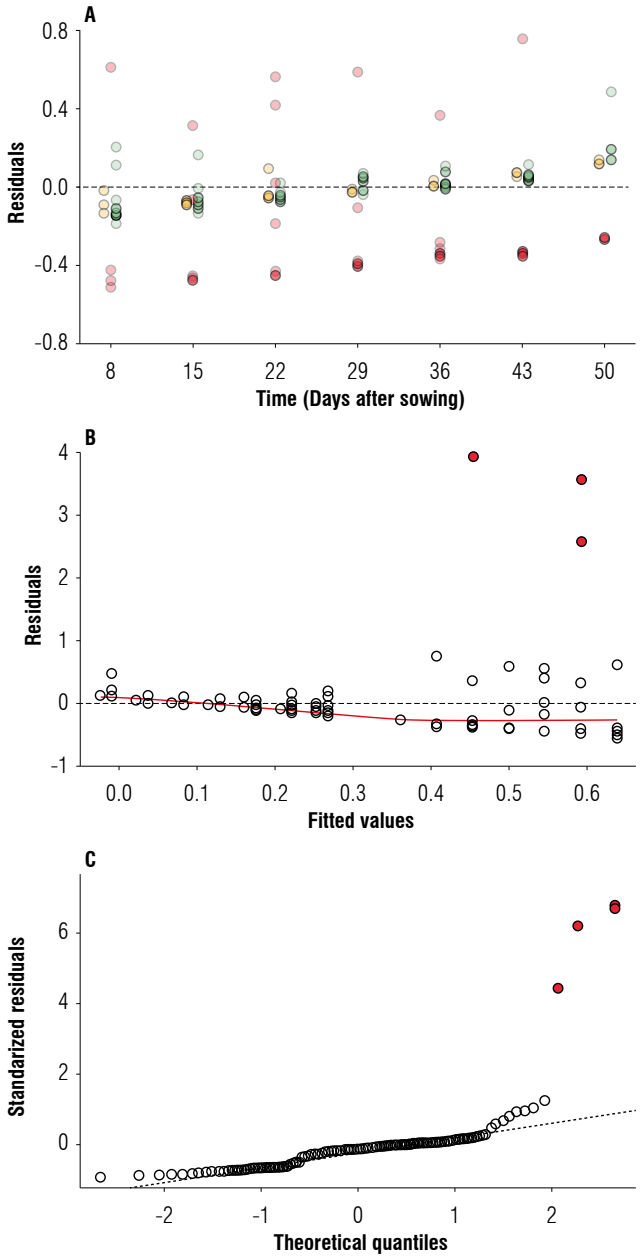

FIGURE S1. Analysis of residuals from the linear model of Ammonium concentration ~ Treatment + Days. A) Residuals vs. Predictor plot. Residuals show a similar distribution without any distinguishable pattern regardless of the treatment (yellow=T3/control, red=T1/Uncoated, green=T2/Coated). Outliers (n=3) omitted to facilitate visualization of differences in the y axis. B) Residual vs. Fitted values plot. Mean is approximately 0. Interestingly, the variance is relatively homogeneous only with slight alterations at higher values of concentration. Outliers shown in red. C) Normality Q-Q plot. Overall, the standardized residuals correspond to the expected quantile without red. Subtle deviations are shown in the tails. Larger deviations in the outliers shown in red.

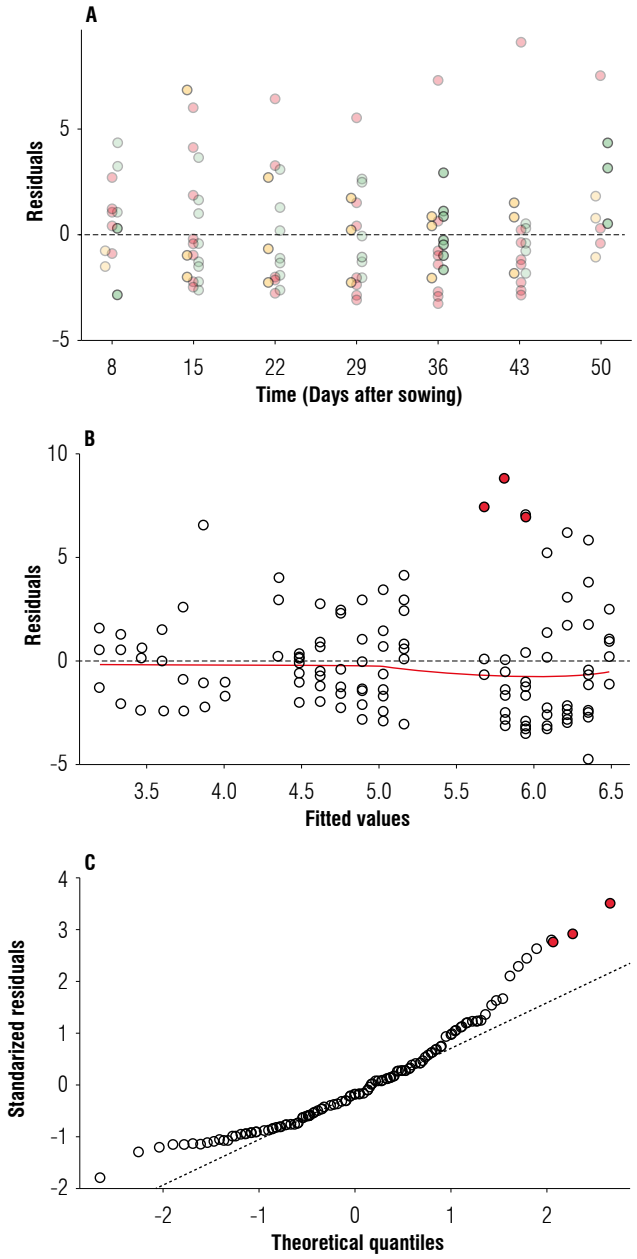

FIGURE S2. Analysis of residuals from the linear model of Potassium concentration ~ Treatment + Days. A) Residuals vs. Predictor plot. Residuals show a similar distribution without a distinguishable pattern regardless of the treatment (yellow=T3/control, red=T1/Uncoated, green=T2/Coated). B) Residual vs. Fitted values plot. Mean is approximately 0. Outliers shown in red. C) Normality Q-Q plot. Overall, the standardized residuals correspond to the expected quantile without red. Subtle deviations are shown in the tails. Outliers shown in red.

S2. Model selection tables

Each table shows the summary of likelihood and AIC criteria for each possible model that can be constructed with the combination of variables in this experiment. Each row represents one model and the columns represent the following: intercept (Intrc), the estimates for the two variables Days and Treatment (Trtmn), the degrees of freedom (df), log likelihood (LogLik), AIC value, ΔAICC (Difference in AIC with the model of minimum AIC) and the standardized weight of the model.

TABLE S2.1. Model selection table with the concentration of ammonia as a response variable. Model 3 was preferred over model 4 since the ΔAICC < 2. This means that the variable 'Days' does not help to explain the variability in the response variable. The only variable with a considerable effect was Treatment.

Model	(Intrc)	Time	Trtmn	df	logLik	AIC	ΔAICc	weight
4	0.3053	-0.00657	+	5	-111.698	233.4	0	0.554
3	0.1148		+	4	-112.975	234	0.56	0.42
1	0.2771			2	-118.442	240.9	7.49	0.013
2	0.4456	-0.00601		3	-117.46	240.9	7.52	0.013

TABLE S2.2. Model selection table with the concentration of potassium as a response variable. Model 3 was preferred since it has the best AIC (minimum). The only variable with a considerable effect was Treatment.

Model	(Intrc)	Time	Trtmn	df	logLik	AIC	ΔAICc	weight
3	3.593		+	4	-276.909	561.8	0	0.605
4	4.169.	-0.1918	+	5	-276.348	562.7	0.88	0.39
1	5.125			2	-284.143	572.3	10.47	0.003
2	5.744	-0.02212		3	-283.479	573	11.14	0.002

Selection of the minimum indicator set for agricultural sustainability assessments at the plot scale

Selección del conjunto mínimo de indicadores para evaluaciones de sostenibilidad agrícola a escala de parcela

Oscar Iván Monsalve Camacho[1]* and Martha Cecilia Henao Toro[1]

ABSTRACT

Some authors raise concerns about the validity, reliability, and transparency of indicator selection in agricultural sustainability assessments. In this regard, several selection criteria have been put forward for sustainability assessments at the farm, regional, country, or planet levels. However, assessments at the plot or experimental unit level require, in addition to the adaptation of these criteria or the generation of new ones, the construction of a selection methodology. Thus, the aim of this study was to build a framework for selecting the minimum set of indicators that will be part of the agricultural sustainability analyses at the plot or experimental unit level. A hierarchical order of indicators was established, consisting of raw, baseline, and core indicators; the latter made up the minimum indicators set (MIS). Subsequently, selection procedures and criteria were established, consisting of mandatory, main non-mandatory, alternative non-mandatory, and correlation indicators. The selection method was evaluated with the results of a greenhouse tomato fertilization study. Of the 40 raw indicators with which the analysis began, the MIS was made up of eight core indicators: three environmental, four social, and one economic. This indicator selection method uses a rigorous process, with 22 selection criteria, distributed in four hierarchical groups. At the same time, it promotes less subjectivity, by including statistical analysis, algorithms, and mathematical processes.

Key words: experimental unit, greenhouse tomato, indicator selection, selection criteria, core indicators.

RESUMEN

Algunos autores plantean preocupaciones con respecto a la validez, confianza y transparencia al momento de seleccionar indicadores en los análisis de sostenibilidad agrícola. En ese sentido, se han planteado una serie de criterios de selección orientados a evaluaciones de sostenibilidad a escala finca, región, país o planeta. Sin embargo, las evaluaciones a escala de parcela o unidad experimental requieren, además de la adaptación de esos criterios o la generación de unos nuevos, la construcción de una metodología de selección. El objetivo de este estudio fue, por lo tanto, construir un marco de selección del conjunto mínimo de indicadores que harán parte de los análisis de sostenibilidad agrícola a escala de parcela o unidad experimental. Se estableció un orden jerárquico de indicadores, compuesto por indicadores crudos, base y centrales; estos últimos conforman el conjunto mínimo de indicadores (CMI). Posteriormente, se establecieron los procedimientos y criterios de selección, conformados por: obligatorios, no obligatorios principales, no obligatorios alternativos y de correlación. Para evaluar el marco de selección propuesto, se utilizaron los resultados de un estudio de fertilización en tomate bajo invernadero. De los 40 indicadores crudos con que se inició el análisis, el CMI se conformó por ocho indicadores centrales: tres ambientales, cuatro sociales y uno económico. Esta metodología de selección de indicadores utiliza un riguroso proceso, con 22 criterios de selección, distribuidos en cuatro grupos jerárquicos. Al mismo tiempo, promueve una menor subjetividad, al incluir análisis estadísticos, algoritmos y procesos matemáticos.

Palabras clave: unidad experimental, tomate bajo invernadero, selección de indicadores, criterios de selección, indicadores centrales.

Introduction

When performing agricultural experiments in the field, many variables are usually measured. To estimate the sustainability generated by the treatments evaluated in such experiments, it is necessary to choose which of these variables are indicators and, therefore, be part of the minimum set of sustainability analyses indicators.

Although there is consensus on the use of indicators to assess the sustainability of agricultural production systems, there is still no agreement on how to select these indicators

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with a wide diversity of approaches (Parris & Kates, 2003; Bell & Morse, 2008; Bockstaller *et al*., 2009; de Olde, Moller *et al*., 2016; Alaoui *et al*., 2022). This implies the possibility that the indicators evaluated may not be focused on the objective of the study, increasing measurement costs and raising concerns about the validity of the approach and the usefulness and reliability of the evaluation (Bockstaller *et al*., 2009; Schader *et al*., 2014; de Olde, Moller *et al*., 2016). In response, many authors have highlighted the importance of establishing indicator selection procedures with transparent and well-defined criteria that lead to relevant, reliable, comprehensive, meaningful assessments that comprehensively represent the agricultural production system under study (Hunnemeyer *et al*., 1997; Binder *et al*., 2010; Lebacq *et al*., 2013; Marchand *et al*., 2014).

The criteria for selecting the most relevant indicators have been as follow: the indicator must be measurable (Dantsis *et al*., 2010; Gómez-Limón & Sánchez-Fernández, 2010; Roy & Chan, 2012), sensitive to variations (Bélanger *et al*., 2012), relevant to the case study (Dantsis *et al*., 2010; Bélanger *et al*., 2012), and directly related to the topic of study (van Asselt *et al*., 2014). The selection and prioritization of the criteria used to define the indicators differ widely among the sustainability assessment tools (Reed *et al*., 2006; Bell & Morse, 2008; de Olde, Oudshoorn, *et al*., 2016; Pereira *et al*., 2022).

Agricultural sustainability assessments can be conducted at different scales. Depending on the geographic projection to which these assessment results are to be scaled, opinions may be generated that reflect differences in worldviews, *e.g*., reductionist versus more holistic perspectives to understanding a multivariate activity such as agriculture (de Olde, Oudshoorn, *et al*., 2016). When agricultural sustainability assessments are made to compare different treatments in scientific experimentation, *i.e*., when the assessment scope is at the plot or experimental unit scale, indicators should reflect changes in production systems in response to the treatments being assessed.

Based on the above, this study aims to propose a selection framework for the minimum set of indicators, adapted to the plot or experimental unit scale that combines qualitative and quantitative criteria.

Materials and methods

Indicator hierarchical order

The variables considered indicators are assigned according to their hierarchy in raw, base, or central indicators.

Raw indicators: These are all the variables measured and estimated in the experiment in question from which significant differences are expected due to the application of the treatments evaluated and, in some way, their relation to the sustainability of the agricultural production system.

Baseline indicators: These are those raw indicators that have met the mandatory selection criteria outlined below. In this group, all the indicators obtained a score higher than zero after running the mandatory criteria checklist.

Core indicators: Those baseline indicators that scored the most after running the entire selection criteria checklist are discussed below.

Minimum Indicator Set (MIS): The MIS is composed of the core indicators that obtained the highest score. The indicators that make up the MIS are used for sustainability analysis.

Selection criteria

The list of indicator selection criteria, synthesized by de Olde, Oudshoorn, *et al*. (2016), was used as a reference point.

A scoring system was established based on the total or partial fulfillment of the different selection criteria to quantitatively select indicators. A checklist type "Not meets = 0 or Yes meets = 1" and rating (from 0 to 3) was established according to the rules corresponding to the selection criterion that gives the indicator a score according to the partial or total compliance with the criterion.

The selection criteria were grouped into direct mandatory criteria, main non-mandatory criteria, alternate non-mandatory criteria, and correlation criteria. The evaluation of each criterion for each indicator is done sequentially, obeying the following order:

Mandatory criteria: These are strict compliance criteria, *i.e*., if the indicator does not meet any of the criteria in this group, it will obtain a total score of zero, will be discarded, and will not continue with the agricultural sustainability assessment. They are the first criteria to be considered. All raw indicators must meet these criteria to move to the next step, becoming baseline indicators. The mandatory selection criteria are the following:

a) Related to the agricultural sustainability objective. The indicator has a highly significant (2), significant (1), or non-significant (0) relationship to the agricultural sustainability objective. Note that the sustainability objective may

be different from the study objective. For example, the effect of treatments on soil carbon stock is related to both the study objective and the agricultural sustainability objectives. The concentration of nutrients in plant tissues would not directly correlate with the sustainability objectives;

b) Quantifiable. Counts and continuous variables are more exact than ranges (ordinal scales) or 'yes/no' scores (binary); any form of quantification is more recommended than entirely qualitative assessment (de Olde, Oudshoorn, *et al*., 2016). It is quantifiable (1), not quantifiable (0);

c) Specifically interpretable. The change in the indicator can be interpreted by modifying the system in applying the treatments. It is specifically interpretable (1), not specifically interpretable (0);

d) Transparent and standardized. The indicator is based on clearly defined, verifiable, and scientifically acceptable data, collected through standardized and affordable methods to be reliably replicated and contrasted with each other. It is transparent and standardized (1), not transparent and standardized (0);

e) Not redundant. The indicator is not obtained from another variable that is part of the analysis or is not a variable within an aggregation function (FAg). A FAg is an indicator that aggregates two or more variables within itself and, through an equation, obtains the interaction of the variables that make up the function. If the first case is presented, priority is given to the independent variable. In the second case, the FAg is chosen because it generates a more significant amount of information. It is redundant (0), not redundant (1), a FAg (1), or is a variable within a FAg included in the study (0);

f) Significantly different. The indicator is sensitive, changes substantially, and it is within the assessment threshold with the treatments applied. Statistically, the indicator presents significant differences between the treatments evaluated. An analysis of variance (ANAVA) and a comparison test is performed. If at least 20% of the treatments have significant differences (P<0.05), a score of one (1) is assigned; between 20-40%, two (2); 40-60%, three (3); 60-80%, four (4); and 80-100%, five (5). If it turns out that they are not significant, a score of zero (0) is assigned and the indicator does not continue in the process.

Main non-mandatory criteria. These are criteria whose compliance is highly recommended, as they provide greater validity, transparency, and confidence in the analysis but are not strictly mandatory. The main non-mandatory selection criteria are as follow:

a) Affordable measurement. Direct measurement (field or laboratory) or estimation of the indicator through functions or models is easy and cheap (3), easy but expensive (2), cheap but complicated (1), or complicated and expensive (0) for most stakeholders. Affordable assessment increases participation and constancy of monitoring (de Olde, Oudshoorn, *et al*., 2016);

b) Parameterized. The indicator has pre-set ranges or thresholds (3). It is highly recommended that indicators be parameterized rather than comparing treatments; thresholds clearly define whether the application of the treatment results in an increase or decrease in the system's sustainability;

c) Measured or estimated. The indicator is measured directly in the field or estimated from variables measured directly in the field (2), is measured in the laboratory or estimated from variables measured in the laboratory (1), or is estimated through functions (*e.g*., pedo-transfer) or modeling (0). Actual (observed) values are generally preferable to estimates;

d) Related to the objective of the study. The indicator has a highly significant (2), significant (1), or non-significant (0) relationship with the objective of the study. It should be noted that the objective of the study may be different from the objective of the sustainability analysis;

e) Variable between repetitions. The indicator shows differences between repetitions of the same treatment (1). Some indicators obtained using estimates generate exactly the same value for all the repetitions of the treatment; this could detract from the validity of the statistical analysis.

Alternative non-mandatory criteria. These are criteria whose fulfillment is recommended, but they are used more as a means of tiebreaking if two or more indicators that meet the other criteria obtain the same score. The alternative non-mandatory selection criteria are as follow:

a) Acceptance. The indicator is either accepted (1), widely accepted (2), or not accepted (0) by the main stakeholders or decision-makers (*e.g*., producers, government officials, scientists, etc.);

b) Participatory development. The indicator was chosen in a participatory way (1) or not (0). It is more probable

that indicators and tools will be relevant, reliable, practical, heard, and used when stakeholders chose them (de Olde, Oudshoorn, *et al*., 2016);

c) Present and future balance. The indicator can be used to assess current and future sustainability. Some of the indicators should monitor potential new menaces and opportunities in the future (de Olde, Oudshoorn, *et al*., 2016).

d) Aggregate. The indicator is a FAg representing a set of variables (1) or not (0). This type of indicator is preferable since a single value explains the behavior of two or more variables or components of the production system;

Correlation criterion. This criterion allows us to choose between indicators that present a significant correlation. The following algorithm must be followed to assign the score:

1. A correlation matrix is made between the indicators to be compared;

2. The selection factor one (FS_1) is estimated for each indicator, applying Equation 1:

$$FS_1 = 1 - [\overline{X}(Ck_{1-n})] \quad (1)$$

Where $\overline{X}$ = Average, Ck_{1-n} = correlation coefficient value, in absolute values (0 to 1), between the evaluated indicator and the other indicators in the correlation matrix. FS_1 determines the degree of global correlation of the indicator, *i.e*., its degree of correlation with the other indicators within the correlation matrix. The higher the FS_1, the lower the overall correlation of the indicator;

3. The selection factor two (FS_2) is estimated for each indicator, based on Equation 2:

$$FS_2 = \left[\sum_{k=1}^{k=n} k - \left(\sum_{FL=1}^{FL=n} FL\right)\right] - 1 \quad (2)$$

Where k = indicator, FL = logic function, ranging from 1 to n, according to the following expression:

FL = If $FS_1 > 0.7$, then 0, otherwise 1

FS_2 relates the magnitude of the correlation coefficient to the significance of each indicator. In this way, highly correlated indicators are eliminated that are assigned a value of zero;

4. The selection factor three (FS_3) is estimated for each indicator, based on the following logical functions:

If Maximum value [(FS_2(k1) … FS_2 (kn)] > 0, then FS_3 = FS_2. If Maximum value [(FS_2(k1) … FS_2 (kn)] = 0, then {If FS_1 < Maximum value [(FS_1(k1) … FS_1 (kn)], then FS_3 = 0, otherwise, FS_3 = FS_1]}.

FS_3 allows choosing an indicator when all the matrix indicators are correlated in a highly significant way;

5. FS_3 is normalized using Equation 3:

$$FS_N = \frac{FS_3}{\text{Maximun value } [FS_3(k_{1-n})]} \quad (3)$$

Where FS_N = Normalized value of FS_3, FS_3(k1-n) = Maximum value of FS_3 from indicator one ton.

If FS_N = 0, then the indicator is removed;

6. Two indicators may correlate significantly with the other indicators in the correlation matrix and that, in turn, have a highly significant correlation with each other. In this case, one of the indicators must be eliminated. To define which indicator is eliminated, the correlated indicators must first be identified using the following logical function:

If [FS_1(k1) = FS_1(k2), and FS_1(k1) = FS_1(k3), and … FS_1(k1) = FS_1(kn)], then the correlated indicators (k) are identified with this symbol "†". This comparison must be made with all the indicators [FS_1(k1) to FS_1(kn)];

7. For each indicator that has been assigned an FS_N value, the sum of the scores obtained in the other selection criteria is made, namely: ∑CS = ObDr + NbPr + NbAt. If two correlated indicators were identified in step six with this symbol (†), the one that has obtained the highest ∑CS is chosen;

8. CrLc is calculated for each selected indicator by multiplying ∑CS * WCS, where WCS is the weighting value assigned for the selection criteria.

At the end of the process, a sum of the scores obtained in each category is calculated (ObGt + NbPr + NbAt + CrLc). The indicators that obtain a score higher than zero will be part of the MIS.

Case study

This methodology was used with the data of an experiment that evaluated the effect of different mixtures of organic fertilizers and chemical synthesis in the pre-plant application on a greenhouse tomato crop. The research was carried out in the Bio-Systems Center of the Jorge Tadeo Lozano University of Bogotá, located in the municipality of Chia (Colombia) (4°53'3.62" N, 74°00'50" W) at an altitude of 2650 m a.s.l. Tomato (*Solanum lycopersicum* L) cv. Sheila was used. Five treatments were evaluated: 1) Chemical control (ChC) (100% chemical pre-planting fertilization formula); 2) organic control (OrC) (100% organic pre-planting fertilization formula; 3) mixture 1, Mx1): 25% organic - 75% chemical pre-planting fertilization formula; 4) mixture 2 (Mx2) (50% organic - 50% chemical pre-planting fertilization formula); 5) mixture 3 (Mx3); 75% organic - 25% chemical pre-planting fertilization formula. A randomized complete block design was established with five treatments and 15 experimental units (EU) (three replicates per treatment). Each EU had an area of 12.3 m^2 for a total of 185 m^2. Table 1 shows the variables evaluated in the experiment.

Statistical analysis

The statistical analyses were performed with the R software version 3.6.2 (R Core Team, 2020). For all the variables studied, a descriptive analysis was performed, detecting extreme values using boxplot graphs, using the mvoutlier library (Filzmoser & Gschwandtner, 2017), and performing normality tests (Shapiro test) and variance homogeneity tests (Bartlett test) from the normtest library (Gavrilov & Pusev, 2014). In the cases where mismatches were detected, to find the appropriate transformation of the data, the boxcox tool from the MASS library was used (Ripley *et al*., 2017). Pearson's correlation analysis was performed with R's source aids. Anava and the Tukey's multiple comparison test (HSD) were performed with the Agricolae library (Mendiburu, 2017) to determine the differences between treatments.

Results

After running the mandatory criteria list for each raw indicator (Tab. 1), SQ_{PCA}, LU, W-kg, N-kg, FWT, MWT, EP, AP, GWP, and ODP were defined as the baseline

TABLE 1. Raw environmental, social, and economic indicators evaluated in the case study.

Environmental		Social	
Raw indicator	**Abbreviation**	**Raw indicator**	**Abbreviation**
Carbon stock	StockC	Yield	Yd
pH	pH	Percentage of first category	PCat
Electrical Conductivity	EC	Wages per cycle per hectare	WC
Effective Cationic Exchange Capacity	ECEC	Wages per year per hectare	WY
Phosphorus	P	Work effort indicator	WE
Bulk density	Db	High and maximum work effort	$WE_{4.5}$
Available Water Capacity	AWC	Formation of photochemical oxidants	PO
Texture	Txt	Toxicity for humans	TH
Weighted Average Diameter	WAD		
Nutrient concentration in plant tissue	Ntr-Veg	**Economic**	
Soil Management Assessment Framework	SQ_{SMAF}	Variable costs	VC
Soil Quality Indicator with Principal Component Analysis	SQ_{PCA}	Fixed Costs	FC
Land Use	LU	Investment	IV
Amount of water per kilogram produced	W-kg	Gross Income	GI
Quantity of nitrogen per kilogram produced	N-kg	Net Income	NI
Fresh Water Toxicity	FWT	Net Present Value	NPV
Marine Water Toxicity	MWT	Benefit-Cost ratio	B/C
Eutrophication Potential	EP	Opportunity Rate Obtained	ORO
Potential Acidification	PA	Internal Rate of Return	IRR
Global Warming Potential	GWP	Breakeven Point by Quantity	BPQ
Ozone depletion	ODP	Breakeven Point by Price	BPP

indicators of the environmental dimension (Tab. 2). The raw indicators StockC, pH, EC, ECEC, P, Db, AWC, and Txt are aggregated in the soil quality functions SQ_{SMAF} and SQ_{PCA}. For this reason, they were assigned a score of zero for the redundancy criterion (NoRd). Ntr-Veg has no significant direct relationship to the sustainability objective (ObSt) and was assigned a zero score for that criterion (Tab. 2). The SQ_{SMAF} indicator did not show significant differences.

As for the social dimension, the raw indicators WY and WE scored zero for being redundant with WC and $WE_{4.5.}$ Similarly, PCat did not present significant differences and was also eliminated (Tab. 2). In this dimension, Yd, WC, $WE_{4.5}$, PO, and TH continued as baseline indicators. In the economic dimension, the raw indicators VC, FC, IV, and GI are aggregated in the functions of the profitability indicators B/C, NPV, ORO, and IRR, while BPP did not show significant differences, so they obtained a score of zero. In this dimension, the following continued as baseline indicators: NI, B/C, NPV, ORO, IRR, and BPQ (Tab. 2).

Once the base indicators for each dimension were defined, the next step was to select the core indicators. To do this, first, the checklist of non-mandatory criteria was run on all the core indicators. Finding that the measurement of these indicators was affordable, although none were parameterized, all indicators were assigned a zero score for this criterion.

In the environmental dimension, only LU and W-kg indicators were directly measured in the field, while N-kg was measured in the laboratory. The other baseline indicators of this dimension were estimated by functions or modeling. In the social dimension, it was necessary to make measurements directly in the field to calculate Yd and WC, while $WE_{4.5}$, PO, and TH were estimated through functions or modeling. All the baseline indicators were obtained from field measurements.

Within the environmental dimension, the baseline indicators FWT, MWT, AP, GWP, and ODP did not have a significant relationship with the study's objective. The same happened for WC, $WE_{4.5}$, PO, and TH in the social

TABLE 2. Score obtained by the raw indicators for the mandatory selection criteria (MnTr).

Raw Indicator	StOb	QuAt	SpIn	TrSt	NoRd	SgDf	MnTr		Raw Indicator	StOb	QuAt	SpIn	TrSt	NoRd	SgDf	MnTr	
	0 - 2	0 or/a 1				0 - 5	W_{CS} =	0.5		0 - 2	0 or/a 1					W_{CS} =	0.5
Environmental dimension									**Social dimension**								
StockC	2	1	1	1	0	3	0.00		Yd	2	1	1	1	1	1	1.00	0.50
pH	1	1	1	1	0	0	0.00		PCat	1	1	1	1	1	0	0.00	
EC	1	1	1	1	0	3	0.00		WC	2	1	1	1	1	1	1.00	0.50
ECEC	1	1	1	1	0	2	0.00		WY	2	1	1	1	0	1	0.00	
P	2	1	1	1	0	0	0.00		WE	1	1	1	1	0	1	0.00	
Db	1	1	0	1	0	0	0.00		$WE_{4.5}$	1	1	1	1	1	1	0.86	0.43
CAD	2	1	1	1	0	0	0.00		PO	1	1	1	1	1	1	0.86	0.43
Txt	0	0	0	1	0	0	0.00		TH	2	1	1	1	1	1	1.00	0.50
Ntr-Veg	0	1	1	1	1	2	0.00		**Economic dimension**								
SQ_{SMAF}	2	1	1	1	1	0	0.00		VC	2	1	1	1	0	1	0.00	
SQ_{PCA}	2	1	1	1	1	2	0.73	0.36	FC	2	1	1	1	0	0	0.00	
LU	2	1	1	1	1	1	0.64	0.32	IV	2	1	1	1	0	0	0.00	
W-kg	2	1	1	1	1	1	0.64	0.32	GI	1	1	1	1	0	1	0.00	
N-kg	1	1	1	1	1	2	0.64	0.32	NI	2	1	1	1	1	1	1.00	0.50
FWT	2	1	1	1	1	5	1.00	0.50	B/C	2	1	1	1	1	1	1.00	0.50
MWT	2	1	1	1	1	5	1.00	0.50	NPV	1	1	1	1	1	1	0.86	0.43
EP	2	1	1	1	1	5	1.00	0.50	ORO	1	1	1	1	1	1	0.86	0.43
AP	1	1	1	1	1	5	0.91	0.45	IRR	1	1	1	1	1	1	0.86	0.43
GWP	2	1	1	1	1	5	1.00	0.50	BPQ	2	1	1	1	1	1	1.00	0.50
OLD	1	1	1	1	1	5	0.91	0.45	BPP	2	1	1	1	1	0	0.00	

StOb: related to the agricultural sustainability objective; QuAt: quantifiable; SpIn: specifically interpretable; TrSt: transparent and standardized; NoRd: not redundant; SgDf: significantly different; and WCS: weighting value assigned for the selection criteria. The conventions of the variables can be seen in Table 1.

dimension. The only raw indicator that was not variable between repetitions was WC since all the experimental units within the same treatment obtained the same value for this indicator.

The only indicator that is considered not yet accepted is $WE_{4.5}$, since it has not been used in other studies. At the same time, the only indicator chosen in a participatory way is Yd. Within the base indicators, it was considered that SQ_{PCA}, LU, W-kg, N-kg, Yd, WC, and all the economic indicators could be useful for estimates of future sustainability. SQ_{PCA} of the environmental dimension and all economic dimension indicators, except NI, were aggregation functions (Tab. 3).

After constructing the correlation matrix, it was evident that there were significant and highly significant correlations between many base indicators in each dimension. According to the results shown in Table 4, the environmental indicators N-kg, FWT, MWT, EP, AP, GWP, and ODP were significantly and highly correlated with each other and with the other indicators of the environmental dimension. These indicators were eliminated from the process, leaving N-kg as the highest FS_1.

The LU and W-kg indicators also showed a highly significant correlation, besides being correlated equivalently with the other environmental dimension indicators. In this sense, according to the score obtained with the other selection criteria (ΣCS), LU continued in the process. From the environmental dimension's baseline indicators, they became central indicators SQ_{PCA}, LU, and N-kg (Tab. 4).

In the social dimension, the PO and TH indicators presented the same correlation among themselves and their dimension indicators. This last one reached a higher score

TABLE 3. Score obtained by the baseline indicators for the main (NmMn) and alternate (NmAt) non-mandatory selection criteria.

Baseline Indicator	Main					NmMn		Alternative				NmAt	
	AfMs	PrTz	MsEd	ObSt	VrRt			AcTn	PtDv	PrFu	AgGt		
	0-3		0-2		0 or 1	W_{CS} =	0.2	0-2		0 or 1		W_{CS} =	0.2
Environmental dimension													
SQ_{PCA}	3	0	0	2	1	0.55	0.11	2	0	1	1	0.80	0.16
LU	3	0	2	1	1	0.64	0.13	2	0	1	0	0.60	0.12
W-kg	3	0	2	1	1	0.64	0.13	1	0	1	0	0.40	0.08
N-kg	3	0	1	2	1	0.64	0.13	1	0	1	0	0.40	0.08
FWT	2	0	0	0	1	0.27	0.05	1	0	0	0	0.20	0.04
MWT	2	0	0	0	1	0.27	0.05	1	0	0	0	0.20	0.04
EP	2	0	0	1	1	0.36	0.07	2	0	0	0	0.40	0.08
AP	2	0	0	0	1	0.27	0.05	1	0	0	0	0.20	0.04
GWP	2	0	0	0	1	0.27	0.05	2	0	0	0	0.40	0.08
OLD	2	0	0	0	1	0.27	0.05	1	0	0	0	0.20	0.04
Social dimension													
Yd	3	0	2	2	1	0.73	0.15	2	1	1	0	0.80	0.16
WC	2	0	2	0	0	0.36	0.07	2	0	1	0	0.60	0.12
$WE_{4.5}$	3	0	0	0	1	0.36	0.07	0	0	0	0	0.00	0.00
PO	2	0	0	0	1	0.27	0.05	1	0	0	0	0.20	0.04
TH	2	0	0	0	1	0.27	0.05	1	0	0	0	0.20	0.04
Economic dimension													
NI	1	0	2	1	1	0.45	0.09	2	0	1	0	0.60	0.12
B/C	1	0	2	1	1	0.45	0.09	2	0	1	1	0.80	0.16
NPV	1	0	2	1	1	0.45	0.09	2	0	1	1	0.80	0.16
ORO	1	0	2	1	1	0.45	0.09	1	0	1	1	0.60	0.12
IRR	1	0	2	1	1	0.45	0.09	2	0	1	1	0.80	0.16
BPQ	1	0	2	1	1	0.45	0.09	2	0	1	1	0.80	0.16

AfMs- affordable measurement; PrTz- parameterized; MsEd- measured or estimated; ObSt- related to the study objective; VrRt- variable between repetitions; AcTn- acceptance; PtDv- participatory development; PrFu- present and future balance; AgGt- aggregate; and WCS- weighting value assigned for the selection criteria. The conventions of the variables can be seen in Table 1.

TABLE 4. Score obtained by the base indicators for the correlation criterion (CrLc).

Baseline Indicator	Correlation matrix										Selection factors						CrLc	
Environmental dimension																		
	SQ_{PCA}	**LU**	**W-kg**	**N-kg**	**FWT**	**MWT**	**EP**	**AP**	**GWP**	**OLD**	**FS_1**	**FS_2**	**FS_3**	**FS_N**		**∑CS**	**W_{CS} =**	**0.1**
SQ_{PCA}		0.39	0.39	0.76 ***	0.72 ***	0.72 ***	0.72 ***	0.72 ***	0.72 ***	0.72 ***	0.35	1.00	1.00	1.00		1.25	1.00	0.10
LU	0.39		1.00 ***	0.54 **	0.77 ***	0.77 ***	0.77 ***	0.77 ***	0.77 ***	0.77 ***	0.27	1.00	1.00	1.00	†	0.00	1.00	0.10
W-kg	0.39	1.00 ***		0.54 **	0.77 ***	0.77 ***	0.77 ***	0.77 ***	0.77 ***	0.77 ***	0.27	1.00	1.00	1.00	†	0.00		
N-kg	0.76 ***	0.54 **	0.54 **		0.95 ***	0.95 ***	0.95 ***	0.95 ***	0.95 ***	0.95 ***	0.16	1.00	1.00	1.00		0.00	1.00	0.10
FWT	0.72 ***	0.77 ***	0.77 ***	0.95 ***		1.00 ***	1.00 ***	1.00 ***	1.00 ***	1.00 ***	0.09	0.00	0.00					
MWT	0.72 ***	0.77 ***	0.77 ***	0.95 ***	1.00 ***		1.00 ***	1.00 ***	1.00 ***	1.00 ***	0.09	0.00	0.00					
EP	0.72 ***	0.77 ***	0.77 ***	0.95 ***	1.00 ***	1.00 ***		1.00 ***	1.00 ***	1.00 ***	0.09	0.00	0.00					
AP	0.72 ***	0.77 ***	0.77 ***	0.95 ***	1.00 ***	1.00 ***	1.00 ***		1.00 ***	1.00 ***	0.09	0.00	0.00					
GWP	0.72 ***	0.77 ***	0.77 ***	0.95 ***	1.00 ***	1.00 ***	1.00 ***	1.00 ***		1.00 ***	0.09	0.00	0.00					
OLD	0.72 ***	0.77 ***	0.77 ***	0.95 ***	1.00 ***	1.00 ***	1.00 ***	1.00 ***	1.00 ***		0.09	0.00	0.00					
Social dimension																		
	Yd	**WC**	**$WE_{4.5}$**	**PO**	**TH**													
Yd		0.19	0.41 *	0.77 ***	0.77 ***						0.47	1.00	1.00	0.50		0.00	0.50	0.05
WC	0.19		0.71 ***	0.24	0.24						0.66	2.00	2.00	1.00		0.00	1.00	0.10
$WE_{4.5}$	0.41	0.71 ***		0.70 ***	0.70 ***						0.37	2.00	2.00	1.00		0.00	1.00	0.10
PO	0.77 ***	0.24	0.70 ***		1.00 ***						0.32	1.00	1.00	0.50	†	0.00		
TH	0.77 ***	0.24	0.70 ***	1.00 ***							0.32	1.00	1.00	0.50	†	0.00	0.50	0.05
Economic dimension																		
	NI	**B/C**	**NPV**	**ORO**	**IRR**	**BPQ**												
NI		1.00 ***	1.00 ***	1.00 ***	1.00 ***	0.96 ***					0.01	0.00	0.00					
B/C	1.00 ***		1.00 ***	1.00 ***	1.00 ***	0.96 ***					0.01	0.00	0.00					
NPV	1.00 ***	1.00 ***		1.00 ***	1.00 ***	0.96 ***					0.01	0.00	0.00					
ORO	1.00 ***	1.00 ***	1.00 ***		1.00 ***	0.97 ***					0.01	0.00	0.00					
IRR	1.00 ***	1.00 ***	1.00 ***	1.00 ***		0.97 ***					0.01	0.00	0.00					
BPQ	0.96 ***	0.96 ***	0.96 ***	0.97 ***	0.97 ***						0.04	0.00	0.04	1.00		0.00	1.00	0.10

FS- selection factor; FS_N- normalized value; ∑CS- sum of the scores obtained in the other selection criteria; WCS- weighting value assigned for the selection criteria; ** and ***: high and very highly significant correlation, †: Correlated indicators (k). The conventions of the variables can be seen in Table 1.

for ∑CS. The following were selected as central indicators of the social dimension: Yd, WC, $WE_{4.5}$, and TH (Tab. 4).

In the economic dimension, all the baseline indicators showed highly significant correlations among them. The BPQ indicator obtained the highest FS_3, so it was chosen as the central indicator representing all the economic dimensions (Tab. 4).

Based on the results shown in Table 4, the minimum indicator set (MIS) was made up, at the environmental dimension, of the central indicators SQ_{PCA}, LU, and N-kg. In the social dimension, the central indicators were Yd, WC, $WE_{4.5}$, and TH, and in the economic dimension the indicator was BPQ. The SQ_{PCA}, Yd, LU, and BPQ indicators obtained the highest score (0.87, 0.86, 0.85, and 0.85, respectively), while $WE_{4.5}$ reached the lowest score (0.6) (Fig. 1). From 21 raw indicators (10 environmental, 5 social, and 6 economic), were chosen 8 core indicators (3 environmental, 4 social, and 1 economic).

Discussion

Taking as a reference what Smith and Dumanski (1994) have said, an indicator is a characteristic that measures or reflects the state or condition of a system's change. Likewise, an agricultural sustainability indicator is a variable or a function of aggregation of a set of variables associated with the environmental, social, or economic dimensions of an agricultural production system, established as a reference for reporting on the functioning of that system (Gerdessen & Pascucci, 2013; de Olde, Oudshoorn, *et al.*, 2016). An indicator shows sustainability as a measure of distance to the target; *i.e.*, it measures the distance between

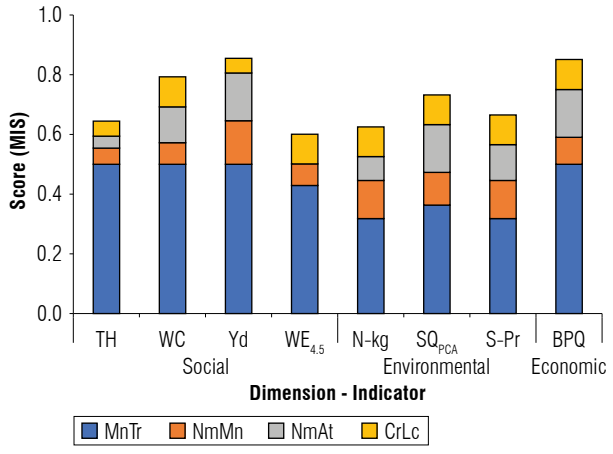

FIGURE 1. Score obtained for each indicator of the minimum indicators set (MIS) for each selection criterion.

the actual or predicted values of the variable and the reference value (representing the value with which sustainability is achieved).

The selection of indicators is a process that involves both qualitative and quantitative analysis. However, although indicators can be quantitative (numbers) or qualitative (*e.g.*, graphics, colors, symbols), they need to be transformed into numerical values and have a unit of measurement (Waas *et al.*, 2014). With the procedure described in this paper, the aim was to reduce as much as possible the level of subjectivity generally associated with the selection of qualitative indicators. However, it is not easy to eliminate subjectivity from selecting agricultural sustainability indicators since decisions must be made closely related to the researcher experience. The first subjective choice made is the weighting assigned to each group of selection criteria. In this work, mandatory criteria are considered to have the highest weight, so they were assigned a WCS of 0.5 (scale 0 to 1) (Tab. 2), while the main and alternate non-mandatory criteria were assigned a WCS of 0.2 each, and the correlation criterion a WCS of 0.1. There could be a consensus that the mandatory criteria are more important than the non-mandatory ones; the question is which WCS value should be assigned to each selection criterion? Similarly, the mandatory criteria are more important because if anyone of them is not met, the indicator is immediately removed from the process, and, therefore, they should be reviewed in more detail.

The second subjective choice is the score assigned to some selection criteria. For example, defining whether an indicator has a significant or highly significant relationship to sustainability's objective could have several points of view. Determining that an indicator has no relationship to the sustainability objective can generate a great deal of discussion. In this regard, there is a lack of consensus on which indicators to include in sustainability analyses, with a wide diversity of approaches (Parris & Kates, 2003; Bell & Morse, 2008; Bockstaller *et al.*, 2009). However, the simple fact of considering these selection criteria increases the study's reliability, despite not reaching an absolute agreement.

This indicator selection methodology is designed to dilute the subjective selection process as one moves from raw indicators to base and core indicators. The degree of subjectivity is diminished by including statistical analysis and absolute (yes/no) selection criteria.

As shown in this paper, many indicators are usually measured, but many are redundant and correlated. The choice

of indicators through a matrix and a correlation algorithm allows selecting the indicators with the most significant statistical weight, avoiding redundancy in the analysis. Likewise, the selection procedure gives higher importance to those indicators that are aggregation functions since a single indicator explains the behavior of all variables that make up the function.

From a technical perspective, an indicator is "a variable or an aggregation function of several variables related to a reference value that gives meaning to the values taken by the variables" (Pintér *et al*., 2012; Singh *et al*., 2012). In that sense, an indicator is related to a reference value since the term comes from the Latin *indicare* that means to point to something (Waas *et al*., 2014). None of the base indicators were parameterized. Therefore, the definition of sustainable must be made in terms of the comparison between the treatments evaluated, assuming that more or less is better for the indicator in question.

Choosing only the indicators that show significant differences between the treatments evaluated eliminates noise in the analysis. Working with indicators that have the same importance for all the systems under evaluation only increases the work but does not inform about the differences to be identified between the treatments under evaluation. This is more evident at small scales such as plot or experimental units. At these scales, indicators associated with government or macroeconomic policies, for example, would not have a differential effect among the treatments under evaluation, and therefore should not be considered.

Although the methodology proposed in this study promotes less subjectivity for the indicator selection process, it must still be recognized that definitions of sustainability and indicator selection vary with the researcher approaches, contexts, and expectations (Bell & Morse, 2008; Gasparatos, 2010). Nevertheless, this methodology allows the indicators that are part of the minimum set of indicators to show the effect of the treatments evaluated on the production system's sustainability. By applying this methodology, it can be ensured, to a large extent, that all environmental, social, and economic variables measured in the experiment are represented in the minimum set of indicators. This, despite the fact that in some cases, as in the environmental dimension, it began with a significant number of indicators (22) and was reduced to three. This drastic reduction was associated with a high correlation and/or redundancy between the environmental indicators evaluated.

Generally, the criteria for selecting indicators for agricultural sustainability assessments are associated with post-field study evaluations. However, it is highly recommended that the selection criteria proposed in this study (except, of course, those related to statistical analyses) be considered when evaluations are being planned. This would decrease the investment of resources.

Conclusions

The indicator selection process began with the evaluation of 40 raw indicators: 21 environmental, 8 social, and 11 economic. At the end of the process, they were reduced to 8 core indicators: 3 environmental (N-kg, SQ_{PCA}, S-Pr), 4 social (TH, WC, Yd, and $WE_{4.5}$), and 1 economic (BPQ). This indicator selection methodology uses a rigorous process, with 22 selection criteria distributed in four hierarchical groups while promoting less subjectivity by including statistical analysis, algorithms, and mathematical processes. Using this methodology, the probability that all environmental, social, and economic variables measured in the experiment are represented in the minimum set of indicators is increased. Also, it increases the possibility that the selected core indicators will more reliably assess the production system's sustainability. We suggested replicating this work under different environments, species, and treatments.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

OIMC designed the methodology and carried out field sampling. OIMC and MCHT did the processing, data analysis, writing and revision of the final document.

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Dynamics of the weed community during pineapple growth in the Brazilian semi-arid region

Dinámica de la comunidad de malezas a lo largo del crecimiento de piña en la región semiárida brasileña

João Rafael Prudêncio dos Santos[1], Victor Martins Maia[1]*, Bruno Soares da Silva[1], Pedro Mendes Demicheli[1], Ignacio Aspiazú[1], and Germani Concenço[2]

ABSTRACT

The pineapple belongs to the family Bromeliaceae and is a slow-growing succulent monocot with a reduced superficial root system. For this reason, the interference of weeds in competition with this crop can cause significant losses to the production. One of the bases to elaborate a control strategy is the knowledge of the diversity of weeds that occur in the cultivated areas. The objective of this study was to identify the weed community during pineapple growth in a semi-arid climate region of Brazil. Weeds were collected 60, 120, 180, 240, 300, and 360 days after planting (DAP) the pineapple. These collections were made in three different plots every two months until floral induction, composed of three pineapple cultivars. The weed community found in the irrigated pineapple field, in semi-arid climate conditions, was mostly composed by species belonging to the families Amaranthaceae, Asteraceae, Convolvulaceae, Fabaceae, Malvaceae, and Poaceae. The highest diversity of weed species was found at 60 DAP. The species *Ipomoea acuminata* was present throughout the development of the pineapple and showed the highest importance value index in most of the periods evaluated during the pineapple growth.

Key words: *Ananas comosus* var. comosus, phytosociological survey, IAC Fantastic, Perola, Smooth Cayenne.

RESUMEN

La piña pertenece a la familia Bromeliaceae y es una monocotiledónea suculenta de crecimiento lento con un sistema radical superficial reducido. Por este motivo, la interferencia de las malezas en competencia con el cultivo puede provocar pérdidas importantes para la producción. Una de las bases para la elaboración de una estrategia de control es el conocimiento de la diversidad de malezas que se dan en las áreas cultivadas. El objetivo de este trabajo fue identificar la comunidad de malezas durante el crecimiento de la piña en una región de clima semiárido de Brasil. Las malezas se recolectaron 60, 120, 180, 240, 300 y 360 días después de la siembra (DDS) de la piña. Estas recolecciones se realizaron en tres parcelas diferentes cada dos meses hasta la inducción floral, compuestas por tres cultivares de piña. La comunidad de malezas que se encontró en el campo de piña irrigado, en condiciones climáticas semiáridas, estaba compuesta principalmente por especies pertenecientes a las familias Amaranthaceae, Asteraceae, Convolvulaceae, Fabaceae, Malvaceae y Poaceae. La mayor diversidad de especies de malezas se encontró a los 60 DDS. La especie *Ipomoea acuminata* estuvo presente durante todo el desarrollo de la piña y mostró el índice de valor de importancia más alto en la mayoría de los períodos evaluados durante el crecimiento de la piña.

Palabras clave: *Ananas comosus* var. comosus, estudio fitosociológico, IAC Fantástico, Perola, Cayena lisa.

Introduction

The pineapple (*Ananas comosus* var. comosus) is the third most cultivated tropical fruit in the world, and Brazil is the third largest producer with 2.2 million t in an area of 62,116 ha (FAO, 2018).

The semi-arid zones in the world occupy around 15% of the global land surface, including the hot and cool semi-arid regions. These zones are characterized by low average annual rainfall which corresponds to between one-fifth and one-half of the potential evapotranspiration. Hot semi-arid regions have a mean annual temperature above 18°C (Scholes, 2020); therefore, the Brazilian semi-arid zone is classified as hot semi-arid.

As a plant with slow vegetative growth, small size and superficial root system, the pineapple suffers intense competition with weeds, especially in the first months after planting. This contributes to delay the development of the

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crop and reduces productivity and quality of the fruits (Maia *et al*., 2012; Maia *et al*., 2018).

Weeds compete with agricultural crops for water, light and nutrients, and can release allelopathic substances that inhibit crop growth (Zimdahl, 2008; Ghanizadeh *et al*., 2014; Maia *et al*., 2018). Additionally, they can host pests and diseases, thus, hindering crop management and harvesting as well as impairing the quality of the marketable product (Swanton *et al*., 2015; Mureithi *et al*., 2017; Ocimati *et al*., 2018).

The degree of weed interference depends on the weed community, environment and the period and time of the crop's coexistence with weeds. In this sense, it is essential to identify the species present in the cultivation areas and determine the main periods of interference, indicating the appropriate time to carry out weed control in the fields (Zimdahl, 2008; Swanton *et al*., 2015; Marques *et al*., 2017).

The knowledge of the diversity of weeds in the cultivation areas is the basis for the elaboration of a weed control proposal. In addition to the identification of weed species, a survey needs to include a quantitative analysis of these species, *i.e.,* a phytosociological study or method (Braun-Blanquet, 1979; Swanton *et al*., 2015). This method provides data that are specific to the species present, such as frequency, density, and abundance, and their relationship with the whole weed population. This tool allows making many inferences about the weed in question to define what will be done, how and when.

A phytosociological study for weed management is justified as infestation conditions are varied and the management possibilities are diverse (Sarmento *et al*., 2017; Santos *et al*., 2019) since the establishment and dynamics of a weed community depend on edaphoclimatic conditions, crop practices, seed bank, etc. (Adegas *et al*., 2010; Swanton *et al*., 2015; Korres *et al*., 2019). As phytosociological studies in irrigated crops in the hot semi-arid region showing weed community fluctuations along the year are scarce, especially in the pineapple crop, the objective of this study was to identify the dynamics of the weed community during pineapple growth in a semi-arid climate region of Brazil.

Materials and methods

The experiment was carried out in the municipality of Janaúba, MG, under the geographical coordinates of 15°43'48'' S, 43°19'23'' W and 533 m a.s.l. The region has "Aw" climate (Sá Júnior *et al*., 2012). The climatic conditions during the period of the experiment are shown in Figure 1.

Weeds were collected 60, 120, 180, 240, 300, and 360 days after planting (DAP) of pineapple, in a plot composed of three pineapple cultivars (Perola, IAC Fantastic, and Smooth Cayenne) planted in double rows spaced 1.2 x 0.3 x 0.20 m, totaling 66,666 plants ha^{-1}. The three cultivars occupied the same proportion in the sample plot.

The area was prepared by plowing at a depth of 0.40 m with two harrowings, furrowing and pre-planting fertilization with chemical and organic fertilizers. Before planting, a composite soil sample from the 0-20 cm layer was collected to determine the soil chemical properties.

The soil of the area was an eutrophic Red Latosol (Oxisol), medium/clayey texture (EMBRAPA, 2006), and the physical and chemical properties in the 0-20 cm layer were as follows: pH(H_2O) = 6.5; organic matter = 15 g kg^{-1}; P = 8 mg dm^{-3}, K = 374 mg dm^{-3}, Ca^{2+} = 3.2 $cmol_c$ dm^{-3}; Mg = 1.2 $cmol_c$ dm^{-3}; Al^{3+}= 0 $cmol_c$ dm^{-3}; H+Al = 1.4 $cmol_c$ dm^{-3}; Zn = 1.5 mg dm^{-3}; Fe = 23.4 mg dm^{-3}; Mn = 33.3 mg dm^{-3}; Cu = 0.9 mg dm^{-3}; B = 0.3 mg dm^{-3}; sum of base cations (SB) = 5.5 $cmol_c$ dm^{-3}; base saturation (V) = 80%; aluminum saturation (m) = 0%; effective cation exchange capacity (CEC) = 5.5 $cmol_c$ dm^{-3}; total CEC = 6.8 $cmol_c$ dm^{-3} and remaining phosphorus (P- rem) = 33.3 mg L^{-1}.

The crop was planted in August 2017 at a depth of 0.20 m and, after furrowing, a micro-sprinkler irrigation system was implemented. After the adjustments and adaptations of the irrigation system, preliminary tests were carried out to determine the flow rate of the micro-sprinklers and the water distribution uniformity coefficient. Slip, slip-sucker, and sucker propagules were used for planting with the same kind of propagule in the same plot to maintain crop uniformity.

For weed collection, the standard method of the square inventory (0.5 m x 0.5 m) was employed, randomly thrown once in the useful area of each plot. The square was thrown three times per collection, collecting all plants as described by several authors (Curtis & McIntosh, 1950; Odum, 1971; Braun-Blanquet, 1979; Swanton *et al*., 2015). Only the weed shoots were collected. The identification of the species in each square was carried out by comparison, according to the classification of Lorenzi (2008) and quantified by family, genus and species, with later determination of the phytosociological values.

The samples of each species were then packed in paper bags and put into a forced air circulation oven (model TE 394/3, Tecnal, Piracicaba, Brazil) at 65°C for 72 h, for later weighing of the dry matter mass on a precision scale,

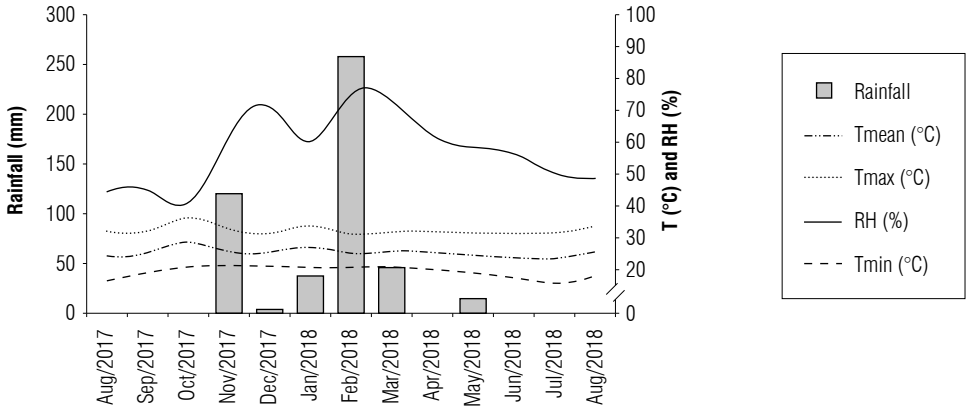

FIGURE 1. Rainfall, minimum, maximum, and mean temperatures (T, °C) and relative humidity (RH, %) during the experiment.

with the result expressed in g. The total dry matter of the weed community was used to adjust a growth model and estimate the absolute and relative growth rate of the weed community as a function of growth sampling dates in the pineapple crop (Hunt, 2012; Soltani & Sinclair, 2012).

The number of individuals per species in each plot and the total number per collection were determined. From the identification and counting of species, we carried out the calculations and descriptive analysis of the following phytosociological variables: frequency (FR), density (DE), abundance (AB), relative frequency (FRR), relative density (DER), relative abundance (ABR), relative dominance (DOR), importance value index (IVI) and coverage value index (CVI) (Mueller-Dombois & Ellenberg, 1974; Braun-Blanquet, 1979; Tuffi Santos *et al*., 2004).

The relative indexes were used to calculate the importance value indexes expressed as constants. To perform the calculations, the following formulas were used:

FR = number of squares containing the species / total number of squares obtained;

FRR = FR of the species × 100 / total frequency of all species;

DE = total number of individuals per species / total area occupied by the squares;

DER = DE of the species × 100 / total density of all species;

AB = total number of individuals per species / total number of squares containing the species;

ABR = AB of the species × 100 / total abundance of all species;

DOR = biomass of the species / ∑ of total biomass of all species × 100;

IVI = FRR + DER + ABR;

CVI = DOR + DER.

All results were presented using descriptive statistics done with Microsoft Excel. The graphs were made using the software Sigma Plot 12.5 Demo Version.

Results and discussion

Throughout the pineapple growth cycle, the weed community obtained by the surveys showed a distribution of 22 species and 12 families. Among the species, a population of 68.2% of dicotyledonous plants and 31.8% of monocotyledonous plants was found (Tab. 1). As for the carbon fixation process, 54.6% were plants with C_3 metabolism and 45.4% were with C_4 metabolism. No plants with CAM metabolism were observed.

Regarding the number of species, among the dicots, the families Amaranthaceae, Asteraceae, Convolvulaceae, Fabaceae and Malvaceae stood out. These families corresponded to 45.5% of the identified species. Among the monocotyledonous plants, the family Poaceae stood out, with five species, equivalent to 22.7% of the identified species (Tab. 1). Sarmento *et al*. (2017) cultivated pineapple under similar climatic conditions in the spring-summer period of Brazil and identified ten weed species distributed in nine genera and eight families, with the families Euphorbiaceae and Poaceae standing out, with two individuals each. In the autumn-winter period, nine species, seven genera and six families were identified, with Euphorbiaceae and Poaceae standing out again with three and two individuals, respectively.

Model *et al*. (2008) reported that 40 weed species were identified in an area of pineapple cultivation in Maquiné (RS, Brazil), a region with a humid subtropical climate. The authors highlighted the presence of the families Asteraceae

(27.5%), Poaceae (25%), Cyperaceae (7.5%), Fabaceae (7.5%), Apiaceae (5%) and others with 2.5% each. In the following year, Model and Favreto (2009) identified 74 weed species belonging to 25 botanical families, with the families Poaceae (23%), Asteraceae (28%), Cyperaceae (5%), and Fabaceae (5%) standing out. Similar results were found in pineapple areas located in the humid tropics of India (Kerala) (Girija & Menon, 2019).

The high number of species and families shows the great botanical diversity of the weed flora with the potential to compete with the pineapple. The favorable climate, soil productive potential, pH, and nutrient corrections are factors that can influence the diversity of species and the development of weeds (Model & Favreto, 2009). In addition to these factors is the use of irrigation, which allows for the germination and growth of weeds to occur in all months of the year.

The Asteraceae and Poaceae families have been commonly reported and found in several weed studies (Jakelaitis *et al*., 2003; Erasmo *et al*., 2004; Murphy *et al*., 2006; Duarte *et al*., 2007; Maia *et al*., 2018). This is certainly because they show high dissemination and colonization of different environments (Pedrotti & Guarim Neto, 1998).

Most of the species of the family Poaceae are perennial and produce large amounts of seeds, which considerably increases their dissemination power and the colonization of different types of environments, even under adverse conditions (Holm *et al*., 1991).

The species of the family Asteraceae have similar characteristics and are easily established in different environments, being the first weeds in the cultivation area after soil preparation (Lorenzi, 2008). The species of this family are considered among the most infesting plants of annual and perennial crops (Holm *et al*., 1991; Zimdahl, 2008). The identification of weed species is important and necessary, as each species has the potential to establish itself in the area and can interfere differently, depending on the crop (Korres *et al*., 2019).

The dry matter of the weed community increased during the entire survey period. There was an exception at 180 d, with dry matter of 1,780.58 g m^{-2}, *i.e*., slightly lower than

TABLE 1. Botanical and photosynthetic classification of weeds found during pineapple cultivation in the semi-arid climate region of Brazil.

Family	Genus	Scientific name	Class	Photosynthesis
Malvaceae	*Sida*	*S. rhombifolia*	Magnoliopsida	C_3
Amaranthaceae	*Amaranthus*	*A. viridis*		C_4
	Bidens	*B. pilosa*		C_3
Apocynaceae	*Calotropis*	*C. procera*		C_3
Asteraceae	*Galinsoga*	*G. parviflora*		C_4
	Acanthospermum	*A. hispidum*		C_3
Commelinaceae	*Commelina*	*C. benghalensis*	Liliopsida	C_3
Convolvulaceae	*Merremia*	*M. aegyptia*	Magnoliopsida	C_3
	Ipomoea	*I. acuminata*		C_3
Euphorbiaceae	*Euphorbia*	*E. hirta*		C_4
Fabaceae	*Senna*	*S. obtusifolia*		C_3
	Mimosa	*M. pudica*		C_4
Malvaceae	*Malva*	*M. sylvestris*		C_3
Poaceae	*Brachiaria*	*B. plantaginea*	Liliopsida	C_4
	Sorghum	*S. bicolor*		C_4
	Cenchrus	*C. echinatus*		C_4
	Panicum	*P. maximum*		C_4
	Eleusine	*E. indica*		C_3
	Brachiaria	*B. decumbens*		C_3
	Dactyloctenium	*D. aegyptum*		C_4
Portulacaceae	*Portulaca*	*P. oleracea*	Magnoliopsida	C_4
Turneraceae	*Turnera*	*T. subulata*		C_3

the previously evaluated period. As a result, the maximum accumulated dry matter (3,436.77 g m^{-2}) of the weed community was reached 360 DAP (Tab. 2). This is probably due to the greater adaptation and competition potential of these species compared to the pineapple plants.

Weed species can harm the crop by competing for the essential resources for plant development (water, light, and nutrients), especially nutrient extraction. Considering the highest value of dry matter obtained at 360 DAP (3,436.77 g m^{-2} = 34.4 t ha^{-1}), it can be estimated that 515.5 kg of N

TABLE 2. Dry matter (DM, g m^{-2}) and number of individuals per species (NIS) of weeds found during pineapple cultivation in the semi-arid climate region of Brazil.

Sampling moments					
60 d after planting			**120 d after planting**		
Weed	**DM**	**NIS**	**Weed**	**DM**	**NIS**
Amaranthus viridis	92.26	7	*Amaranthus viridis*	49.45	6
Brachiaria plantaginea	3.45	92	*Calotropis procera*	52.6	7
Cenchrus echinatus	53.59	5	*Cenchrus echinatus*	206.2	26
Commelina benghalensis	42.3	5	*Galinsoga parviflora*	61.12	8
Eleusine indica	15.4	3	*Ipomoea acuminata*	1173.5	35
Ipomoea acuminata	849.8	16	*Merremia aegyptia*	156.3	3
Malva sylvestris	10.36	3	*Panicum maximum*	125.9	5
Merremia aegyptia	264.05	14	*Portulaca oleracea*	31.2	6
Mimosa pudica	53.3	9	*Sida rhombifolia*	37.9	5
Senna obtusifolia	12.69	53			
Sida rhombifolia	115.23	6			
Sorghum bicolor	233.9	2			
Turnera subulata	37.6	2			
Total	1783.93	217		1894.2	101
180 d after planting			**240 d after planting**		
Weed	**DM**	**NIS**	**Weed**	**DM**	**NIS**
Amaranthus viridis	13.9	5	*Amaranthus viridis*	36.5	26
Cenchrus echinatus	128	18	*Bidens pilosa*	0.06	1
Euphorbia hirta	43.92	3	*Commelina benghalensis*	3.62	4
Ipomoea acuminata	1395	41	*Ipomoea acuminata*	1718.9	37
Malva sylvestris	35.1	7	*Malva sylvestris*	18.66	4
Merremia aegyptia	79.6	12	*Merremia aegyptia*	215.8	13
Mimosa pudica	31.96	3			
Sida rhombifolia	53.1	2			
Total	1780.58	91		1993.54	85
300 d after planting			**360 d after planting**		
Weed	**DM**	**NIS**	**Weed**	**DM**	**NIS**
Amaranthus viridis	5.6	2	*Brachiaria decumbens*	1021	69
Cenchrus echinatus	199.5	6	*Ipomoea acuminata*	1997.21	27
Ipomoea acuminata	1937.8	41	*Acanthospermum hispidum*	6.2	1
Merremia aegyptia	142.5	6	*Bidens pilosa*	0.9	1
Calotropis procera	369.4	1	*Malva sylvestris*	85.8	8
			Dactyloctenium aegyptum	315.3	39
			Senna obtusifolia	10.36	3
Total	2654.8	56		3,436.77	148

(1.5%), 61.85 kg of P (0.18%), 580.79 kg of K (1.69%), 154.6 kg of Ca (0.45%), 164.96 kg of Mg (0.48%), and 68.73 kg of S (0.20%) are extracted from the soil by weeds at the expense of pineapple plants.

Those values were estimated based on a study by Souza *et al.* (1999), who evaluated the levels of macro and micronutrients and the C:N ratio of various weed species of the families Commelinaceae, Poaceae, Cyperaceae, Amaranthaceae, Compositae, Convolvulaceae, Euphorbiaceae, Malvaceae, and Rubiaceae.

The accumulation of dry matter in the weed community over the periods of evaluation showed exponential growth, with an increase in the amount of dry matter accumulated as a function of time. The highest dry matter value (3,436.77 g m^{-2}) was observed on the last collection date. An increment of 0.0128 g per d was observed from the sampling times of the weed community in the pineapple crop (Fig. 2A).

From the adjusted model, the absolute and relative growth rates of the weed community were estimated. The absolute growth rate (AGR) is a physiological index used to obtain the average growth speed over the observation period (Hunt, 2012; Soltani & Sinclair, 2012). On the other hand, the relative growth rate (RGR) expresses the increase in dry matter per unit of the initial weight over a period of time (g g^{-1}/d) (Hunt, 2012; Soltani & Sinclair, 2012).

The relative and absolute growth rates showed the same exponential behavior observed for dry matter accumulation (Fig. 2B). The maximum observed AGR value was 22.29 g m^{-2}/d, while the maximum observed RGR was 0.00644 g g^{-1} m^{-2}/d, both observed on the last collection date.

The growth rate is an important feature to describe the ecological strategies of plants. Weeds, in general, show rapid initial growth, allowing them to absorb mineral nutrients and develop in environments without limitations, thus, being able to stabilize in the environment (Ravindra *et al.*, 2008; Korres *et al.*, 2019).

In the analysis of plant growth, the absolute growth rate usually shows an increase until it reaches a peak, with a subsequent fall. The relative growth rate, in turn, decreases over the time of collection. The higher the values of this rate and the longer the time it remains high, the greater the competition capacity of the species in question. Therefore, the weed community showed great competitive capacity with the studied species (pineapple) and that this capacity increased over time. This indicates that, the longer the pineapple is subjected to weed competition, the greater the competition capacity of these species.

During the pineapple growth period, the species that showed the best carbon fixation capacity, in terms of dry matter, were *Ipomoea acuminata*, *Merremia aegyptia*, and *Brachiaria decumbens* (Tab. 2). The other species collected showed the lowest number of individuals and lower dry matter values than those previously mentioned. However, these values can be considered representative since the capacity to extract resources from the soil tends to increase possibly with the higher number of individuals.

Ipomoea acuminata and *Merremia aegyptia* have a climbing habit, which can make certain growing practices and pineapple harvesting difficult. *Brachiaria decumbens*, being a C_4 metabolism plant, tends to be more efficient in the use of scarce resources, especially water, considering the semi-arid climate conditions (Zimdahl, 2018).

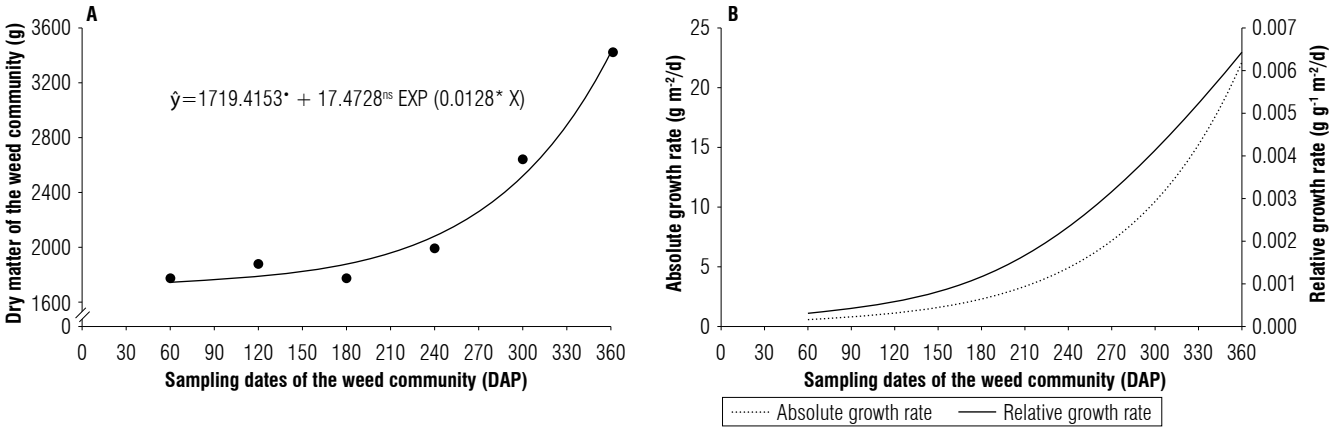

FIGURE 2. A) Dry matter and B) absolute growth rate (g m^{-2}/d) and relative growth rate (g g^{-1} m^{-2}/d) of the weed community as a function of growth sampling dates in the pineapple crop. DAP - days after planting.

At 60 DAP, 217 weed specimens were identified (Tab. 2), with *Brachiaria plantaginea* and *Senna obtusifolia* having the highest number of individuals per species (NIS) and, consequently, the highest relative density (DER), relative abundance (ABR) and importance value index (IVI) (Fig. 3). However, *Ipomoea acuminata* and *Merremia aegyptia* showed higher dry matter compared to the other species.

When the pineapple reached 120 and 180 DAP, 101 and 91 weed specimens were observed, respectively, with the largest number of individuals and amount of dry matter observed for the species *I. acuminata* and *Cenchrus echinatus* (Tab. 2). For these two species, higher values of DER, ABR and IVI were also observed (Fig. 3). However, the highest relative frequency (FRR) was observed only for *I. acuminata*, 180 DAP. At 240 and 300 DAP, there was a reduction in the number of specimens. However, *I. acuminata* remained with a larger number of individuals and showed higher dry matter in this period. This behavior remained similar for FRR, DER, ABR, and IVI (Fig. 3).

Sarmento *et al.* (2017) observed higher frequency (FR) values for the species *Euphorbia heterophylla*, *Cynodon dactylon*, *Dactyloctenium aegyptium,* and *Amaranthus hybridus*

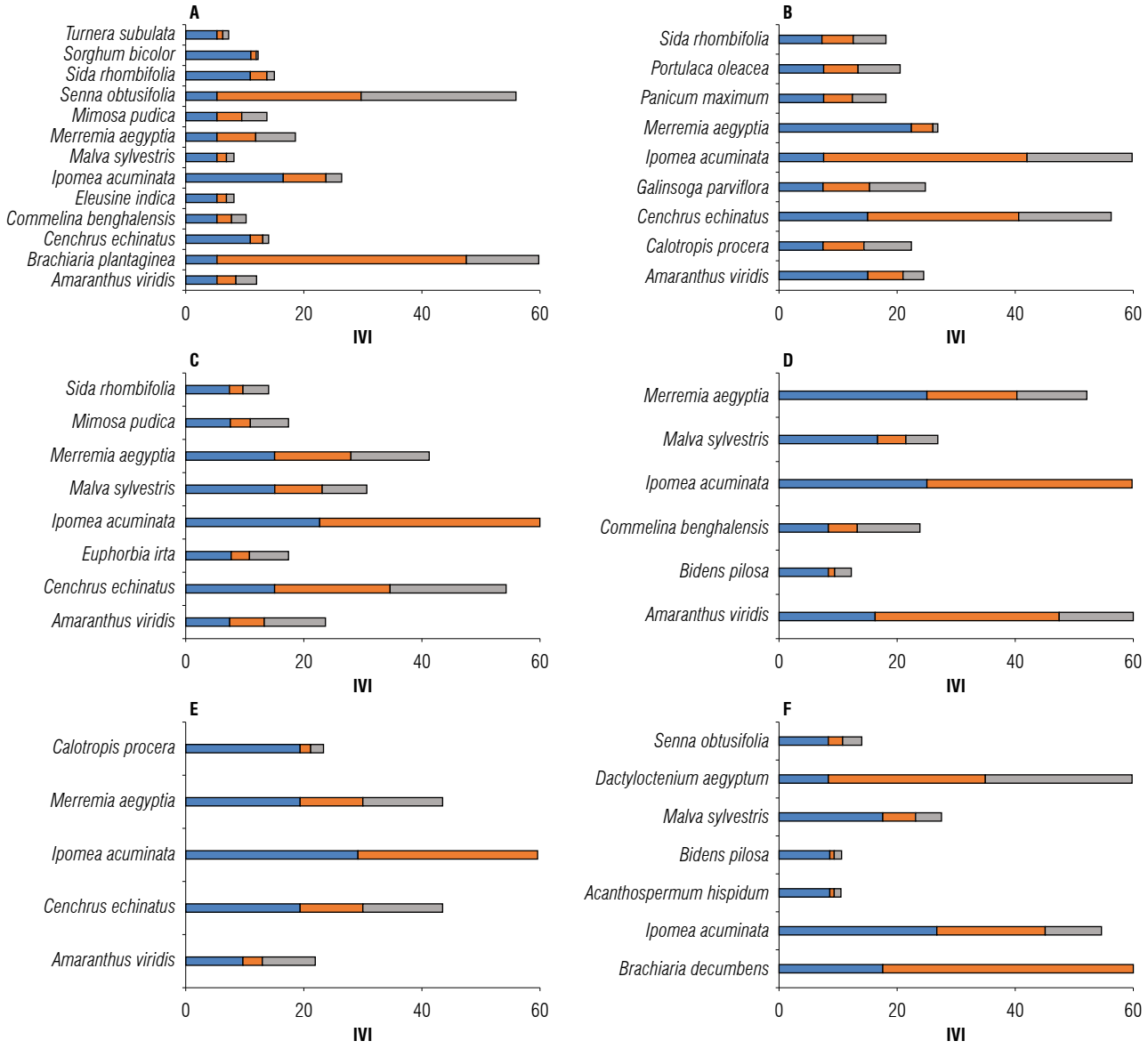

FIGURE 3. Importance value index (IVI) of the main species of weeds collected during the development of pineapple, in days after planting (DAP), cultivated in the semi-arid climate region of Brazil. Blue bar = relative frequency (FRR); orange bar = relative density (DER); gray bar = relative abundance (ABR). A) 60 DAP; B) 120 DAP; C) 180 DAP; D) 240 DAP; E) 300 DAP; F) 360 DAP.

in the spring-summer period of Brazil. The greatest distribution in the autumn-winter period was observed in the species *Euphorbia hirta*, *Amaranthus hybridus*, *Euphorbia heterphylla,* and *Portulaca oleracea*. Unlike this study, the same authors found that the species that showed the highest IVI values were *Cyperus iria*, *Cynodon dactylon*, *Euphorbia heterophylla*, *Amaranthus hybridus*, *Euphorbia hirta,* and *Boerhavia diffusa*. This is because, although the study was conducted under similar climatic conditions, the spatial variation in the distribution of weed species was large, confirming that other factors influence the diversity of the weed community.

Brachiaria decumbens and *Dactyloctenium aegyptum* were found only at 360 DAP, while *I. acuminata* was identified at all survey times (Tab. 2). A greater number of individuals at 360 DAP was observed in the species *B. decumbens*, *D. aegyptum,* and *I. acuminata*, respectively, as well as the greater FRR, DER, ABR, and IVI (Fig. 3).

The highest weed density during the pineapple development cycle was verified at 60 DAP, when 217 individuals per m^2 were counted. The lowest density was observed at 300 DAP, with 56 individuals per m^2 (Tab. 2). According to Radosevich *et al.* (2007), as the density and development of weeds that germinate and emerge at the beginning of the crop cycle increases, interspecific and intraspecific competition intensifies, so that more developed weeds become dominant, and the rest are suppressed or die.

The diversity and density of weeds were higher in the first days after planting, *i.e.*, crop still establishing itself. At this stage, the plant has not yet reached the ideal height of the shoot; therefore, its potential to shade the soil is low, allowing weeds to receive sunlight. Additionally, the pineapple has a slow growth pattern, which makes it uncompetitive against weeds.

At 360 DAP, weed diversity was reduced, as in other survey periods, compared to the first 60 DAP. This probably occurred due to competition between weeds, as some are more aggressive than others, resulting in reduced emergence and/or growth of weeds.

The species that stand out from the others generally have high seed production (to supply the soil seed bank), combined with other mechanisms, such as dispersion, longevity, and dormancy for a long period. Species developing these mechanisms can survive even under adverse conditions (Zimdahl, 2008; 2018; Korres *et al.*, 2019).

Regarding relative dominance (DOR), *I. acuminata* stood out in relation to the other species in all survey periods, except for 300 DAP, when *M. aegyptia* showed the highest DOR value (Tab. 3). At 360 DAP, the species *I. acuminata* (58.11), *B. decumbens* (29.70), and *D. aegyptum* (9.17) stood out. These same species produced a higher number of individuals per species and higher dry matter in the same period.

TABLE 3. Relative dominance (DOR) and weed cover value index (CVI) during the pineapple cultivation cycle in the semi-arid climate region of Brazil.

Sampling moments					
60 d after planting			120 d after planting		
Weed	**DOR**	**CVI**	**Weed**	**DOR**	**CVI**
Amaranthus viridis	5.17173	8.398	*Amaranthus viridis*	2.610656	8.55125
Brachiaria plantaginea	0.19339	42.59	*Calotropis procera*	2.776957	9.70765
Cenchrus echinatus	3.00404	5.308	*Cenchrus echinatus*	10.88609	36.62867
Commelina benghalensis	2.37117	4.675	*Galinsoga parviflora*	3.22676	11.14755
Eleusine indica	0.86326	2.246	*Ipomoea acuminata*	61.95306	96.60652
Ipomoea acuminata	47.6364	55.01	*Merremia aegyptia*	8.251679	11.22198
Malva sylvestris	0.58074	1.963	*Panicum maximum*	6.646746	11.59724
Merremia aegyptia	14.8016	21.25	*Portulaca oleracea*	1.647168	7.587762
Mimosa pudica	2.98779	7.135	*Sida rhombifolia*	2.000887	6.951382
Senna obtusifolia	0.71135	25.14			
Sida rhombifolia	6.45933	9.224			
Sorghum bicolor	13.1115	14.03			
Turnera subulata	2.10771	3.029			
Total	100	200		100	200

Continue

Sampling moments					
180 d after planting			240 d after planting		
Weed	**DOR**	**CVI**	**Weed**	**DOR**	**CVI**
Amaranthus viridis	0.780645	6.27515	*Amaranthus viridis*	32.4191	32.42
Cenchrus echinatus	7.188669	26.96889	*Bidens pilosa*	1.17948	1.179
Euphorbia hirta	2.466612	5.763315	*Commelina benghalensis*	4.88747	4.887
Ipomoea acuminata	78.34526	123.4002	*Ipomoea acuminata*	129.753	129.8
Malva sylvestris	1.971268	9.663575	*Malva sylvestris*	5.64191	5.642
Merremia aegyptia	4.470453	17.65727	*Merremia aegyptia*	26.1191	26.12
Mimosa pudica	1.794921	5.091624			
Sida rhombifolia	2.982174	5.179977			
Total	100	200		200	200
300 d after planting			360 d after planting		
Weed	**DOR**	**CVI**	**Weed**	**DOR**	**CVI**
Amaranthus viridis	5.624597	5.624597	*Brachiaria decumbens*	29.70813	76.32975
Cenchrus echinatus	10.71766	10.71766	*Ipomoea acuminata*	58.113	76.35624
Ipomoea acuminata	73.41792	73.41792	*Acanthospermum hispidum*	0.180402	0.856078
Merremia aegyptia	107.4045	107.4045	*Bidens pilosa*	0.026187	0.701863
Calotropis procera	2.835362	107.4045	*Malva sylvestris*	2.49653	7.901936
			Dactyloctenium aegyptum	9.174312	35.52566
			Senna obtusifolia	0.301446	2.328473
Total	200	200		100	200

The species that stood out in relation to the coverage value index (CVI) were *I. acuminata* and *B. plantaginea* (55.01 and 42.59) at 60 DAP. *Ipomoea acuminata* and *C. echinatus* showed higher CVI values (96.60 and 36.62, respectively) at 120 and 180 DAP. *Merremia aegyptia* showed CVI equal to 17.65 at 180 DAP. In the last survey period, 360 DAP, *I. acuminata* (76.35), *B. decumbens* (76.32), and *D. aegyptium* (35.52) showed higher CVI values compared to the other weeds. According to the CVI, the species *I. acuminata* covered most of the study area during the collection periods.

Known as a common morning-glory, the species *I. acuminata* was present on all collecting dates. This plant has a climbing growth habit, as do the species of the *Merremia* genus, which was also present in almost all surveys but in a smaller number. As these weeds are herbaceous climbing plants that reproduce by seeds and, in general, prefer tilled, fertile soils with good humidity, they can establish easily. As for competition for nutrients, the common morning-glories can be great extractors, as observed by Souza *et al.* (1999) in the sugarcane crop.

Conclusions

The weed community found in irrigated pineapple cultivation under semi-arid climate conditions was composed mostly of species belonging to the families Amaranthaceae, Asteraceae, Convolvulaceae, Fabaceae, Malvaceae, and Poaceae. The highest diversity of weed species was found 60 d after planting. The diversity of weed species decreased over the pineapple growth cycle. The species *Ipomoea acuminata* was present throughout the growth of the pineapple and showed a higher index of importance value in most periods evaluated.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

JRPS, VMM and IA conceptualized and designed the experiment; JRPS, BSS and PMD carried out the field experiment and laboratory analysis; VMM, JRPS, IA and GC contributed to the data analysis; VMM, JRPS, IA and GC

wrote the original draft; VMM, JRPS, IA and GC wrote, reviewed, and edited the article; VMM and IA supervised and managed the project. All authors reviewed the manuscript.

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Economic efficiency of biochar as an amendment for *Acacia mangium* Willd. plantations

Eficiencia económica del biocarbón como enmienda en plantaciones de *Acacia mangium* Willd.

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ABSTRACT

Biochar is a product of pyrolysis obtained from any type of biomass and can be used as a soil amendment or conditioner, improving the physical, chemical, and biological properties of the soil. Additionally, it can serve as an alternative to the application of synthetic fertilization in forest species such as *Acacia mangium* Willd. This research was oriented towards the determination of the economic efficiency of the use of biochar in *A. mangium* compared to the use of synthetic fertilizers. Production costs of wood and by-products, income and profits from forestry, economic efficiency of capital (cost-benefit ratio), labor (wood production per worker), and land (wood production ha^{-1}) were considered. We found that the production of wood using biochar increased by 47% per unit area (ha), by 23% per unit of work (worker), and increased earnings by approximately one million Colombian pesos ha^{-1} compared to the use of only synthetic fertilizers.

Key words: costs, income, labor efficiency, land efficiency, profitability.

RESUMEN

El biocarbón es el producto de la pirólisis que se obtiene de cualquier tipo de biomasa y puede ser usado como enmienda o acondicionador para mejorar las propiedades físicas, químicas y biológicas del suelo. Además, se puede utilizar como una alternativa para reemplazar la aplicación de fertilizantes sintéticos en especies forestales como *Acacia mangium* Willd. Esta investigación se orientó hacia la determinación de la eficiencia económica del uso del biocarbón en *A. mangium* frente al uso de fertilizantes sintéticos. Se consideraron costos de producción de madera y subproductos, los ingresos y ganancias de la actividad forestal, la eficiencia económica del capital (relación costo-beneficio), del trabajo (producción de madera por trabajador) y de la tierra (producción de madera ha^{-1}). Se encontró que la producción de madera con biocarbón se incrementó en un 47% por unidad de superficie (ha), en un 23% por unidad de trabajo (trabajador) y las ganancias aumentaron en aproximadamente un millón de pesos colombianos ha^{-1} respecto al uso de sólo fertilizantes sintéticos.

Palabras clave: costos, ingresos, eficiencia del trabajo, eficiencia de la tierra, rentabilidad.

Introduction

Economic efficiency in agriculture, which includes forestry, is reflected in a better production with the same number of resources or the same production with a lower number of resources. Better production refers to a greater quantity, better quality, higher diversity, or a mixture of the above. For economic efficiency, the prices of resources and products at the time of their measurement are important. It is also important to consider the physical, social, environmental, and political context of the agricultural production being analyzed. Globally, from 1990 to 2020, the increase in planted forest area was 123 million ha, reaching 294 million ha (FAO & UNEP, 2020). In Colombia, the registered area of commercial forest plantations for 2016 was approximately 470,000 ha (Martínez *et al*., 2016).

The cultivation and use of forest species in Colombia are mainly aimed at obtaining wood. However, other additional uses are gaining value, such as the use of forest residues. Proper management of forest residues brings some benefits, such as avoiding contamination *in situ* and the nearby ecosystems. Additionally, these residues constitute a good source of improvement that, in turn, can reduce the use of synthetic fertilizers (Arvanitoyannis *et al*., 2006).

If organic waste, including that of forest species, is subjected to a thermal conversion of biomass in an oxygen-limited

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environment, a solid, fine-grained, and porous product with a high content of organic carbon called biochar is obtained from the pyrolyzed material (IBI, 2013; Ippolito, Donnelly, & Grob, 2015). Biochar stands out for absorbing nutrients and water and reducing the bulk density of the soil (Lehmann, 2007; Reddy *et al*., 2013). Given its stability in the environment (Ippolito, Spokas, *et al*., 2015), biochar generates environmental benefits associated with the reduction of CO_2 in the atmosphere through carbon sequestration in the soil increasing the organic matter content of the soil, and economic benefits by generating emission quantifiers for GHG or CERTS and carbon credits (Antle & McCarl, 2002; Lehmann *et al*., 2003; Post *et al*., 2004). These benefits have allowed biochar to be currently considered as an environmental alternative to the use of synthetic fertilizers in forestry production.

Forest crops such as *Acacia mangium* generate organic residues that are not yet being used properly. In the department of Meta, nine years after cultivation was established, 1 t of biomass was produced for every four usable trees (CONIF, 2013). Thus, in a plantation with a density of 400 plants ha^{-1}, an average of 100 t ha^{-1} of biomass is obtained, whereas in the studied plot this biomass is obtained after 12 years under the same conditions. Of the total biomass, 40% remains in the field as waste, 40% remains in the sawmill as waste, and only 20% is used as wood (SIOC, 2018). These 80 t ha^{-1} of unused residue could be converted into 24 t ha^{-1} of biochar at an efficiency of 30%. The use of this biochar in the same forest crop to replace synthetic fertilizers could have consequent favorable economic and environmental effects.

The economic efficiency of biochar as an amendment was analyzed in a commercial forest plantation of *A. mangium* located in Colombia. This research studied the costs, income, and efficiency of labor, land (yield) and capital (profitability) for the use of biochar vs. the use of synthetic fertilizers in *A. mangium* to determine whether the application of biochar in the soil of an *A. mangium* agroecosystem is viable in economic terms, compared to the conventional agronomic practices of the same plantation.

Materials and methods

Location and characteristics of the study area

The study was carried out in the Planas village, located in the municipality of Puerto Gaitán, department of Meta (Colombia), (between 3°05' and 4°08' N, and between 71°05' and 72°30' W). The area has an average annual temperature of 30°C and a total annual rainfall of around 2,300 mm with a bimodal pattern. The soils that dominate the region are Oxisols and Ultisols. In the Planas village, the soils are Typic Troporthents, shallow and low in bases (IDEAM, 2013). In Planas, there is a commercial *A. mangium* crop belonging to an associative forestry company. At the time of the study, the *A. mangium* crop had an area of 2,100 ha in different stages of development. The first plantations were established in 2008. Between 2017 and 2018, a field trial was carried out on this company's facilities, which served as the basis for the elaboration of a doctoral thesis from which the data for the present article were taken (Reyes Moreno, 2018). Under the same edaphoclimatic conditions of the forest farm, two comparative forms of timber production with different nutrition models were considered: a "standard" crop (ST), with the use of a synthetic fertilizer ("Triple 15" or 15-15-15: nitrogen (N) 15%, phosphorus (P_2O_5) 15%, potassium (K_2O) 15%, YARA, Colombia) and an "optimal" crop (OP) with biochar and synthetic fertilizer applications. The biochar was applied once at the beginning, while the synthetic fertilizer was applied every year in both scenarios. A "real" analysis was performed for ST, and a projection, with data that came from a statistical analysis of response surface (Reyes-Moreno *et al*., 2019), was performed for OP of management and harvesting activities (pruning, thinning, and cutting). The first pruning was carried out in the first year of establishment and then every 15 months. The first thinning was performed in the fifth year and the second in the ninth year. The thinnings provided saleable timber. After the two thinnings, the crop was left with a density of 400 trees ha^{-1} until the time of cutting, which was carried out in year 12. The projected cultural activities of the trial were those corresponding to commercial cultivation and consisted of pruning and thinning. The first pruning was carried out in the first year of establishment, then every 15 months. The thinning was carried out in the fifth and ninth years. Thinning also provided saleable timber material. After thinning, the crop was left with 400 trees ha^{-1} until the time of cutting.

The biochar was obtained from the same plantation according to the methodology of Jouiad *et al*. (2015). Thinning and pruning residues from the commercial *A. mangium* plantation were subjected to slow pyrolysis with a residence time of 14 h and temperatures between 350°C and 400°C in two pyrolytic furnaces (made with local technology) located in the same plantation.

The field information was obtained in two different phases: during the nursery phase, which lasted three months (April

to June 2017), and during the initial growth phase in the field with a duration of one year (July 2017 to July 2018). The field trial consisted in a comparison of the effects of synthetic fertilization and the application of biochar on the growth and biomass gain of the *A. mangium* crop, allowing a projection of future production. The treatments with three replicates are shown in Table 1.

TABLE 1. Comparative trial with three replicates of synthetic fertilizer vs. biochar in the *Acacia mangium* plantation during the establishment and early growth.

Biochar (t ha^{-1})	Synthetic fertilizer 15-15-15 (g/plant per year)		
	0	50	100
0	T_1	T_2	T_3
40	T_4	T_5	T_6
80	T_7	T_8	T_9

Estimated volume of OP wood

Estimates of the wood volume in OP were made using the volume equation of a truncated cone, using the height and radii of the lower and upper bases of the trunk (Eq. 1). Measurements were carried out with a caliper for the radii and tape measure for the height. This approach was confirmed with a destructive pilot sampling to discard the use of the form factor and use the convenience of the truncated cone instead of the oblique one, since it is the most similar three-dimensional geometric shape in practice for the age of the plantation. For the process of optimizing the volume of *A. mangium* wood, two applications of fertilizer were carried out to the soil, each at two concentrations, adjusting a second order model design. In the model, two treatment levels were used, namely 40 and 80 t ha^{-1} of biochar and 50 and 100 g of synthetic fertilizer per plant. Finally, in this analysis, data were obtained for the application of 63.1 t ha^{-1} of biochar at transplanting (seeding) and 84.4 g/plant per year of synthetic "Triple 15" fertilizer. This was done once at crop establishment (Reyes-Moreno *et al*., 2019).

$$A = \pi r^2 \quad (1)$$

where A = area, π = 3,141592, and r = stem radius.

Estimated volume of wood in the standard system (ST)

Projected timber production in the ST was calculated through a non-linear regression developed from the information collected in the above-mentioned trial. To calculate the projection of wood volume (Tab. 2) in the ST, a non-linear regression was used (Eq. 2):

$$E(V|t) = (65{,}7753 + 206{,}741 * Ln(t))^2 \quad (2)$$

where V was the estimated volume (cm^3/plant), E(V|t) was the expected value of the volume given the explanatory variable associated with time (*Ln* is the natural logarithm), and t was time in years.

In the ST crop, 100 g/plant per year of synthetic fertilizer was applied as a crown at the base of each tree.

TABLE 2. Projection of the volume of wood produced (m^3 ha^{-1}) during a cycle under the standard production system in a 600-ha crop of *Acacia mangium*.

Year	Accumulation of biomass (m^3/plant)	Wood available per each plant (%)	Trees harvested ha^{-1}	Wood harvested (m^3 ha^{-1})	Wood harvested (m^3 600 ha^{-1})
1	0.004	0.20	0	0	0
2	0.044	0.20	0	0	0
3	0.086	0.20	0	0	0
4	0.124	0.20	0	0	0
5	0.159	0.20	300	9.53	5,718
6	0.190	0.20	0	0	0
7	0.219	0.20	0	0	0
8	0.246	0.20	0	0	0
9	0.270	0.20	300	16.23	9,738
10	0.294	0.20	0	0	0
11	0.315	0.20	0	0	0
12	0.336	0.20	400	26.88	16,128
Total			1,000	52.64	31,584

The plant density in the plantation in the first year was 1,000 plants ha^{-1}.

Apart from logging, the plantation provides indirect services associated with carbon fixation. The carbon credits corresponded to one metric ton of CO_2 verified by an entity governed by ICONTEC standards. Regarding the carbon reservoir, biomass above ground was considered (only living wood), where 240,000 t were quantified with a value per ton of 15,000 Colombian pesos (in 2018).

Studied variables

The variables used were production costs, income and, therefore, profits and profitability. Additionally, the efficiency of labor and land use for the crops under study were compared (Tab. 3).

TABLE 3. Differences in production costs (millions of Colombian pesos in 2018) between a standard system (ST) and an optimal system (OP) (600 ha).

	ST	OP
Production factors	**Quantity**	
Fixed assets		
Seeding machine	1	1
Chainsaw	3	3
Tractor	3	3
Vehicle	1	1
Sawmill	3	3
Finger machine	1	1
Power plant	2	2
Biochar furnaces	2	4
Facilities	1	1
Inputs		
Pellets (unit)	660,000	660,000
Pesticides (L)	1,220	1,220
Fertilizers (kg)	132,600	112,008
Gasoline (gal)	1,300	2,150
Labor		
Wages	1,557	1,350
Services		
Technical consultant	20	18
Accountant	1	1
Manager	1	1
Secretary	1	1
Soil analysis	10	10
Maintenance of machinery and equipment	31	29
Transportation (gasoline gallons)	2,200	2,200
Land (ha)	600	600

Table 4 shows the differences between the standard and optimal systems in terms of production.

TABLE 4. Differences in production between a standard system (ST) (600 ha) and an optimal system (OP) (600 ha).

Products and by-products	ST	OP
Charcoal (12 kg)	40,000	40,000
Wood (m^3)	31,578	47,052
Carbon credits (t)	38,921	73,710

Results

Production costs

Production costs by stages

Production in the entire cycle in OP is approximately 2% less expensive than in ST (Tab. 5). On average, 1 ha of the *A. mangium* crop costs approximately 30 million pesos per 12-year cycle (2.5 million pesos per year).

Production costs by factors

Direct costs, made up of inputs and labor, are 20% and 23% of total costs for OP and ST, respectively; indirect costs, made up of fixed assets, services and land, are 80% and 77% for OP and ST, respectively. Thus, this activity is high in demand for investment (capital), with a return in the medium (5 years) and long term (9-12 years). Fixed assets are the costs with the highest proportion (64-65%), followed by inputs (13-15%), services (10%), labor (7-8%) and land (4%) (Tab. 6).

Regarding production factors, OP and ST differ fundamentally in labor and the use of fertilizer and biochar. The OP uses more labor than the ST in harvesting and pyrolysis due to higher production, and the ST uses more labor than the OP in the annual application of fertilizer.

Income

Income from forestry is generated by producing charcoal, wood and by fixing CO_2 (carbon credits). The main business is the production of wood.

According to the projected yields, the OP obtains 47% more wood production (78 $m^3\ ha^{-1}$) than the ST (53 $m^3\ ha^{-1}$). The first harvest at year 5 generates 18% of the total wood production, the second at year 9 generates 31%, and the third at year 12 generates 51% (Tab. 7). The production of charcoal from year 5 generates income to cover part of the labor costs (Tab. 8).

TABLE 5. Costs (millions of Colombian pesos in 2018) of production by stages. Standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium.*

Year	Nursery		Establishment of crop		Management of crop		Harvest		Wood produced		Pyrolysis		Total	
	ST	OP	ST	OP	ST	OP	ST	OP	ST	OP	ST	OP	ST	OP
1	172	171	2,189	2,157	0	0	524	521	1,375	1,367	150	298	4,411	4,516
2	0	0	0	0	1,155	1,121	0	0	0	0	0	0	1,155	1,121
3	0	0	0	0	1,155	1,121	0	0	0	0	0	0	1,155	1,121
4	0	0	0	0	1,155	1,121	0	0	0	0	0	0	1,155	1,121
5	0	0	0	0	811	711	194	236	253	254	72	126	1,329	1,327
6	0	0	0	0	1,009	999	0	0	0	0	200	159	1,209	1,158
7	0	0	0	0	1,009	999	0	0	0	0	200	159	1,209	1,158
8	0	0	0	0	996	983	22	25	0	0	198	156	1,215	1,164
9	0	0	0	0	757	695	235	287	236	248	150	111	1,378	1,342
10	0	0	0	0	996	983	22	25	0	0	198	156	1,215	1,164
11	0	0	0	0	1,009	999	0	0	0	0	200	159	1,209	1,158
12	0	0	0	0	691	629	412	466	216	225	137	100	1,456	1,420
Total	172	171	2,189	2,157	10,742	10,361	1,409	1,561	2,080	2,094	1,505	1,424	18,098	17,769

ST: 100 g/plant per year of 15-15-15 used for fertilization. OP: 63.1 t ha^{-1} of biochar plus 84.4 g/plant per year of 15-15-15 used for fertilization.

TABLE 6. Costs (millions in 2018) of production by factors. Standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium.*

Year	Fixed assets		Inputs		Labor		Services		Land		Total	
	ST	OP	ST	OP	ST	OP	ST	OP	ST	OP	ST	OP
1	3,244	3,364	291	260	70	78	152	149	667	667	4,424	4,518
2	750	750	218	186	42	38	154	149	0	0	1,164	1,123
3	750	750	218	186	42	38	154	149	0	0	1,164	1,123
4	750	750	218	186	42	38	154	149	0	0	1,164	1,123
5	750	750	218	195	179	204	154	149	0	0	1,301	1,298
6	750	750	218	186	95	76	154	149	0	0	1,217	1,161
7	750	750	218	186	95	76	154	149	0	0	1,217	1,161
8	750	750	218	186	95	76	154	157	0	0	1,217	1,169
9	750	750	218	195	240	231	154	167	0	0	1,362	1,343
10	750	750	218	186	95	76	154	154	0	0	1,217	1,166
11	750	750	218	186	95	76	154	149	0	0	1,217	1,161
12	750	750	218	195	312	303	154	175	0	0	1,434	1,423
Total	11,494	11,614	2,689	2,333	1,402	1,310	1,846	1,845	667	667	18,098	17,769

Fixed assets (ST): seeder machine (1), chainsaws (3), tractors (3), vehicles (1), sawmills (1), finger machine (1), power plant (2), biochar furnaces (2) and facilities (3 houses, 4 cabins and a dining room).

Fixed assets (OP): seeder machine (1), chainsaws (3), tractors (3), vehicles (1), sawmills (1), finger machine (1), power plant (2), biochar furnaces (4) and facilities (3 houses, 4 cabins and a dining room).

Inputs (ST): pesticides 25 L ha^{-1}/12-year cycle, synthetic fertilizer 900 kg ha^{-1}/12 years, gasoline (18,500 gallons/12-year cycle).

Inputs (OP): pesticides 25 L ha^{-1}/12-year cycle, synthetic fertilizer 747 kg ha^{-1}/12 years, gasoline (2050 gallons/12-year cycle).

Workforce (ST): workers: 84 salaries/12 years.

Labor force (OP): workers: 102 salaries/12 years.

Services (ST): consulting (6), maintenance (5), secretary (1), manager (1) and accountant (1).

Services (OP): consulting (6), maintenance (5), secretary (1), manager (1) and accountant (1).

Land: purchase of land.

TABLE 7. Wood production for a standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium*.

Year	ST		OP	
	Harvested wood (m^3 ha^{-1})	Harvested wood (m^3 600 ha^{-1})	Harvested wood (m^3 ha^{-1})	Harvested wood (m^3 600 ha^{-1})
5	10	5,718	14	8,520
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	16	9,738	24	14,510
10	0	0	0	0
11	0	0	0	0
12	27	16,122	40	24,022
Total	53	31,572	78	47,042

TABLE 8. Income (Colombian pesos in 2018) by forest production for a standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium*.

Year	Products	Unit	Production		Unit price		Income	
			ST	OP	ST	OP	ST	OP
5	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
	Wood	m^3	5,718	8,520	470,000	470,000	3,259,260,000	4,856,400,000
6	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
7	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
8	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
9	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
	Wood	m^3	9,738	14,510	470,000	470,000	5,550,660,000	8,270,700,000
	Carbon credits	t	38,921	73,710	15,000	15,000	583,815,000	1,105,650,000
10	Charcoal	Package	5,000	5,000	12,000	12,000	60,000.000	60,000,000
11	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
12	Charcoal	Package	5,000	5,000	12,000	12,000	60,000,000	60,000,000
	Wood	m^3	16,122	24,022	470,000	470,000	9,189,540,000	13,692,540,000
Total							19,063,275,000	28,405,290,000

Economic efficiency

Economic efficiency of capital

The OP has higher income due to higher production and lower production costs due to less use of synthetic fertilizers. Its profitability is approximately 1.60 compared to 1.05 for the ST (Tab. 9).

Economic efficiency of work

The OP is also superior to the ST in terms of labor efficiency. Thus, a worker in the OP produces approximately 23% more wood than in the ST in a 12-year cycle (Tab. 10).

TABLE 9. Profit and profitability (cost-benefit ratio) (Colombian pesos in 2018) by forest production for a standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium.*

Year	Income		Costs		Earnings	
	ST	OP	ST	OP	ST	OP
1	0	0	4,424,000,000	4,518,000,000	-4,424,000,000	-4,518,000,000
2	0	0	1,164,000,000	1,123,000,000	-1,164,000,000	-1,123,000,000
3	0	0	1,164,000,000	1,123,000,000	-1,164,000,000	-1,123,000,000
4	0	0	1,164,000,000	1,123,000,000	-1,164,000,000	-1,123,000,000
5	3,319,260,000	4,916,400,000	1,301,000,000	1,298,000,000	2,018,260,000	3,618,400,000
6	60,000,000	60,000,000	1,217,000,000	1,161,000,000	-1,157,000,000	-1,101,000,000
7	60,000,000	60,000,000	1,217,000,000	1,161,000,000	-1,157,000,000	-1,101,000,000
8	60,000,000	60,000,000	1,217,000,000	1,169,000,000	-1,157,000,000	-1,109,000,000
9	6,194,475,000	9,436,350,000	1,362,000,000	1,343,000,000	4,832,475,000	8,093,350,000
10	60,000,000	60,000,000	1,217,000,000	1,166,000,000	-1,157,000,000	-1,106,000,000
11	60,000,000	60,000,000	1,217,000,000	1,161,000,000	-1,157,000,000	-1,101,000,000
12	9,249,540,000	13,752,540,000	1,434,000,000	1,423,000,000	7,815,540,000	1,232,954,000
Total	19,063,275,00	28,405,290,000	18,098,000,000	17,769,000,000	9,652,750,000	10,636,290,000
Mean (Colombian pesos ha^{-1} per year)	2,647,677	3,945,179	2,513,611	2,467,917	134,067	147,7263
				Profitability	1.05	1.60

TABLE 10. Work performance (m^3/worker) for a standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium.*

Year	Production of wood (m^3)		*Number of workers		Work performance (m^3/worker)	
	ST	OP	ST	OP	ST	OP
1	0	0	22	28	0	0
2	0	0	12	11	0	0
3	0	0	12	11	0	0
4	0	0	12	11	0	0
5	5,718	8,520	42	33	136	258
6	0	0	22	31	0	0
7	0	0	22	31	0	0
8	0	0	22	31	0	0
9	9,738	14,510	44	56	221	259
10	0	0	22	31	0	0
11	0	0	22	31	0	0
12	16,122	24,022	48	60	336	400
Total/mean	31,572	47,042	302	365	105	129

A worker is active 44 h a week with a monthly salary of $900,000 Colombian pesos (in 2018). Year 1 is dedicated to the nursery and establishment. Years 2 to 8, 10 and 11 are dedicated to management. Year 5 is the first thinning and year 9 the second thinning (wood harvest). Year 12 is of wood harvest.

Economic efficiency of the land

The expected average production of wood ha^{-1} is 53 m^3 and 78 m^3 in the ST and OP respectively; that is, the OP is 47% more efficient in land use than ST. Additionally, OP earnings are approximately 10 times more than ST earnings (Tab. 11).

TABLE 11. Land yield (m^3 ha^{-1} and Colombian pesos in 2018 ha^{-1}) for standard system (ST) vs. optimal system (OP) of a 600-ha crop of *Acacia mangium.*

Year	Wood production (m^3)		Earnings	
	ST	OP	ST	OP
1	0	0	-4,424,000,000	-4,518,000,000
2	0	0	-1,164,000,000	-1,123,000,000
3	0	0	-1,164,000.000	-1,123,000,000
4	0	0	-1,164,000,000	-1,123,000,000
5	5,718	8,520	2,018,260,000	3,618,400,000
6	0	0	-1,157,000,000	-1,101,000,000
7	0	0	-1,157,000,000	-1,101,000,000
8	0	0	-1,157,000,000	-1,109,000,000
9	9,738	14,510	4,832.475,000	8,093,350,000
10	0	0	-1,157,000,000	-1,106,000,000
11	0	0	-1,157,000,000	-1,101,000,000
12	16,122	24,022	7,815,540,000	12,329,540,000
Total	31,572	47,042	965,275,000	10,636,290,000
Mean (ha per cycle)	53	78	1,608,804	17,727,156
Mean (ha per year)	4.4	6.5	134,067	1,477,263

Discussion

The cost difference between the ST and OP systems is relatively small. The OP costs are 2% lower than ST for 185 USD ha^{-1}. However, this small difference is part of the economic advantage of the OP system over the ST system. The application of biochar, like the application of fertilizers, has a cost. Although this study did not focus on this, Williams and Arnott (2010) give us an idea in this regard. Depending on the quantity (2.5 - 50 t ha^{-1}) and the application method (broadcast-and-disk and trench-and-fill), the costs found were between 29 and 300 USD ha^{-1}. The great advantage of using biochar is the increased yield; the OP system has 25 m^3 more production (47%) than the ST system. Higher production and a lower cost lead to an even higher profit, with 60% in the OP system and only 5% in the ST system. The economic advantages of using biochar are also reflected in the efficiency of the use of land and labor resources. The OP system needs more work, but by producing more, it obtains 23% more wood per worker and 47% more per ha of land than the ST system. The economic efficiency of capital is measured through profitability. In our case, the difference between both systems is remarkable. In other studies, such as those of Maraseni (2010), positive results were also found with the addition of biochar. The researchers found that the income per kilogram of wheat went from USD$1098.84 to USD$1741 t ha^{-1} when biochar was applied to the soil. However, in other trials such as those of Ringius (2002), the financial returns of different agricultural practices with the application of various biofuels oscillated between 4.1 and -1.3, values below those found in this research.

Conclusions

The cost of producing *A. mangium* wood under an optimal system (with the use of biochar) is slightly lower than that of a conventional system (with the use of a synthetic-based fertilizer). The production cost in the optimal system includes the purchase of equipment and machinery for the pyrolysis of the organic remains of the plantation as well as the production and use of biochar as a basic addition. Fixed assets make up a large part of the costs.

The higher production of wood (about 50% more) in the optimal system (with the use of biochar) compared to the conventional system increases income and, therefore, profit. Thus, the economic profitability (cost-benefit ratio) of the *A. mangium* crop is 1.60 under an OP whereas under the ST it reaches only 1.05.

The efficiency of labor in the OP is 23% higher than in the ST. OP land efficiency is also higher since the OP produces 47% more wood per ha than the ST.

Thus, from the economic point of view, the OP production of *A. mangium* is more favorable than the ST production; thus, the system becomes an economic and environmentally friendly alternative to produce wood of this species.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

GRM participated in the conceptualization of the initial project from which this article originates, as well as in the data curation, supervision, formal analysis, and research. JCBF wrote, reviewed, and edited the manuscript, and supervised the research activity. EDC participated in the formal analysis and supervision of the research.

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Design and development of a mixed alcoholic beverage kinetics using asaí (*Euterpe precatoria*) and copoazú (*Theobroma grandiflorum*)

Diseño y desarrollo de una cinética de bebida alcohólica mixta de asaí (*Euterpe precatoria*) y copoazú (*Theobroma grandiflorum*)

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ABSTRACT

Copoazú (*Theobroma grandiflorum*), a fruit from the same genus as cacao, and asaí (*Euterpe precatoria*) a palm fruit, both of Amazonian origin, could promote local economic growth through fruit processing to increase the added value. This study aimed to identify the kinetics of alcoholic fruit beverages made from copoazú and asaí pulp or seeds, *i.e.*, the fermentation kinetics in the case of copoazú drinks and the diffusion kinetics in the case of asaí drinks. Additionally, the feasibility of generating a milky mixture with the liquor obtained from the copoazú fruit processing was evaluated. Statistical analysis was performed by ANOVA tests and modeling of kinetics parameters with an evolutionary algorithm and optimization. Copoazú pulp was fermented with 15% Prestige Turbo Yeast®. Fermentation was separated into two stages: controlled fermentation during the first 5 d and a maturation process in the following 25 d. According to the modeling, the greatest efficiency was observed with 600 g L^{-1} pulp concentration and soluble solids adjusted at 35°Brix, with alcohol contents of up to 20% (w/v) after 30 d of processing and evidence that there may be inhibition of fermentation due to glycerol. The whole fruit and pulp of asaí were extracted with ethanol to obtain a liquor with the micronutrients and flavors of the fruit, and the anthocyanin content was used as a degradation process marker. Modelling showed that the optimum point that yielded maximum anthocyanin concentration was achieved at 60 d of maturation by extracting pulp in a 45% (w/v) ethanol solution resulting in a maximum anthocyanin content of 94.2 ± 15.3 mg of cyanidin-3-glucoside kg^{-1} of liquor. After that, a degradation process was observed as anthocyanin content diminished.

Key words: Amazonian fruits, modelling, fermentation, diffusion.

RESUMEN

El copoazú (*Theobroma grandiflorum*), una fruta del mismo género que el cacao, y el asaí (*Euterpe precatoria*) una fruta de palma, ambas de origen amazónico, podrían promover el crecimiento económico local a través de su procesamiento para aumentar el valor agregado. El objetivo de este estudio fue identificar la cinética de las bebidas alcohólicas elaboradas con pulpa o semillas de copoazú y asaí, es decir, la cinética de fermentación en el caso de las bebidas de copoazú y la cinética de difusión en el caso de las bebidas de asaí. Además, se evaluó la viabilidad de generar una mezcla láctea con el licor obtenido del procesamiento del fruto del copoazú. El análisis estadístico se realizó con pruebas ANOVA y el modelamiento de los parámetros de las cinéticas con un algoritmo evolutivo y optimización. La pulpa de copoazú se fermentó con levadura Prestige Turbo® al 15%. La fermentación se separó en dos etapas: fermentación controlada en los primeros 5 d y un proceso de maduración en los siguientes 25 d. De acuerdo con el modelamiento, la mayor eficiencia se obtuvo con una concentración de 600 g L^{-1} y sólidos solubles ajustados a 35°Brix, con contenidos de alcohol de hasta 20% (p/v) después de 30 d de procesamiento y evidencia de la inhibición de la fermentación debida al glicerol. Un proceso de extracción etanólica de los frutos completos y pulpa de asaí se utilizó para obtener un licor con los micronutrientes y sabores de la fruta, y se usó el contenido de antocianinas como marcador del proceso de degradación. El modelamiento mostró que el punto óptimo se alcanzó tras 60 d de maduración al extraer la pulpa en una solución de etanol al 45% (p/v), alcanzando una concentración máxima de antocianinas de 94.2 ± 15.3 mg de cianidina-3-glucósido kg^{-1} de licor. Luego de esto, se observó un proceso de degradación al disminuir el contenido de antocianinas.

Palabras clave: frutos amazónicos, modelamiento, fermentación, difusión.

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Introduction

A mixed alcoholic drink or cocktail is obtained by mixing one or more alcoholic liquids or food-grade ethyl alcohol, either with an agricultural origin of simple alcoholic distillates or with other beverages, such as fruit juice, macerated fruits, syrups, milk, eggs, or other animal or plant-based substances. The alcohol level in these drinks can range between 0.4 and 40 alcoholic degrees (Wardencki, 2019). In general, skills, supplies, and knowledge of food technology and other disciplines are needed to prepare these drinks and achieve a suitable mixture with pleasant sensory properties. Alternatively, an alcoholic drink could be mixed in a bottle dispenser, possibly with two different alcoholic beverages designed for subsequent mixing. In this study, the first drink was made with asaí, a neotropical palm fruit of Amazon origin with grape-shaped berries and a dry and oleaginous pulp that has a high content of antioxidants, such as anthocyanins, and a low amount of carbohydrates (Castillo *et al*., 2012). This means that alcoholic fermentation cannot be carried out without adding sugars; therefore, this fruit was used to make an infused liquor. On the other hand, copoazú, a fruit from the *Theobroma* genus, like cacao and also of Amazon origin, has a higher content of carbohydrates in its pulp than fruits in the same class, which allows alcoholic fermentation (Duarte *et al*., 2010). Its profile of acids and sugar contents allows for mixing milk with the resulting liquor according to the bromatological composition (Tab. 1). The asaí and copoazú liquor extracts can be mixed in a cocktail.

This study aimed to identify the kinetics of alcoholic fruit beverages made from copoazú and asaí pulp, *i.e*., the fermentation kinetics in the case of copoazú drinks and the diffusion kinetics in the case of asaí drinks. Predicting the behavior of these drinks during preparation would facilitate correct decision-making for production conditions and provide the best quality product for subsequent cocktail preparation, thus avoiding an excessive number of trials and wasting raw materials (Wardencki, 2019).

A fermentation kinetic model was developed from the Monod model for copoazú alcoholic beverages because of its ability to represent microbial behavior, providing modifications for particular processing conditions (Gao *et al*., 2018; Miller & Block, 2020). On the other hand, a diffusive process was used for the asaí alcoholic beverages, which is the most appropriate for modeling with Fick's law, using the continuity equation and considering degradation factors, interactions, and diffusion of the main component as a follow-up to the variable anthocyanin content (Chung *et al*., 2016; 2017; Miller & Block, 2020).

Materials and methods

Fruits

Asaí and copoazú fruits were purchased from local producers in the Department of Amazonas. Ripe, washed, packed whole, or pulped fruits were selected at the agroindustry pilot plant of the SINCHI Institute in the city of Leticia, and sent to Bogotá properly refrigerated to perform the experiments.

Copoazú must preparation

Copoazú fruit pulp was diluted with water at varying concentrations between 400 and 600 g L^{-1} and adjusted with a

TABLE 1. Nutritional content of asaí and copoazú (modified from Cuellar Álvarez *et al*. (2017), Carrillo Bautista *et al*. (2016); Carrillo Bautista *et al*. (2017), Castillo *et al*. (2012) and Castillo Quiroga *et al*. (2017)).

Nutrient	Unit	Asaí		Copoazú	
		Pulp	Seed	Pulp	Seed
Fat	g/100 g	36.96	15.04	3.60	32.80
Fiber	g/100 g	42.43	36.29	16.00	22.00
Carbohydrates	g/100 g	18.28	NS	52.30	30.90
Protein	g/100 g	0.03	0.06	10.90	11.50
Calories	kcal	284.90	464.50	284.90	464.50
Ash	mg/100 g	2290	2600	232.10	689.90
Anthocyanins (Cyanidin 3-glucoside)	mg kg^{-1}	1136.31 ± 204		12.84 ± 0.39	
β-Carotene	mg/100 g	7.45		2.44 ± 0.47	
Citric acid	mg/100 g	NS		2186.90 ± 198.70	
Malic acid	mg/100 g	NS		49.20 ± 43.20	

NS - Not specified.

sucrose solution to 15, 25, or 35°Brix (Duarte *et al*., 2010; Dias *et al*., 2017; Wardencki, 2019) as shown in Table 2. These tests were performed in triplicate.

TABLE 2. Design of the copoazú alcoholic beverage fermentation experiment.

Trial	Substrate concentration (g L^{-1})	Soluble solids (°Brix)
A	400	15
B	400	25
C	400	35
D	600	15
E	600	25
F	600	35

Fermentation trials

Each must was fermented in 3 L batches for 5 d using 15% Prestige Turbo Yeast, *Saccharomyces cerevisiae*, controlling the temperature at 22°C. The pH was adjusted to 4.5 by adding calcium carbonate, and potassium metabisulfite to inhibit bacterial growth at a concentration of 100 g L^{-1} of free sulfur dioxide (SO_2) (Duarte *et al*., 2010; Dias *et al*., 2017). The fermented must was then matured for 25 d and the slurry was removed every 5 d, adding fresh water to replace the retired volume, and keeping the batch always at 3 L volume.

Infusion trials

Asaí fruit or pulp was mixed with extra neutral alcohol at a concentration of 15% (w/v), varying the concentration: 15, 30, or 45% (w/v) for the diffusion process as seen in Table 3. The infusion was kept in a maturation process for 90 d, and samples were collected on days 1, 2, 4, 8, 15, 30, 60, and 90 since the phenomenon of diffusion lessens over time, meaning that more time is needed between measurements to observe changes in the concentration of the analytes of interest in the beverage.

TABLE 3. Experiment design for the asaí alcoholic beverage formulation.

Formula	Asaí pulp (%)	Asaí fruit (%)	Water (%)
A	15	0	75
B	30	0	60
C	45	0	45
D	0	15	75
E	0	30	60
F	0	45	45

Analytical methods

Fermentation kinetics

The Monod model was used to model the behavior of the sugar contents, biomass, and ethanol concentration, with some variations to inhibit microorganisms with ethanol and glycerol. The following differential equations were used to observe variations in the three factors in fermentation and an equation for inhibition of the specific growth rate (Kumar *et al*., 2013; Comelli *et al*., 2016; Miranda Castilleja *et al*., 2017):

Biomass: $$\frac{dx}{dt} = x\mu e^{-\frac{K_L S_a}{t}} \quad (1)$$

Substrate: $$\frac{dS}{dt} = -\frac{\mu}{Y_{\frac{x}{S}}} x e^{-\frac{K_L S_a}{t}} \quad (2)$$

Ethanol: $$\frac{de}{dt} = \left(Y_{\frac{e}{x}}\mu x + \gamma x\right) e^{-\frac{K_L S_a}{t}} \quad (3)$$

Specific growth rate: $$\mu = \frac{\mu_{max} S}{S + K_S + K_i S^2} \quad (4)$$

Where K_i is the inhibition constant expressed in L g^{-1}, K_s is the saturation constant expressed in g L^{-1}, K_L is the lag constant expressed in L h g^{-1}, μ is the specific growth rate expressed in h^{-1}, μmax is the maximum specific growth rate expressed in h^{-1}, $Y_{x/S}$ is the interaction between the biomass and substrate (adimensional), $Y_{e/x}$ is the interaction between the ethanol and substrate (adimensional), S is the substrate content expressed in g L^{-1}, x is the biomass content expressed in g L^{-1}, is the ethanol content expressed in g L^{-1}, γ is the ethanol production kinetic constant expressed in h^{-1}, S_a is the amount of sugar added expressed in g L^{-1}, and t is the time expressed in h.

Regression was used on the experimental data, finding the inhibition constant, the saturation constant, the lag constant, and the maximum specific growth rate with an evolutionary model that minimized the differences between theoretical and experiment data (Kumar *et al*., 2013; Comelli *et al*., 2016; Miranda Castilleja *et al*., 2017). Subsequently, linear regression was used to calculate the interaction between the biomass and substrate and the interaction between the ethanol and substrate with the graphical representation x_i-x_0 vs. S_i-S_0, where the slope of the line was $Y_{x/S}$ and graphing e_i-e_0 vs. x_i-x_0 showed $Y_{e/x}$; these graphs resulted in several lines, each corresponding to a processing condition, which was proportional to the amount of added sugar (Comelli *et al*., 2016).

Total soluble solids and biomass content

The fermentation kinetics were observed by measuring the total soluble solids (°Brix) every 30 min with an Atago PAL-1 pocket refractometer (Atago, Japan). For the biomass, the optical density of the must was measured every 30 min by taking 1 ml of sample and placing it in a quartz cell, with the measurement taken at a wavelength of 600 nm in a Thermo Scientific Evolution 60S UV-Visible spectrophotometer (Thermo Fisher Scientific, USA), following the adjusted methodologies of Bermejo *et al*. (2011).

Alcohol content

A sample of the liquid phase was taken from the fermenter and placed in a cylinder, covering the alcoholometer (ECO, Spain) but allowing the contents to move around freely. The alcoholometer was immersed, gently rotated, and left to stabilize. The corresponding reading was taken in the lower meniscus, according to Miller (2019), as adapted for fruit wines.

Infusion kinetics

Anthocyanin content

The infusion kinetics were observed by measuring the anthocyanin content of the asaí infusion, following the methodology of Li *et al*. (2017), modified for the measurement of infusion liquors. One hundred mg of the sample was weighed in triplicate and 5 ml of analytic grade methanol, acidified with 5% formic acid, was added. Each mixture was vortexed for 10 min and centrifuged for 45 min at 15,000 rpm. Two hundred µl of supernatant was taken and the pH was adjusted to 1.0 (0.025 M KCl buffer) and 4.5 (0.4 M Sodium acetate buffer), adding 2 ml of the buffer in each case. This measurement was obtained in triplicate using a Thermo Scientific Evolution 60S UV-Visible spectrophotometer (Thermo Fisher Scientific, USA) at the wavelengths 520 nm and 700 nm. Finally, the anthocyanin content was calculated using the following equation:

$$C = \frac{PM \times V \times (A_{520nm} - A_{700nm})_{pH=1.0} - (A_{520nm} - A_{700nm})_{pH=4.5}}{P \times \varepsilon} \quad (5)$$

where PM is the molar weight of cyanidin-3-glucoside (449.2 g /mol), ε is the molar extinction coefficient (25.964 /mol cm^{-1}), P is the sample's fresh weight, V is the volume of added methanol, A is the absorbance at each pH and wavelength, and C is the anthocyanin content [mg of cyanidin-3-glucoside kg^{-1}] (Salaha *et al*., 2008; Li *et al*., 2017; Li *et al*., 2018).

Oxidative rancidity (Fat acidity index)

The methodology described by the United States Pharmacopeial Convention (2013) was followed to determine this index, in which 2.5 g of the fresh sample was weighed in an Erlenmeyer flask, to which 50 ml of a mixture of alcohol and ether was added at a 1:1 w/w ratio (this mixture was neutralized with 0.1 N potassium hydroxide, observing the turning point with phenolphthalein). The homogeneous mixture was titrated with 0.1 N potassium hydroxide until the solution was slightly pink for 30 sec. The acid number was the number of hydroxide ions necessary to neutralize 10 g of sample, calculated as follows:

$$Oleic\ Acid\ \% = \frac{282.4 \times V \times N}{W} \times 100 \quad (6)$$

where 282.4 is the molecular weight of oleic acid, V is the volume in ml, N is the normality of the potassium hydroxide solution, and W is the fresh weight of the sample (United States Pharmacopeial Convention, 2013).

Profile of sugar and organic acids

High-Performance Liquid Chromatography (HPLC) was used to measure the organic acids. First, 100 mg of sample was weighed and extracted with 3 ml of 5 mM sulfuric acid for 10 min in a Vortex. Then, the solution was centrifuged at 10,000 rpm for 45 min. Finally, the remaining liquid was removed, which was filtered with 0.45 µm PTFE membranes before injection in an Aligent 1200 Series HPLC (Aligent, USA). A 300 mm x 7.8 mm HPX-87H column was used, with a refractive index detector (RID) and diode array detector (DAD), 5 mM sulfuric acid as the mobile phase, and a flow of 0.5 ml/min. The peaks were detectable at 243 nm (Topalovic & Mikulic-Petkovsek, 2010).

Statistical data analysis

For the analysis of variance, the data obtained in triplicate in each variable were processed in IBM SPSS Statistics 25 with an ANOVA test finding significant differences between the treatments with a probability of 95%. Matlab 2019b was used for the parameters of the kinetic equations, optimizing the parameters with an evolutionary algorithm and the optimization toolbox sandbox, which progressively minimized the differences between the theoretical and experimental data.

The data were analyzed to determine the parameters of each kinetic, fermentation kinetics for the copoazú, and diffusion kinetics for the asaí. The kinetics were determined for

the most appropriate process parameters in each case to subsequently mix both liquors.

Results and discussion

Fermentation kinetics

Sugar and organic acid profile

The liquors had a relatively high content of citric acid (Tab. 4), which was consistent with these fruits, while the content of other acids was due to the acid transformation in the reactions of the citric acid cycle activated in the reoxidation of NADH to NAD^+. This is also because the glycolysis reactions were maintained since, under anaerobic conditions, NAD^+ is regenerated through the conversion of pyruvate to lactate by lactate dehydrogenase. This conversion was probably the result of lactic acid bacteria present in most naturally. This production can significantly increase the acidity of a wine as well as result in soft aromas and a sweet flavor, improving the sensory profile for subsequent mixing with milk for cream liqueurs (Vasantha Rupasinghe *et al*., 2017). These components can affect the quality of the product, and volatile components can disturb the taste and smell of the final product, leading to possible applications of the final liquor in cocktails and general consumption (Pugliese *et al*., 2013; Reboredo-Rodríguez *et al*., 2015).

The amounts of citric acid, succinic acid, and acetic acid showed significant differences between the treatments with different concentrations of copoazú, while the concentrations of sugars glucose, ribose, and glycerol significantly differed between the treatments with different amounts of sugar. These results denote a stronger relationship between the amount of added sugar and complex sugars in copoazú and the amount of ethanol produced, including glycerol, as observed by other authors when fermenting fruit must (Vasantha Rupasinghe *et al*., 2017). The amount of sucrose could have been due to incomplete fermentation because of the inhibitory factors mentioned above. However, this value was lower than that of glucose possibly because of the affinity of the strain used by the glucose, resulting in accelerated fermentation in the treatments with more added sugar. Similar results were found by Vasantha Rupasinghe *et al*. (2017) in fruit wines when studying the inhibition of by-products.

The chromatogram (Fig. 1) shows that the retention time of 16.8 min had a peak that corresponded to glycerol, leading to the question of whether everything that was fermented became ethanol or if glycerol was favored at some point in the metabolic pathway.

In kinetics, this serves as an inhibitory factor to produce ethanol and biomass (Fig. 2) as well as for substrate consumption (Vasantha Rupasinghe *et al*., 2017), as reflected in the specific growth rate (Fig. 3).

Fermentation process

During the first stage of the fermentation process, the number of microorganisms and the amount of soluble solids remained stable. This phase is known as the adaptation phase of microorganisms, which is followed by a reduction in the number of soluble solids in the must (Fig. 2) and an increase in microorganisms. This is the exponential phase of the process, where both parameters stabilized in the final stage.

TABLE 4. Analysis of variance of the sugar and organic acids profile in the copoazú alcoholic beverages during the fermentation process.

Compound	Treatment (substrate concentration (g L^{-1}) / Soluble solids (ºBrix))					
	A (400 /15)	B (400/25)	C (400/35)	D (600/15)	E (600/25)	F (600/35)
Organic acids						
Citric	15.44 ± 0.43a	15.46 ± 0.82a	15.22 ± 1.23a	23.36 ± 0.9b	23.13 ± 0.31b	22.97 ± 0.03b
Pyruvic	0.55 ± 0.04a	0.57 ± 0.02a	0.54 ± 0.03a	0.83 ± 0.02a	0.81 ± 0.05a	0.81 ± 0.01a
Malic	1.85 ± 0.13a	1.84 ± 0.13a	1.88 ± 0.08a	2.87 ± 0.2a	2.69 ± 0.1a	2.72 ± 0.01a
Succinic	3.63 ± 0.23a	3.61 ± 0.24a	3.56 ± 0.08a	5.55 ± 0.31b	5.28 ± 0.33b	5.45 ± 0.06b
Lactic	0.54 ± 0.03a	0.56 ± 0.04a	0.53 ± 0.02a	0.83 ± 0.04a	0.84 ± 0.06a	0.84 ± 0.01a
Acetic	5.67 ± 0.30a	5.87 ± 0.05a	5.64 ± 0.25a	8.54 ± 0.29b	8.63 ± 0.33b	8.93 ± 0.01b
Sugars						
Sucrose	6.10 ± 0.28ab	4.22 ± 0.07a	0.65 ± 0.08c	3.25 ± 0.03b	1.45 ± 0.05d	0.72 ± 0.01c
Glucose	0.44 ± 0.04a	0.30 ± 0.01a	0.05 ± 0.01b	0.24 ± 0.01a	0.11 ± 0.00b	0.05 ± 0.01b
Ribose	2.94 ± 0.21a	2.10 ± 0.12a	0.31 ± 0.03b	1.59 ± 0.07c	0.71 ± 0.02b	0.35 ± 0.01b
Glycerol	14.13 ± 1.12a	9.70 ± 0.57b	1.54 ± 0.26c	7.44 ± 0.10c	3.32 ± 0.12d	1.66 ± 0.01c

n = 3. Measurements with the same letter do not show significant differences according to the ANOVA ($P<0.05$). All results are expressed in g of the substance fresh weight L^{-1}.

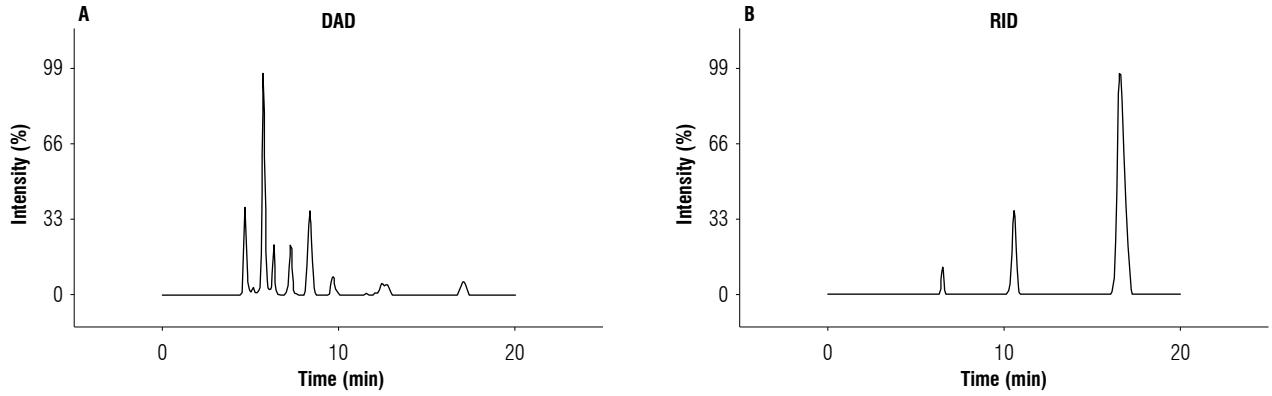

FIGURE 1. HPLC chromatogram for the profile of sugars and organic acids in the copoazú alcoholic beverages. A) DAD - Diode array detector; B) RID - refraction index detector.

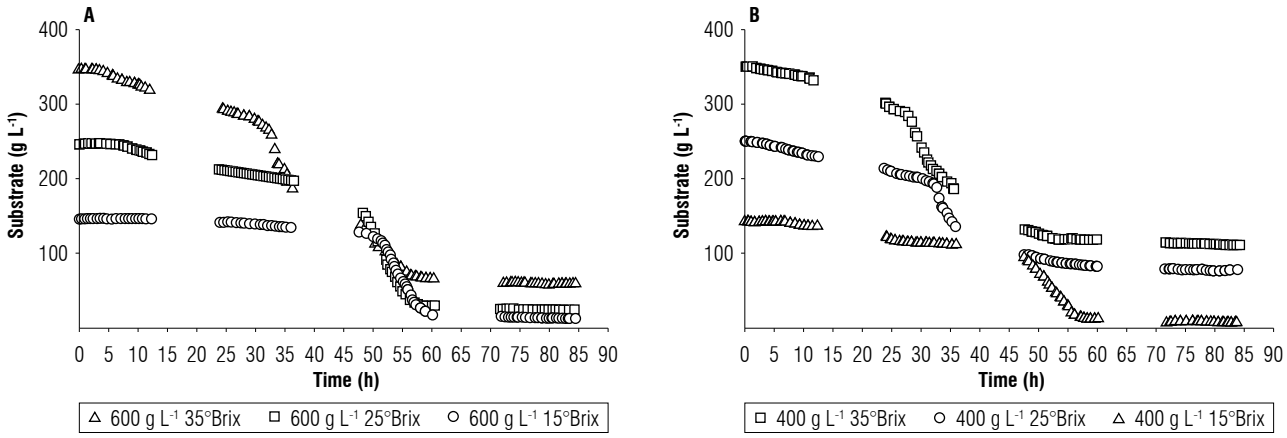

FIGURE 2. Comparison of fermentation performance with substrate variations at A) 600 g L^{-1} and B) 400 g L^{-1} of copoazú of different soluble solid content.

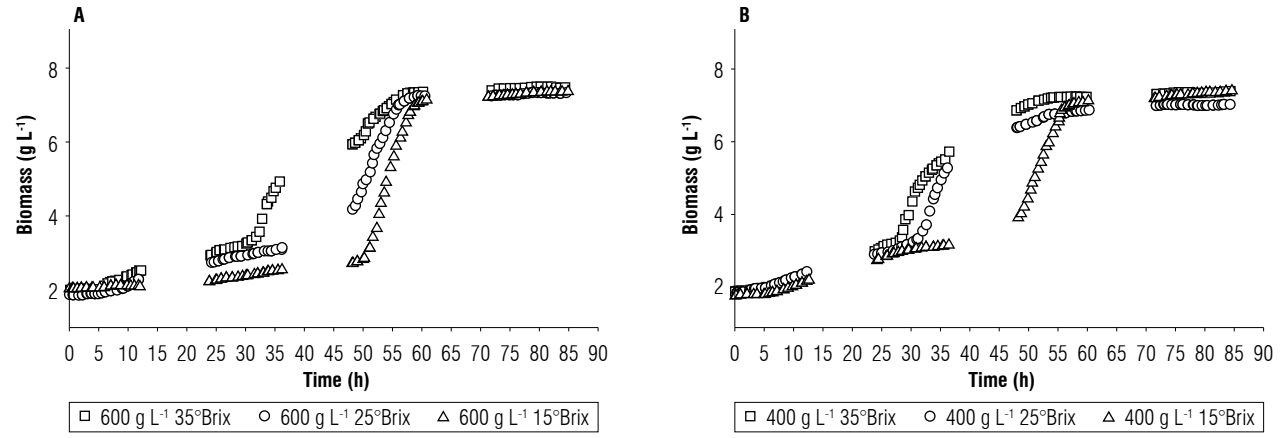

FIGURE 3. Comparison of fermentation performances for biomass variations at A) 600 g L^{-1} and B) 400 g L^{-1} of copoazú.

Figure 2 shows that the content of dissolved solids decreased considerably in the tests with less substrate because the microorganisms initially assimilated less complex sugars. Also, glucose is more easily assimilated by yeast than fructose, making it more effective at generating alcohols from these sugars and inhibiting the production of glycerol. Similar results were found by Gao *et al.* (2018) and Zinnai *et al.* (2013) when working with *Saccharomyces cerevisiae* strains to produce wines. These authors observed that glycerol is generated under alkaline conditions, where

the metabolic pathway of glycerol-3-phosphatase is modified, causing phosphatase to react, forming glycerol. This means that the yeast needed a large amount of energy to convert glycerol into pyruvate and, in turn, pyruvate to ethanol (Arroyo-López, Querol, & Barrio, 2009; Vasantha Rupasinghe *et al*., 2017).

For biomass (Fig. 3), growth followed the phases of microbial growth; in the exponential phase, growth was relatively fast when compared to the same types of fermentation because the biomass production was approximately 1% of the amount of substrate consumed, with an average of 2.89 ± 0.81%. This was possible because of the higher energy consumption caused by the increase in the amount of glycerol during fermentation (Arroyo-López, Orlić, *et al*., 2009).

For the ethanol production (Fig. 4), the growth in relation to substrate consumption followed the following stoichiometry:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

The ethanol yield, on average, varied between 47 and 65%, depending on the amount of substrate used. These results were relatively low because the yield of the conversion of sugars to ethanol was 95% on average (Zinnai *et al*., 2013; Vasantha Rupasinghe *et al*., 2017; Miller & Block, 2020), further indicating that glycerol may be inhibiting the metabolic process of fermentation. Nevertheless, fermentation reached contents higher than 20% ethanol; this allows subsequent mixing with dairy ingredients because the mixture did not need to be "fortified" with extra neutral alcohol to maintain the final alcohol content (Wardencki, 2019).

Fermentation kinetics parameters

Figures 5 and 6 show that the slope changed depending on the amount of added sugar (S_a), demonstrating the interdependence between these two variables. Therefore, linear regressions with these slopes were proposed to consider the sugar content (Arroyo-López, Querol, & Barrio 2009; Miller & Block, 2020).

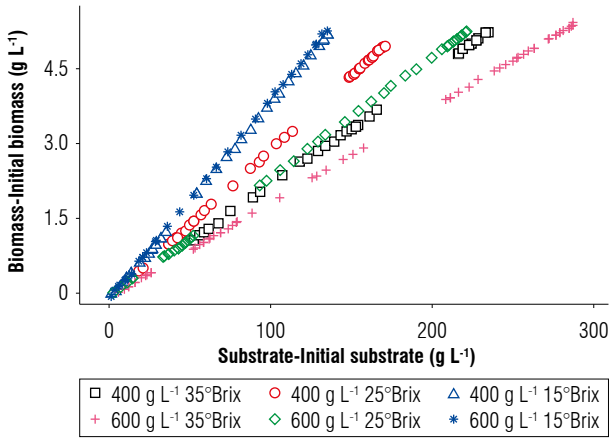

FIGURE 5. Evaluation of the experiment yields for the biomass/substrate interaction ($Y_{x/S}$) with a diagram of biomass formation versus substrate consumption.

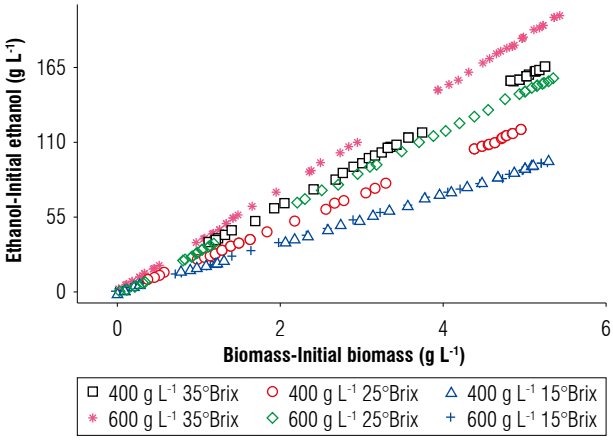

FIGURE 6. Evaluation of the experiment yields for the interaction of ethanol/biomass ($Y_{e/x}$) with a diagram of the formation of ethanol versus biomass.

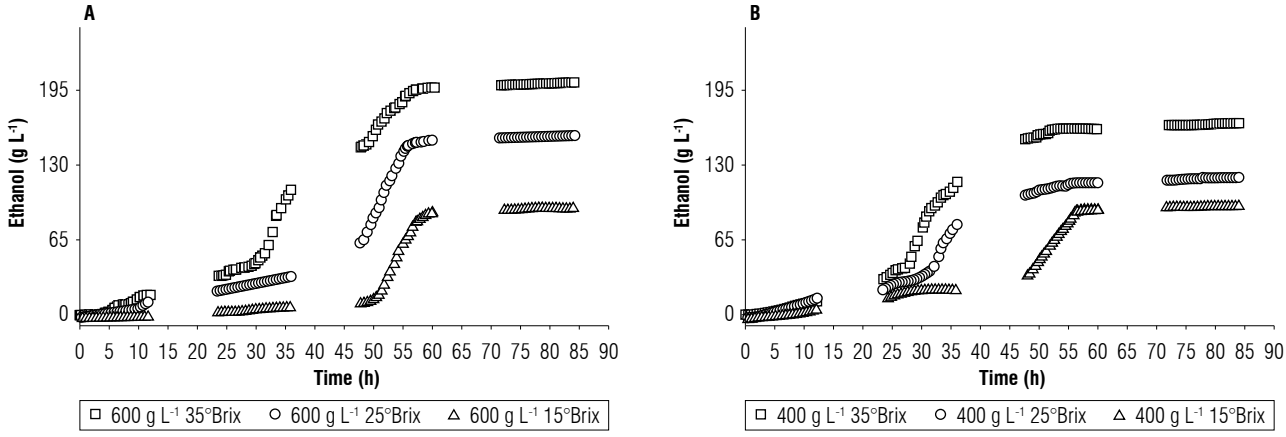

FIGURE 4. Comparison of fermentation yields for variations of ethanol at A) 600 g L^{-1} and B) 400 g L^{-1} of copoazú.

Interaction between substrate and biomass:

$$Y_{\frac{x}{s}} = m_{\frac{x}{s}} S_a + b_{\frac{x}{s}} \quad (7)$$

Interaction between ethanol and biomass:

$$Y_{\frac{e}{x}} = m_{\frac{e}{x}} S_a + b_{\frac{e}{x}} \quad (8)$$

The data predicted the behavior of the biomass and substrate, calculating the missing factor, *i.e.*, the kinetic constant of the ethanol production (γ) through the reduction of least squares (Merger *et al.*, 2016).

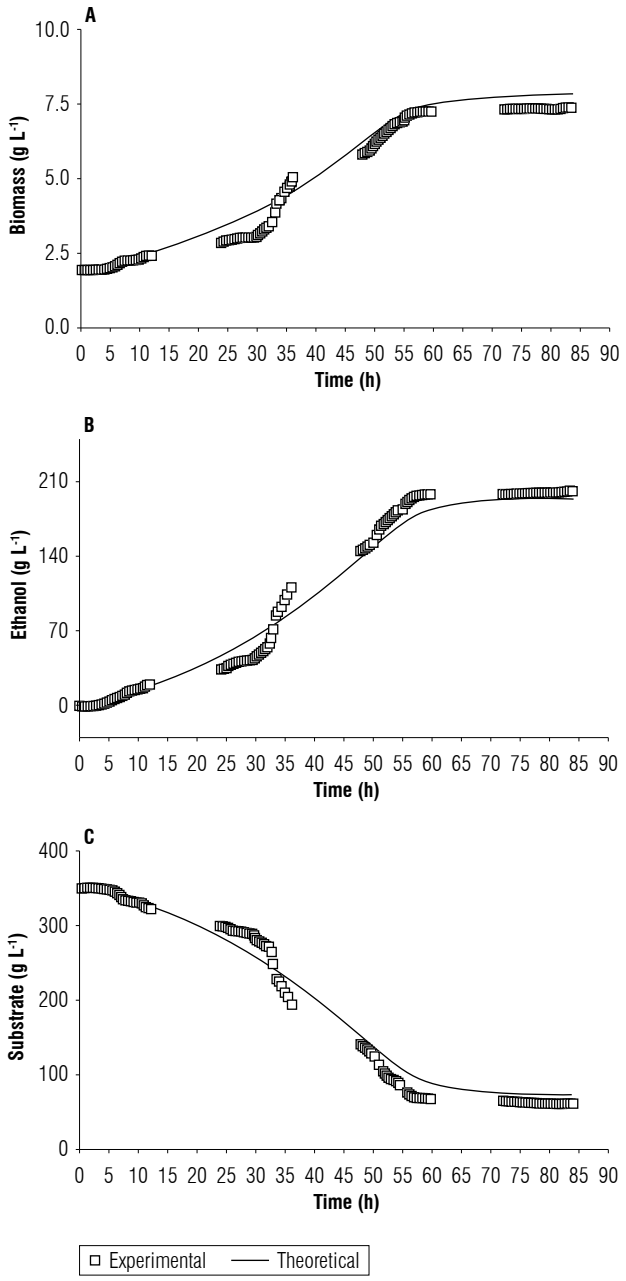

FIGURE 7. Evaluation of the variation between the kinetic model of fermentation and the experiment data for the contents of A) biomass, B) alcohol, and C) substrate in the fermentation of copoazú.

Fermentation kinetics parameters (Tab. 5) showed that the model adjusted favorably to the prediction of the experiment behavior of the substrate, biomass, and ethanol (Fig. 7). Additionally, the best yield was obtained with the treatment with 600 g L^{-1} of the substrate, adjusted with glucose at 35°Brix, and ending fermentation at approximately 60 h for subsequent maturation.

TABLE 5. Constants of the fermentation kinetics.

Constant			Value
Interaction between biomass and substrate	$Y_{x/S}$	$m_{x/S}$	-8.800E-05
		$b_{x/S}$	4.522E-02
Interaction between ethanol and biomass	$Y_{e/x}$	$m_{e/x}$	7.680E-02
		$b_{e/x}$	1.216E01
Ethanol production kinetic constant		γ	4.151E-03
Maximum specific growth rate		μ_{max}	6.061E-01
Saturation constant		K_s	1.328E03
Inhibition constant		K_i	3.861E-02
Adaptation constant		K_L	6.369E-05

Diffusion kinetics

Anthocyanin content and peroxide value of the asaí liqueur

The anthocyanin content initially increased and subsequently decreased (Fig. 8). For the asaí pulp, these readings resulted from the degradation of anthocyanins over time and the interaction processes with the pulp, reaching diffusive equilibrium. The lower anthocyanin content may have been due to the smaller transfer surface, although the drastic drop in a much shorter time compared to that of the pulp suggested that this behavior resulted from something in addition to the balance in the diffusion (Fig. 8) (Li *et al.*, 2017; Li *et al.*, 2018; Boeira *et al.*, 2020).

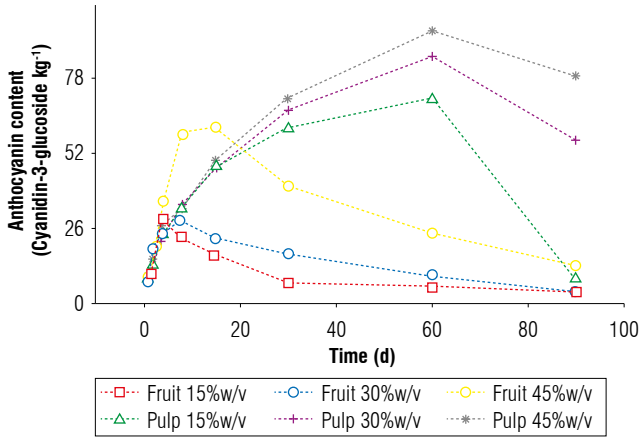

FIGURE 8. Anthocyanin content during the diffusive process of the asaí drink.

A lipid layer of fat was observed on the surface of the drinks because the fruits have a fat content of approximately 53% on a dry basis (Yuyama *et al*., 2011; Castillo *et al*., 2012). These lipids could contribute to oxidative rancidity, which was confirmed by measuring the acidity index of the fat in the beverages throughout the infusion, with changes in the index when the anthocyanin content decreased (Fig. 9). This demonstrated that the decrease in the antioxidant capacity and discoloration of the alcoholic beverages were strongly related to the rancidity of the fats in the liquor (Salaha *et al*., 2008; Andersen & Skibsted, 2010; Peixoto *et al*., 2016). This is why whole fruits were not utilized for the infusions and only the pulp was used, which had a lower percentage of fat that did not have a considerable negative effect on the drinks.

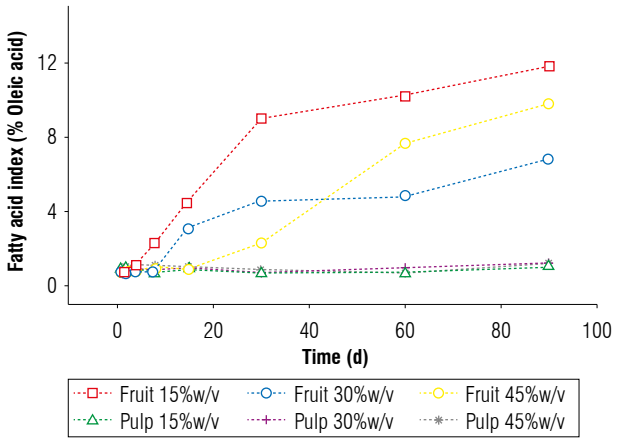

FIGURE 9. Fatty acid index during the diffusive process of the asaí drink.

Diffusion kinetics parameters

It was not possible to adjust the anthocyanin content data during diffusion (Fig. 8) to Fick's law, which is the most common model for diffusive processes since it has variables that affect the content of this metabolite. Therefore, a mathematical model was proposed to understand diffusion kinetics that considers the saturation or interaction of anthocyanins in the system and the deterioration of anthocyanins, either by lipids or light, using the following equation:

$$dC/dt = A + B + D + E \tag{9}$$

where A refers to the diffusive process originating from the pulp; this phenomenon is inversely proportional to time and depends on the initial pulp quantity, which is inversely proportional to the anthocyanin concentration in the beverage and to time. B refers to the interaction of anthocyanins within the drink, which is inversely proportional to time; D is the degradation of these anthocyanins over time, which is inverse to the concentration of anthocyanins in the drink, and E is the transfer constant of this process (Shafirstein *et al*., 2004; Chung *et al*., 2016; 2017; Miller & Block, 2020). Each of these terms is expressed mathematically as follows, where C is the concentration expressed in mg of cyanidin-3-glucoside kg^{-1}, t is time in days, and dC/it is the variation of the concentration over time.

$$A = \frac{C_0 m_0 D_{PW}}{t} \tag{10}$$

where C_0 is the initial concentration of anthocyanins in the pulp, m_0 is the initial fresh mass of the pulp in kg, and D_{PW} is the diffusion constant.

$$B = \frac{K_I C}{t} \tag{11}$$

where K_I is the interaction or saturation constant.

$$D = -\frac{K_D t}{C} \tag{12}$$

where K_D is the decay constant.

$$E = K_t \tag{13}$$

where K_t is the transfer constant.

Replacing these terms in the equation provides:

$$\frac{dC}{dt} = \frac{C_0 m_0 D_{PW}}{t} + \frac{K_I C}{t} - \frac{K_D t}{C} + K_t \tag{14}$$

An evolutionary model was used to find the constants, which minimized the differences between the theoretical and experimental data. The initial values used for the estimation were obtained from the mean values of the segmented slopes of the experiment data, using the interaction constant for the diffusion constant from d 1 to 4, the transfer constant from d 4 to 15, the interaction constant from d 15 to 60, and the decay constant from d 60 to 90, obtaining the values in Table 6.

TABLE 6. Diffusion kinetics constants of the asaí infusion process.

Constant		Value
Diffusion constant	D_{PW}	1.090E-06
Interaction constant	K_i	5.091E-01
Decay constant	K_D	2.190
Transfer constant	K_t	8.418E-01

These parameters, when applied and compared to the experiment data, showed no significant difference (Fig. 10).

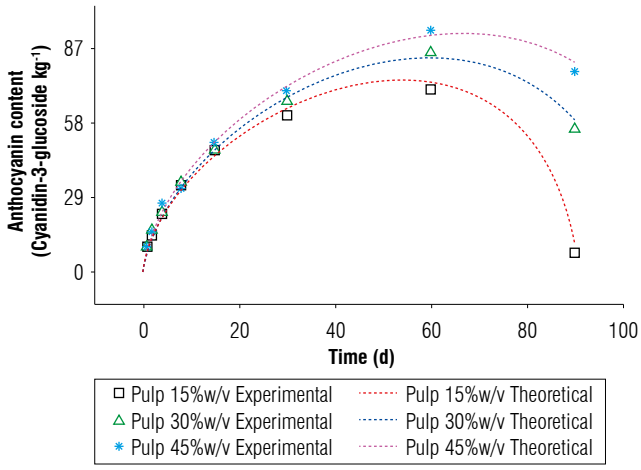

FIGURE 10. Evaluation of the variation between the diffusive model and the experiment data for the diffusion of asaí in the alcoholic beverage.

Conclusions

This study developed kinetic models for copoazú fermentation and asaí diffusion process, to obtain optimum liquor in each case.

In the copoazú liquor, ethanol production was favored by more complex sugars since they delay the inhibition process, either with ethanol or glycerol. The dependence of both products was evidenced in the values of the inhibition and saturation constants that defined the specific growth rate that, in turn, considerably affected the rate of change for the biomass, substrate, and alcohol. The best yield was observed with 600 g L^{-1} of copoazú pulp and glucose at 35 °Brix, achieving yields of up to 20% w/v in the alcohol content.

In the asaí liquor, when the fruit was used instead of the pulp, the fruit lipids caused unfavorable reactions that deteriorated the organoleptic and sensory properties of the liquor. This is an important factor in the relationship between the interaction constant and the decay constant since they define the turning point in the predictive behavior of the anthocyanin content, with the maximum near d 60. The best behavior was seen with the pulp at 45% (w/v) since the greater surface facilitated the exchange and transfer of mass. Additionally, a lower fat content was recorded, thus avoiding rancidity that deteriorates organoleptic characteristics. Anthocyanin content proves to be a good marker of beverage properties deterioration.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

WQM formulated the overarching research goals and aims, conducted the research process, performed the experiments, managed and coordinated the research activity planning and execution, analyzed the study data, designed the methodology, and created the models. RODS designed the methodology and carried out the manuscript's critical review. MSHG obtained the financial support for the project leading to this publication, designed the methodology, and carried out the manuscript's critical review.

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SCIENTIFIC NOTE

Abundance of *Beauveria* spp. and *Metarhizium* spp. in maize and banana agroecosystems in central Cuba

Abundancia de *Beauveria* spp. y *Metarhizium* spp. en agroecosistemas de maíz y banano en el centro de Cuba

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ABSTRACT

Entomopathogenic fungi are an ecological alternative for the control of agricultural pests. These fungi live in organic matter in the soil and can cause natural epizootics in many arthropods associated with the rhizosphere. The aim of this study was to evaluate the abundance of *Beauveria* and *Metarhizium* spp. in maize and banana agroecosystems in central Cuba. Selective medium and insect baiting methods were used to isolate the entomopathogenic fungi from the soil. *Metarhizium* spp. were significantly more abundant than *Beauveria* spp. in both types of fields of agroecosystems. The abundance of *Metarhizium* spp. was higher in Sagua la Grande than in Santa Clara and Camajuaní municipalities. The insect bait method resulted as the most successful way to isolate entomopathogenic fungi from soil. These results show the composition of the entomopathogenic fungi in different agroecosystems, and they are an advance in the understanding of their ecology.

Key words: entomopathogenic fungi, fungal diversity, insect bait method, selective medium.

RESUMEN

Los hongos entomopatógenos son una alternativa ecológica para el control de plagas agrícolas. Estos hongos viven en la materia orgánica contenida en el suelo y pueden causar epizootias naturales a muchos artrópodos asociados a la rizosfera. El objetivo de este estudio fue evaluar la abundancia de *Beauveria* y *Metarhizium* spp. dentro de los agroecosistemas de maíz y banano en el centro de Cuba. Se utilizaron los métodos de medio selectivo e insecto cebo para aislar los hongos entomopatógenos del suelo. *Metarhizium* spp. fue significativamente más abundante que *Beauveria* spp. en ambos agroecosistemas. La abundancia de *Metarhizium* spp. fue mayor en Sagua la Grande que en los municipios de Santa Clara y Camajuaní. Además, el método de insecto cebo constituye el más apropiado para aislar hongos entomopatógenos. Estos resultados muestran la composición de los hongos entomopatógenos en diferentes agroecosistemas y constituyen un avance en la comprensión de su ecología.

Palabras clave: hongos entomopatógenos, diversidad fúngica, método del insecto cebo, medio selectivo.

Introduction

Entomopathogenic fungi constitute an important biotic component in the natural regulation of arthropod populations (Meyling & Eilenberg, 2007). *Beauveria* spp. have been found in several ecosystems worldwide including forest, seminatural habitats, and agricultural fields (Clifton *et al*., 2015). In contrast, *Metarhizium* spp. are more abundant in temperate regions, but not in colder regions (Steinwender e*t al*., 2015).

These entomopathogenic fungi show potential as microbial control agents against different agricultural pests, and they can be artificially reproduced. Among the attributes of these fungi, we can mention a high mortality of the targeted pest population, high genetic diversity across a wide number of strains, infection of multiple life stages, penetration through the integument, and capacity for both horizontal and vertical transmission (Destefano *et al*., 2004; Jaronski, 2014).

The environmental and ecological variations within ecosystems have become a major factor influencing the biocontrol effects of *Beauveria* and *Metarhizium* species. A more detailed understanding of environmental and ecological

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interactions, especially the distributions of these fungi in different ecological areas, is needed to improve consistency in the control capacity of these fungi. In this sense, particular stages in the life cycle of *Beauveria* spp. and *Metarhizium* spp., including their persistence and dispersal in the environment, are unresolved in Cuba. The aim of this study was to evaluate the abundance of *Beauveria* spp. and *Metarhizium* spp. in maize (*Zea mays* L.) and banana (*Musa paradisiaca* L.) fields or agroecosystems. These are the most important crops in Cuba.

Materials and methods

Field sampling

Field samplings were conducted from April to July 2018 in three maize (*Zea mays* (L.), cv. 'Jibara') and three banana (*Musa paradisiaca* (L.), cv. 'Grande Naine') fields located in three municipalities in Villa Clara province, Cuba. The selected municipalities were Camajuaní (22°28'4" N, 79°43'26" W), Santa Clara (22°24'49" N, 79°57'58" W) and Sagua la Grande (22°48'24" N, 80°4'32" W), where five collection points spaced 20 m apart were selected in each of the maize and banana fields. Two soil samples of 500 g each were collected with a garden spade around selected points to a depth of about 15 cm after removal of surface litter. The garden spade was disinfected with 70% ethanol between every collection to avoid contamination (Klingen *et al*., 2002). The soil samples from each point were placed into polyethylene bags and transferred to the Microbiology Laboratory at the Universidad Central "Marta Abreu" de Las Villas. Collected soil samples were thoroughly homogenized by hand and stored at 4°C until processing.

Isolation methods

The selective medium and the insect bait methods were used to isolate entomopathogenic fungi from soil samples. The first method was used through serial dilutions of soil in a culture medium, and the insect bait method employed the use of *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae. *Galleria mellonella* larvae were used for this purpose due to their high susceptibility to many fungal pathogens and because they are commercially reproduced in the Entomophagous and Entomopathogenic Reproduction Center in Cuba. A growth selective medium for *Beauveria* spp. and *Metarhizium* spp. was formulated using saboraud dextrose agar (SDA) (BioCen, Cuba) according to Meyling and Eilenberg (2007). The SDA culture medium was mixed with 1 mg L^{-1} (w/v) of thiabendazole, 0.05% streptomycin sulfate, and 250 mg L^{-1} (w/v) of chloramphenicol to avoid bacterial and some saprophytic fungi.

One g of each soil sample was placed in 20 ml of sterile distilled water with 0.01% Tween 80® in a 40 ml flat bottom glass tube. The tubes were mixed by vortexing for 1 min, and 100 µl of the soil solution was serially diluted to 10^{-3} conidia/ml and then inoculated into Petri dishes (9 cm diameter) with the selective medium described above. The Petri dishes with the soil dilution were incubated at 25 ± 1°C, and 75% relative humidity (RH) in the dark, for the emergence of fungal colonies. There were four replicates for each sample.

The insect bait method was conducted with the use of *G. mellonella* larvae. Soil samples (500 g) were placed in glass containers (500 ml) and five healthy 5-week-old *G. mellonella* larvae, obtained from the Entomophagous and Entomopathogenic Reproduction Center in Santa Clara, Cuba, were added. To prevent cocoon production and further webbing, *G. mellonella* larvae were conditioned before they were added by immersion of the larvae in water at 56°C for 15 sec, followed by the pouring of cold water at 4°C for 30 sec. Finally, the immobile larvae were placed on paper towels until they regained their movement (Woodring & Kaya, 1988). Containers were covered with lids perforated with 15 holes for aeration and placed at 25°C, 90% RH in the dark. No food was provided for the larvae. Containers were inverted every day to ensure that the larvae remained exposed to the soil. They were checked every two days for mortality until all larvae were dead. All cadavers were rinsed with distilled water and transferred to a moist chamber in Petri dishes (9 cm diameter) with moistened filter paper to stimulate fungal growth. A total of 150 larvae were used and the evaluations lasted 24 d. When larvae showed external fungal growth, the fungi were isolated on SDA chloramphenicol (250 mg L^{-1} (w/v)) and incubated at 25 ± 1°C and 90% RH in the dark. Colony colors were treated according to Kornerup and Wanscher (1984).

Entomopathogenic fungi identification

Entomopathogenic fungal isolates obtained from the soil were mounted on standard microscope slides (7.5 x 2.5 cm) and then mixed with a drop of lactophenol. Glass coverlips (2.5 x 5.0 cm and 0.16 cm thick) were then attached to the slide and sealed with resin. Fungal isolates were morphologically identified under a compound microscope (Motic, USA, 400x magnification) according to morphological characteristics described by Humber (2012) for each fungal species. The fungal isolates were kept in refrigeration at 4°C in tubes with SDA in the culture collections at the Departamento de Agronomía, Universidad Central "Marta Abreu" de Las Villas and the Instituto de Investigaciones Fundamentales en Agricultura Tropical "Alejandro de

Humboldt" (WDCM 853). Abundance was determined through the number of samples in which *Beauveria* and *Metarhizium* were found.

Statistical analysis

Analysis of variance (ANOVA) was applied to evaluate differences in frequencies of entomopathogenic fungi in maize and banana fields as well as to compare the effectiveness of the isolation methods. Means of entomopathogenic fungi were separated using Fisher's least significant difference (LSD) test. ANOVA were run using STATGRAPHICS Plus 5.1 (Manugistics Inc.) with significance level of 0.05.

Results and discussion

A total of 151 fungal isolates were obtained from the different maize and banana fields with both selective medium and insect bait methods. The identified entomopathogenic fungi are described below:

Beauveria spp.

Colonies on SDA attaining 50 mm in 7 d at 25°C, cottony at center, radially sulcate to filamentous toward the filiform margin, white (Fig. 1). Reverse colonies were reddish at the center and yellow around the periphery. Mycelium superficial and immersed. Hyphae septate, branched, hyaline, smooth, 1-2 µm wide. Conidiogenous cells polyblastic, lageniform, integrated or discrete, indeterminate ampulliform to subcylindrical at the base, geniculate, sympodial extended forming a rachis, with several distinct or inconspicuous denticles at the conidiogenous loci, arise from aerial hyphae. Conidia solitary, acropleurogenous, globose, unicellular, smooth-walled, hyaline, dry with 3.1 µm of diameter.

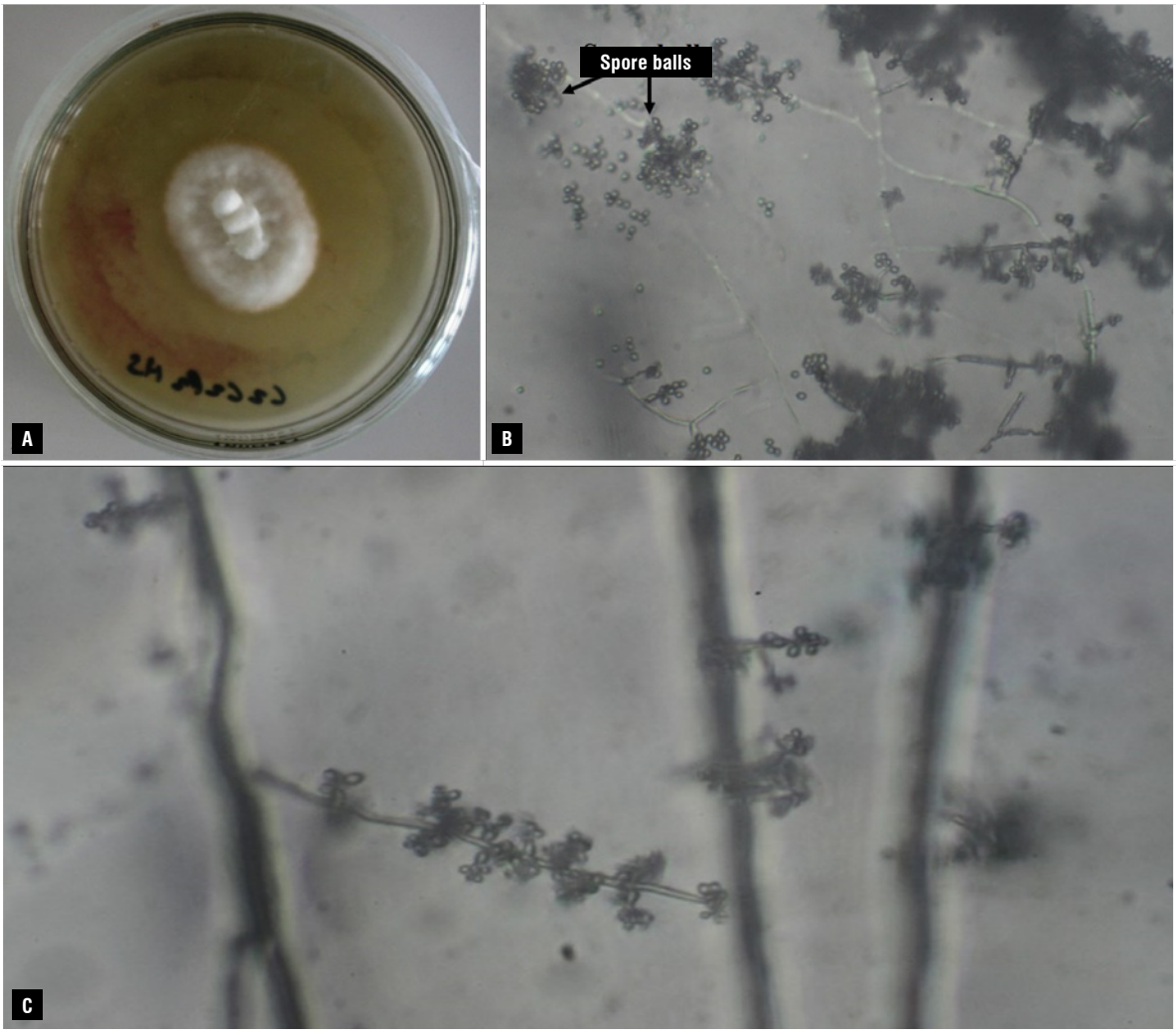

FIGURE 1. *Beauveria* sp. obtained from maize and banana fields. A) Colony of *Beauveria* sp. on SDA culture medium 7 d after inoculation at 25ºC. B) Spore balls representing dense clusters of large numbers of conidiogenous cells and conidia. C) Conidium formed successively on each denticle.

Metarhizium spp.

Colonies on SDA attaining 80 mm in 7 d at 25ºC, cottony to floccose at center, curled toward the slightly filiform margin that is colored white, with several sporodochial conidiomata, green or olivaceous (Fig. 2). Reverse was brownish. Mycelium was superficial and immersed. Hyphae were septate, branched, hyaline, smooth, 1-2 µm wide. Conidiomata were sporodochial, columnar, scattered or confluent, green, olivaceous to olivaceous brown. Conidiophores were macronematous, septate, penicillate or irregularly branched, hyaline, smooth, forming a compact cluster or clumps in the sporodochial conidiomata. Conidiogenous cells were monophialidic, cylindrical, discrete, determinate, smooth, hyaline. Conidia were basocatenulate, cylindrical, truncated at the ends, unicellular, pale olivaceous 6-8 × 1.5-2 µm, accumulating in a columnar, dark olivaceous masses.

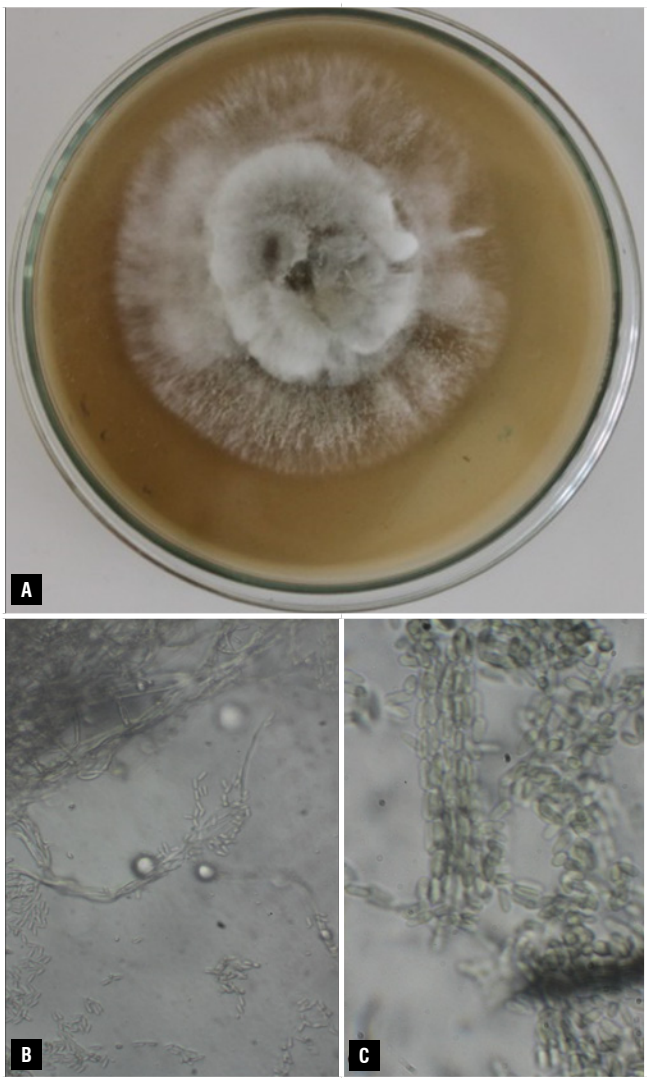

FIGURE 2. *Metarhizium* spp. obtained from maize and banana fields. A) Colony of *Metarhizium* spp. on SDA culture medium 7 d after inoculation at 25ºC. B) Branched conidiophore. C) Conidial chains.

A total of 36 and 52 *Metarhizium* spp. isolates were obtained from maize and banana fields, respectively. This fungal species was significantly (F=15.30; *df*=1; *P*=0.0001) more abundant than *Beauveria* spp., which were represented by 25 and 38 isolates in both the maize and banana fields. *Beauveria* spp. and *Metarhizium* spp. were the most frequently found entomopathogenic fungi in Mexican agroecosystems, and *Beauveria bassiana*, *Beauveria pseudobassiana* and *Metarhizium robertsii* were widely distributed (Pérez-González *et al*., 2014). Our results were in accordance with the results obtained by Korosi *et al*. (2019) who obtained more *Metarhizium* spp. (33%) than *Beauveria* spp. (26) in Australian vineyard soils. The abundance and diversity of entomopathogenic fungi have not been reported in maize and banana fields in Cuba before and, thus, constitutes a new record for the country.

Beauveria spp. isolates obtained from Santa Clara (10), Camajuaní (10) and Sagua la Grande (13) municipalities did not show significant differences (*P*>0.05) in abundance. However, *Metarhizium* spp. isolates in Sagua la Grande (19) were higher in number of infected larvae (F=10.18; *df*=2; *P*=0.0001) than in Santa Clara (13) and Camajuaní (12) (Tab. 1).

TABLE 1. Abundance (number of infected larvae) of *Beauveria* spp. and *Metarhizium* spp. obtained from maize and banana fields in three municipalities in Villa Clara, Cuba.

Location	Entomopathogenic fungi	
	***Beauveria* spp. (mean ± SE)**	***Metarhizium* spp. (mean ± SE)**
Santa Clara	10 ± 0.77 ab	13 ± 1.88 b
Camajuaní	10 ± 0.94 ab	12 ± 1.61 b
Sagua la Grande	13 ± 1.86 a	19 ±1.74 a

Different letters in the same column indicate significant differences in the abundance of *Beauveria* spp. and *Metarhizium* spp. isolates according to the Fisher's test (*P*<0.05).

These results can be supported by the fact that *Metarhizium* is reported to be more abundant than other entomopathogenic fungi in cultivated fields (Tkaczuk *et al*., 2014). In contrast, Pérez-González *et al*. (2014) obtained 112 *Beauveria* spp. and 9 *Metarhizium* spp. isolates from the soil of 11 locations of Guanajuato State, Mexico. These results demonstrated that the abundance and distribution of entomopathogenic fungi is still unclear, and more studies are needed to clarify this aspect. However, the abundance of *Metarhizium* spp. over *Beauveria* spp. in banana and maize fields in Cuba could be explained

through the hypothesis that the association of *Metarhizium* spp. with insect host species has a tropical origin. In addition, *Metarhizium* comprises an assemblage of cryptic species, many of which traverse large geographical barriers (Bidochka & Small, 2005).

Biotic (interaction with other species) and abiotic factors (mainly temperature) are considered primary determinants of abundance and population genetic structure of *Metarhizium* (McGuire & Northfield, 2020). According to these data we infer that the tropical conditions of Cuba allowed a greater abundance of *Metarhizium* spp. in banana and maize fields compared with *Beauveria* spp.

The mean of *Beauveria* spp. (22) and *Metarhizium* spp. (30) isolates recovered with the insect baiting method were higher (F=25.12; *df*=1; *P*=0.0018) than those obtained with the selective medium (10 *Beauveria* spp. and 15 *Metarhizium* spp. isolates) (Tab. 2).

TABLE 2. Abundance of *Beauveria* spp. and *Metarhizium* spp. obtained by selective medium and insect bait methods.

Isolation method	Entomopathogenic fungi	
	***Beauveria* spp. (mean ± SE)**	***Metarhizium* spp. (mean ± SE)**
Selective medium	10 ± 0.70 b	15 ± 0.60 b
Insect bait	22 ± 0.99 a	30 ± 1.35 a

The selective medium indicates the number of colonies per Petri dish, while insect bait shows the number of infected larvae. Different letters in the same column indicate significant differences in the abundance of *Beauveria* and *Metarhizium* isolates obtained with selective medium and insect bait methods according to the Fisher's test ($P<0.05$).

Different results have been obtained about methods of isolating entomopathogenic fungi in the same soil sample (Hernández-Domínguez *et al*., 2016). Our results demonstrated that the insect bait method is better for obtaining entomopathogenic fungi. The selective medium is targeted at particular fungal species, while insect baiting could detect a larger number of species (Keller *et al*., 2003). However, Tkaczuk *et al*. (2014) did not find difference in *Metarhizium* spp. from organic fields using the insect baiting and selective medium methods. The possible explanation for this result is focused on the insect bait method. The absence of water within the plastic boxes could limit the growth of the entomopathogenic fungi. In a similar study conducted by Ramos *et al*. (2017) the authors used sterile water to moisten the soil before introducing it to the plastic boxes. Water contents in the soil helps to maintain a high relative humidity which in turn helps the growth of the entomopathogenic fungi (Lazzarini *et al*., 2006; Jaronski, 2009).

Conclusion

According to our results, the entomopathogenic fungi *Metarhizium* spp. were significantly more abundant than *Beauveria* spp. in both maize and banana plots. The abundance of *Metarhizium* spp. in Sagua la Grande was higher than in Santa Clara and Camajuaní. The insect bait method resulted in the most appropriate method to isolate entomopathogenic fungi from soil. These results contribute to a better understanding of hypocrealean fungi ecology and their composition in both maize and banana fields in central Cuba.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

YR and OP designed the experiment. YR, ADT, CA and AA conducted the experiment. YR performed the statistical analysis. ADT, CA, ALA and RCR wrote the initial draft. YR, RCR and OP wrote the final version of the manuscript. All authors have reviewed the manuscript.

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Acknowledgments

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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