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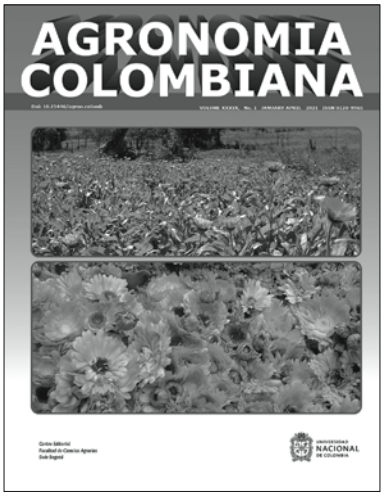

Our cover:

Content and distribution of micronutrients in calendula (*Calendula officinalis*) grown in Valle del Cauca, Colombia. Article on pages: 59-67.

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On behalf of the entire team of the journal *Agronomía Colombiana*, I express my gratitude to Professor Héctor Mauricio Parra Quijano, PhD who served as Editor-in-Chief of this prestigious journal between 2017 and 2020. His important labor in this period allowed *Agronomía Colombiana* to continue being one of the best agricultural science journals in the country. His impeccable editorial work maintained the scientific quality that has always characterized our journal. As a result of his excellent editorial effort, the impact generated has allowed the journal to be re-projected to quartile three (Q3) of the SCImago Journal and Country Rank (SJR) classification, a quartile that we hope to maintain and improve in future indexations. We wish Professor Parra many successes in his new position with our Faculty.

En nombre de todo el equipo de la revista *Agronomía Colombiana*, le expreso mi agradecimiento al Profesor PhD Héctor Mauricio Parra Quijano, quien fue Editor en Jefe de esta prestigiosa revista entre los años 2017 y 2020. Su importante labor en este periodo permitió que *Agronomía Colombiana* continuara siendo una de las mejores revistas en ciencias agrarias del país. Su impecable trabajo editorial mantuvo la calidad científica que siempre ha caracterizado a nuestra revista. Como resultado de su excelente labor como Editor, el impacto generado ha permitido proyectar nuevamente la revista al cuartil tres (Q3) en la clasificación del SCImago Journal and Country Rank (SJR), cuartil que esperamos mantener y mejorar en futuras indexaciones. Le deseamos muchos éxitos al Profesor Parra en su nuevo cargo en nuestra Facultad.

STANISLAV MAGNITSKIY
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Commercial substrates and nutrient concentrations in the production of arugula microgreens

Sustratos comerciales y concentraciones de nutrientes en la producción de microvegetales de rúgula

Albertina Radtke Wieth[1*], Wagner Dutra Pinheiro[1], and Tatiana da Silva Duarte[1]

ABSTRACT

The objective of this study was to evaluate the effects of different substrates and concentrations of nutrient solutions in the production of arugula (*Eruca sativa* Miller) microgreens grown in a protected environment at the campus of the Faculty of Agronomy of the Federal University of Rio Grande do Sul (UFRGS), in Porto Alegre, Brazil. The treatments consisted of the combination of five commercial substrates, CSC® vermiculite (S1), Green-Up phenolic foam (S2), S10 Beifiur® organic (S3), Carolina Soil® seedlings (S4), and Carolina Soil® organic (S5) and three concentrations of nutrients in the nutrient solution (0, 50, and 100%). A 5×3 factorial arrangement was used, in a completely randomized experimental design with three replicates. The addition of nutrients in the irrigation solution favored substrates S1, S4, and S5. Substrate S2 showed better performance with the addition of 50% of the total concentration of nutrients. Substrate S3 without the addition of the nutrient solution showed average values very close to the use of the nutrient solution, which can be considered in the evaluation of production costs of microgreens, generating savings to producers.

Key words: *Eruca sativa* Miller, crops, soilless cultivation, protected environment.

RESUMEN

El objetivo de este estudio fue evaluar los efectos de diferentes sustratos y concentraciones de soluciones nutritivas en la producción de microvegetales de rúgula (*Eruca sativa* Miller) cultivados en un ambiente protegido en el campus de la Facultad de Agronomía de la Universidad Federal de Rio Grande do Sul (UFRGS), en Porto Alegre, Brasil. Los tratamientos consistieron en la combinación de cinco sustratos comerciales, CSC® vermiculita (S1), espuma fenólica Green-Up (S2), S10 Beifiur® orgánico (S3), Carolina Soil® plántulas (S4) y Carolina Soil® orgánico (S5) y tres concentraciones de nutrientes en la solución nutritiva (0, 50 y 100%). Se utilizó un arreglo factorial 5×3, en un diseño experimental completamente al azar con tres repeticiones. La adición de nutrientes en la solución de riego favoreció a los sustratos S1, S4 y S5. El sustrato S2 mostró un mejor desempeño con la adición del 50% de la concentración total de nutrientes. El sustrato S3 sin la adición de la solución nutritiva mostró valores promedio muy cercanos al uso de la solución nutritiva, lo que se puede tener en cuenta en la evaluación de costos de producción de microvegetales, generando ahorros para los productores.

Palabras clave: *Eruca sativa* Miller, cultivos, cultivo de sustrato, ambiente protegido.

Introduction

With the rapid increase in the world population, humanity is faced with the need to develop sustainable ways of living on earth (Walker & Salt, 2006). Rapid urban growth has created serious concerns regarding food production, transport, and consumption, making sustainable production a matter of interest to all sectors, coupled with the increased demand for organic, fresh, and locally-sourced vegetables. Thus, food production in urban centers becomes an alternative, shortening the distance between production and the consumer, reducing carbon emissions and the consumption of fossil fuels for transportation. Also, the shorter distance between cultivation and consumption ensures a higher quality food. Considering the global trends, such as climate change and the scarcity of natural resources, new approaches are needed to make cities more sustainable since the growth of urbanization is inevitable.

The cultivation of microgreens is among the alternatives to mitigate this scenario of unavailability of fresh and healthy food. Microgreens are vegetables that are consumed during the seedling phase, when cotyledon leaves are still turgid and true leaves have not fully expanded (Xiao *et al.*, 2012). They can be grown in small spaces by urban dwellers, as well as by commercial producers. Microgreens are a

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category of new and relatively unknown products on the market, are available in a pleasant variety of colors, textures, and flavors (Pfeiffer *et al*., 2015; Weber, 2017), and have been gaining more and more use in cooking. The main reason for this success is that they are rich in essential elements for the proper functioning of the human body and contain higher concentrations of vitamins and carotenoids when compared to adult vegetables (Xiao *et al*., 2012).

Arugula (*Eruca sativa* Miller) is a leaf-type vegetable that has increased in production in Brazil and has a slightly spicy taste (Menegaes *et al*., 2015). Just like broccoli and cauliflower, arugula has great potential benefits for health thanks to its high content of glucosinolates, vitamins, and polyphenols. This species is a good option for microgreen production since in this growth stage it has a concentrated and attractive taste for the palate, contributing positively to the ornamentation and preparation of dishes, adding color, flavor, and texture.

The production of microgreens serves different consumers and markets, and the adaptation of cultivation techniques allows production in non-traditional spaces for plant cultivation. However, technical information about production systems and the handling of microgreens is scarce. Additionally, there are few scientific studies in the world and in Brazil that validate microgreen production technologies. Thus, this study aimed to evaluate the productivity of arugula microgreens in different commercial substrates and concentrations of nutrient solution, in a closed irrigation system.

Materials and methods

The experiment was conducted on the campus of the Faculty of Agronomy at the Federal University of Rio Grande do Sul (UFRGS), in the Department of Horticulture and Silviculture, located in the city of Porto Alegre, Brazil (30°01' S, 51°13' W). The trial was set up in a protected environment or greenhouse covered with plastic film (low-density polyethylene), arranged in an east-west direction with dimensions of 5.0 m x 10.0 m and 3.0 m height.

The experimental design was completely randomized with a factorial arrangement (5x3) formed by five commercial substrates and three concentrations of nutrients in the solution with three replicates per treatment. The commercial substrates were: CSC® vermiculite (S1), Green-Up phenolic foam (S2), S10 Beifiur® organic (composting waste from the wine industry) (S3), Carolina Soil® seedlings (peat + roasted rice husk + vermiculite) (S4), and Carolina Soil® organic (peat + vermiculite) (S5). The nutrient solution (NS) was prepared according to Santos *et al.* (2004). This solution was suggested for use in hydroponic forage cultivation and had the following composition (100% concentration) of macronutrients (mmol L^{-1}): NO_3^- - 13.89; $H_2PO_4^-$ - 1.41; SO_4^{2-} - 1.09; NH_4^+ - 1.41; K^+ - 6.41; Ca^{2+} - 3.4, and Mg^{2+} - 1.09. Micronutrient composition (mg L^{-1}) was as follows: 5.0 of Fe, 0.05 of Mn, 0.09 of Zn, 0.10 of B, 0.04 of Cu, and 0.02 of Mo. Rainwater was used to prepare the nutrient solution. The initial electrical conductivity (ECi) values were 0 (0% nutrient concentration), 1.20 (50% nutrient concentration), and 2.00 dS m^{-1} (100% nutrient concentration), with pH values from 5.5 to 6.0.

The tested substrates were characterized in terms of chemical and physical properties in the Substrate analysis laboratory at UFRGS/Porto Alegre. Before use, the substrates were sterilized in an autoclave for 120 min at a temperature of 120°C and pressure of 1.5 atm.

Sowing was performed manually on February 22, 2018, using arugula Folha Larga seeds (Sakata®) with lightly cut leaves and bright green coloring, at a density of 0.10 kg m^2 in a previously wetted substrate. The substrates were placed in white polystyrene trays of 0.14 m x 0.21 m and 0.015 m deep, without compartmentation and perforated at the base (Fig. 1). Each tray received a layer of approximately 0.01 m of the substrate, on which the seeds were deposited.

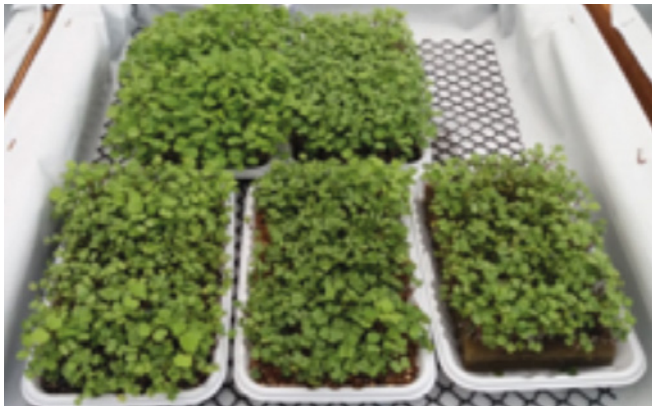

FIGURE 1. Microgreens in white polystyrene trays distributed in rectangular pools.

After sowing, the trays were distributed in rectangular pools, a structure proposed for the production of microgreens, with a sub-irrigation system. The nutrient solution was supplied intermittently (15 min/h) according to the

treatments, from 8 a.m. to 6 p.m. plus two irrigations during the nighttime.

The pools were made of wood and covered with a double face film (white/black) and had a depth of 0.07 m and a slope of 2%. The pool contained a drain for the return of the drained solution to the nutrient solution reservoir; thus, the system was closed, without loss of the drain. During the first 3 d after sowing, the trays were maintained in the dark and covered with paper sheets; then, they were uncovered when seeds were already germinated. This technique was used to favor uniform seed germination.

During the experimental period, the average temperature and the relative humidity of the air were monitored daily, using a temperature and humidity Datalogger (model AK174, AKSO®, São Leopoldo, Brazil), installed inside the cultivation environment, next to the production benches. The harvest point was reached between the 8 to 11 d after sowing when 80% of the microgreen seedlings had the primary leaves in early development and the cotyledons were still turgid. Harvest was carried out by cutting with scissors at the level of the substrate. The fresh and dry mass of the shoots were evaluated; these data were extrapolated for productivity, considering the tray area, and the production cycle (precocity).

The daily averages of air temperature, recorded from February 22 to March 3, 2018, inside the protected environment varied between 24.7 and 30.2°C, and the relative humidity was between 68.0 and 74.5% (min. and max.) (Fig. 2).

Results were subjected to a normality test and analysis of variance by the F test and the means were compared with the Tukey's test at 5% probability of error, using the Sisvar program (Ferreira, 2011).

Results and discussion

For the variables average fresh and dry mass productivity of the shoot of arugula microgreens grown in a hydroponic system, the analysis of variance of the results indicated that there was a significant interaction between the substrate factors and concentration of the nutrient solution according to the F test (<0.05).

The average temperature for the period was approximately 27.4°C. Abreu *et al.* (2012) and Regan (2014) evaluated the effect of different temperatures on the germination of arugula seeds and found that temperatures between 25 and 30°C generated seedlings with greater root length. The temperatures recorded during the experimental period may have contributed to the production of arugula microgreens.

The characterization of the substrates (Tab. 1) demonstrated that the Green-Up phenolic foam (S2) and the S10 Beifiur® organic (S3) have high electrical conductivity (EC), of 1.28 and 1.20 dS m^{-1}, respectively. These values are considered outside the ideal range for substrates.

For Green-Up phenolic foam (S2) and S10 Beifiur® organic (S3), the pH values were 4.05 and 4.86 and are considered less than ideal for this variable. The other substrates, CSC® vermiculite (S1), Carolina Soil® seedlings (S4), and Carolina Soil® organic (S5), showed a range of variation for EC between 0.01 and 0.46 dS m^{-1} and for pH between 5.26 and 6.34. On the other hand, these last substrates proved to be suitable for use for plant production, since these values are in the recommended range for cultivation on substrates (Abreu *et al.*, 2012; Regan, 2014).

Regarding dry density (Tab. 1), the values of commercial substrates ranged from 11.50 to 302.74 kg m^{-3}. According

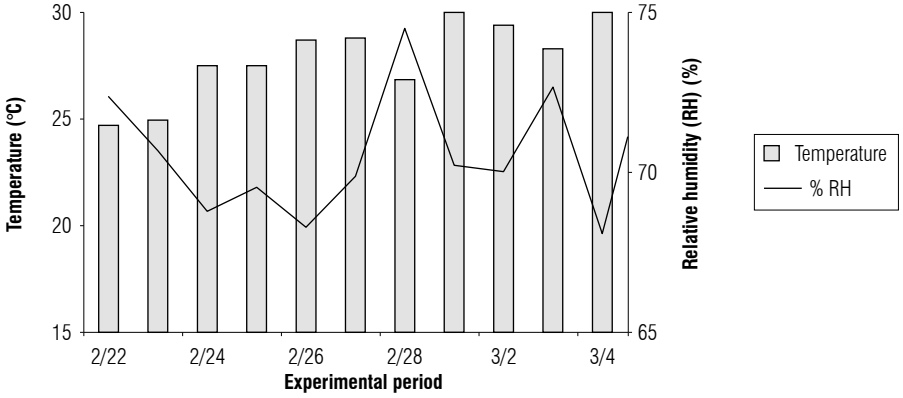

FIGURE 2. Air temperature and relative air humidity during the experimental period. Porto Alegre, UFRGS. 2020.

TABLE 1. Chemical and physical characterization of commercial substrates used in the production of arugula (*Eruca sativa* Miller) microgreens. CSC® vermiculite (S1), Green-Up phenolic foam (S2), S10 Beifiur® organic (S3), Carolina Soil® seedlings (S4), and Carolina Soil® organic (S5).

Chemical characteristics	Substrates				
	S1	S2	S3	S4	S5
EC (dS m^{-1})	0.01	1.28	1.20	0.46	0.28
pH (H_2O)	6.34	4.50	4.86	5.26	5.98
Physical characteristics					
Wet density (kg m^{-3})	181.36	13.80	582.85	262.57	313.58
Dry density (kg m^{-3})	177.90	11.50	302.74	122.41	113.44
TP %	73.23	-	81.33	87.73	91.62
AS %	23.24	-	21.48	38.91	29.32
RAW %	7.21	-	15.50	13.07	21.48
WRC-10 %	49.99	-	59.84	48.82	62.63

EC - electrical conductivity in a 1:5 solution (v/v); pH - in H_2O, dilution of 1:5 (v/v); TP - total porosity; AS - aeration space; RAW - readily available water; WRC-10 - water retention capacity under suction of a 10 cm water column, determined on a volumetric basis (v/v).

to Kämpf (2000), adequate values are between 100-300 kg m^{-3} for cultivation in seedling trays, which are containers with a volume closer to that used for the production of microgreens. The substrate S3, although with a dry density very close to that recommended, showed a higher wet density than the other substrates (Tab. 1). This led to the visual observation of resistance to root penetration, preventing seedling fixation. However, according to Table 2, the average productivity of shoot fresh matter (SFM) and shoot dry matter (SDM) in substrate S3 with concentrations of nutrients C1 and C2 obtained the best responses for these variables.

There is a change in this behavior only in the concentration of 100% of nutrients (C3) in the solution. Thus, the physical characteristic and the density of S3 did not limit the production of arugula microgreens, but the concentration of the nutrient solution and the chemical characteristics of the substrate influenced the increase of mass. This can be seen by the negative influence on the average productivity of SFM and SDM, with a reduction of 7% and 24%, respectively (Tab. 2), when the concentration of the nutrient solution was increased from 50% to 100%.

Chemical characteristics are not generally considered to be exclusive when choosing a substrate since they can be easily modified during preparation and cultivation (Fermino & Kämpf, 2003). Due to the organic composition of S3 originating from the decomposition of residues from the winemaking process, the use of nutrients in the nutrient solution must be adequate to avoid the reduction in microgreen productivity.

According to Silva *et al.* (2013), arugula cultivation is classified as moderately sensitive to salinity with 2.75 dS m^{-1} indicated as without loss of relative yield for a cycle of 30 to 40 d. As substrate S3 showed high initial EC, the use of the conductivity of 2.0 dS m^{-1} in the nutrient solution with 100% of the concentration may have caused an excess of salts for the cultivation. According to Ribeiro *et al.* (2001), the high concentration of nutrients is a stress factor for plants, as it reduces the osmotic potential and provides the action of ions on the protoplasm.

Another substrate that showed high EC was phenolic foam. However, this material is considered inert, and the negative effects of the chemical characteristics were less than that of S3 that accumulates more nutrients with the addition of NS irrigation, and salinization may occur. In this study, the phenolic foam showed a much lower dry density than that recommended for substrates (Tab. 1), but this physical characteristic did not disrupt the response of arugula microgreen productivity in SFM and SDM in this substrate

TABLE 2. Effect of cultivation substrate and nutrient solution concentration on shoot fresh matter (SFM) and shoot dry matter (SDM) of arugula (*Eruca sativa* Miller) microgreens in a recirculating system.

Substrate	SFM (g m^{-2})			SDM (g m^{-2})		
	C1**	C2	C3	C1	C2	C3
S1	567.7 c C *	1534.4 ab B	1989.8 a A	46.6 b B	65.3 ns	67.4 a A
S2	641.2 bc B	1545.6 a A	1567.5 b A	39.1 b C	63.6	52.7 b B
S3	1115.9a AB	1564.3 ab A	1034.7 c B	71,1 a A	68.0	53.8 b B
S4	948.0 ab B	1155.8 bc B	1669.0 ab A	61.9 a AB	67.0	55.4 ab B
S5	581.0 bc C	1026.5 c B	2013.9 a A	43.9 b B	60.2	66.3 a A
CV	12.7	11.3	8.4	9.2	8.8	10.1

ns - not significant according to the Tukey test at 5% probability. *Means followed by the same lowercase letter in the column and uppercase in the row do not differ statistically according to the Tukey test at 5% probability ($P<0.05$). **Initial electrical conductivity (ECi) established for the three evaluated nutrient concentrations (C1 - 0%, C2 - 50%, and C3 - 100%) in the nutrient solution: C1 - 0.0, C2 - 1.20, and C3 - 2.0 dS m^{-1}, respectively. CSC® vermiculite (S1), Green-Up phenolic foam (S2), S10 Beifiur® organic (S3), Carolina Soil® seedlings (S4), and Carolina Soil® organic (S5). CV - coefficient of variation.

(Tab. 2) compared to the other substrates and nutrient concentrations in the nutrient solution. However, a negative point of substrate S2 was the difficulty of accommodating seeds uniformly during sowing when compared to the other substrates. The surface caused an irregular distribution of seed, generating an excess of seeds in some points of the substrate. In these places, seeds showed difficulty in fixing the roots to the substrate. A possible alternative for overcoming this problem in phenolic foam is to use perforated foams for the production of microgreens. However, this visually observed behavior did not negatively affect the productivity of SFM and SDM (Tab. 2) in the phenolic foam since this substrate did not show the lowest results. The sowing density of arugula microgreens used in this study may have not provided seed saturation at the points of accumulation for influencing productivity results.

The average productivity of SFM between all treatments ranged from 567.7 to 2013.9 g m^{-2}. These results were similar to those that Paradiso *et al.* (2018) found with chicory (*Cichorium intybus*, cultivar Italico) microgreens, curly lettuce (*Lactuca sativa*, cultivar Bionda da Taglio) and cabbage (*Brassica oleracea*, cultivar Natalino), grown on peat substrate in which fresh mass varied between 659 g m^{-2} and 1548 g m^{-2}. In the study by Paradiso *et al.* (2018), microgreens were grown in a growth chamber with controlled temperature and relative humidity, making production more expensive. It can be inferred that the system proposed in this work, without control of these variables, responded adequately to the production of arugula microgreens without affecting the productivity of SFM. It should be noted that these authors worked with other species; however, in the literature there is still a lack of data on the production of arugula microgreens.

Without the use of fertilizers (C1) for irrigation, substrate S1 obtained the lowest average yield of SFM (567.57 g m^{-2}) when compared to those with better results (S3 and S4) (Tab. 2). The results in S1 may be related to the EC value that was much lower than that in the other substrates (Tab. 1). Combined with the low chemical activity of this material (Caldeira *et al.*, 2013), S1 did not contribute nutrients to the increase in the fresh mass of the microgreen shoots. Although it is a short cycle, the seed reserves were not able to supply the demand for seedling growth, so the addition of fertilizers to this substrate is necessary for greater SFM yield.

The nutrient solution with 100% concentration (C3) favored the highest productivity of SFM and SDM (Tab. 2) of arugula microgreens grown on substrates S1 and S5, without differing statistically from S4 (Tab. 2); they were 250%, 246%, and 76% higher than SFM productivity when only pure water (C1) was used for irrigation (Tab. 2). It is important to point out that these substrates (S1, S4, and S5) showed EC values below 0.46 dS m^{-1} that are much lower than those of S2 and S3 that were close to 1.2 dS m^{-1} (Tab. 1).

The effect of increasing EC in the tested substrates for the SFM average productivity variable (Tab. 2) showed an increased response for substrates S1, S4, and S5. For S2, the addition of a nutrient solution caused a positive response, but differences were not observed between 50% (C2) and 100% (C3). However, they were higher than C1. Thus, the most concentrated solution (C3) was indicated for substrates S1, S4, and S5, while for S2 and S3 the 50% dilution (C2) was recommended for use.

In the phenolic foam, which also showed a high EC, the addition of 50% of the concentration of nutrient solution provided similar results to the addition of 100% (Tab. 2). From these results, it can be inferred that substrates with higher EC are more successful for the production of microgreens when using lower concentrations of nutrient solution or only water. When compared at different levels of EC in the nutrient solution (Tab. 2), S3 generated similar results between the use of 50% (C2) and 0% (C1). However, when the nutrient solution increased to 100% (C3) there was a reduction in the average productivity of 7.28%. Therefore, for this substrate, we recommend using only water or NS at 50%, because the response becomes negative with a higher concentration.

For the variable average SDM productivity (Tab. 2) using only water for irrigation (C1), substrates S3 and S4 showed statistically equal values that were higher than the other tested substrates. However, when using NS with 50% of the concentration of nutrients (C2), this difference was diluted, and the substrates did not show a statistical difference between them.

When the NS concentration became 100% (C3), there was an inversion in the behavior obtained for C1 (Tab. 2). This was expected mainly for S3 since it showed a reduction in SDM productivity of 21% from C2 to C3 (Tab. 2). The only substrates that responded positively to the addition of nutrient solutions for SDM were S1, S2, and S5. For S1 and S5, the increase in the concentration of nutrients in the nutrient solution from 50% to 100% did not show a statistical difference, and it was not necessary to add 100% nutrients to the NS. The substrate S4 tended to increase the amount of SDM with the addition of the nutrient solution. However, it did not differ statistically when it was worked without

fertilizer applications. Observing the average productivity of SFM (Tab. 2), which is the variable that represents the commercial weight of this product, the answer was different. It showed greater fresh weight when the concentration of 100% of nutrients in the nutrient solution (NS) was used. In this case, the use of 100% NS is indicated for this substrate to obtain fresh weight gain.

An explanation for the decrease in SDM productivity with the increase in the concentration of nutrients in the nutrient solution from C2 to C3 (Tab. 2) in substrates S2, S3 and S4 is due to the chemical characteristics of these materials that had the highest EC values (Tab. 1). This showed the negative effect of increasing the salinity of the root medium on the absorption of water and nutrients by plants since it decreased or canceled the effects of the increase of the nutrient concentration on growth (Rattin *et al*., 2003).

From the responses obtained in this study, it appears that commercial substrates for the production of arugula microgreens were more dependent on chemical characteristics, while physical characteristics had less influence on productivity. Thus, studies for each cultivation system are justified, as they can directly influence the productive and qualitative responses of arugula microgreens.

Conclusions

The commercial substrates tested in this study provided good productivity of arugula microgreens grown in a sub-irrigation system, inside a protected environment. However, the concentration of nutrients in the nutrient solution influenced the responses of the substrates.

For production without adding nutrients to the nutrient solution, the use of the substrate S10 Beifiur® organic is recommended.

For greater economic yield, given by the average productivity of fresh mass, we recommend the use of a nutrient solution with 100% of the concentration of nutrients for substrates CSC® vermiculite, Carolina Soil® seedlings, and Carolina Soil® organic. For the Green-Up phenolic foam and the S10 Beifiur® organic, we recommend the use of 50% of the nutrient solution.

When seeking greater yield in dry mass, we recommend the use of 50% diluted nutrient solution for the substrates CSC® vermiculite, Green-Up phenolic foam, and Carolina Soil® organic, or the use of pure water for the S10 Beifiur® organic and Carolina Soil® seedlings.

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Author's contributions

ARW formulated the overarching research goals and aims, developed the methodology, validated the overall replication/reproducibility of results/experiments and other research outputs, conducted the research process, performed the experiments and data/evidence collection, provided the study materials, and prepared the presentation of the published work, specifically writing the initial draft, including substantive translation. WDP formulated the overarching research goals and aims, developed the methodology, conducted the research process, performed the experiments and data/evidence collection, and prepared the presentation of the published work, specifically writing the initial draft, including substantive translation. TSD prepared the presentation of the published work and was in charge of the critical review, commentary or revision.

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Soybean yield components at different densities and planting seasons in Paraguay

Componentes del rendimiento de la soya en diferentes densidades y fechas de siembra en Paraguay

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ABSTRACT

The aim of this research was to evaluate the performance of soybean cultivars using different population densities and planting seasons. The experiments were established using a completely randomized block design with a 14×3×2 factorial arrangement, where factor A consisted of 14 soybean cultivars, factor B were low (177,700 plants ha^{-1}), medium (266,600 plants ha^{-1}), and high (355,500 plants ha^{-1}) population densities, and factor C consisted of early and late planting seasons. We evaluated the number of pods per plant (NPP), number of grains m^{-2} (NG), 1000-grain weight (TGW), and yield (kg ha^{-1}). The interaction between cultivar and planting season affected the NG, TGW, and yield. Cultivars DM-6563-IPRO, TMG-7062-IPRO, 6505-B, NA-5909-RG, M-6410-IPRO, DM-6262-IPRO, SOJAPAR-R19, 6806-IPRO, 6205-B, M-5947-IPRO and SYN-1163-RR showed higher yields in the early planting season and cultivar NS-5959-IPRO in the late planting season. Cultivars 5907-IPRO and DM-5958 showed similar yields for the two planting seasons evaluated. The highest yields were obtained from a density of 266,600 plants ha^{-1}. The cultivar×planting season interaction affected the TGW, with the early planting season showing a greater TGW for most of the cultivars evaluated. The NPP depended on the interaction between cultivar, density, and planting season. The combination of the NG and the TGW showed a more significant influence on the generation of yield in the cultivars. This study highlights the importance of selecting genotypes according to their response to variations in planting date and plant density. This information could help Paraguayan farmers to maximize production in the same area, optimizing the available resources.

Key words: *Glycine max*, genotype-environment interaction, plant density, planting date, soybean cultivars, yield improvement.

RESUMEN

El objetivo de esta investigación fue evaluar el desempeño de cultivares de soya usando distintas densidades y épocas de siembra. Los experimentos se establecieron usando un diseño experimental en bloques completamente al azar con arreglo factorial 14×3×2, donde el factor A consistió en 14 cultivares de soya, el factor B fueron las densidades poblacionales baja (177,700 plantas ha^{-1}), media (266,600 plantas ha^{-1}) y alta (355,500 plantas ha^{-1}), y el factor C consistió en las siembras realizadas en forma temprana y tardía. Se evaluó el número de vainas por planta (NVP), el número de granos por m^2 (NG), el peso de mil granos (PMG), y rendimiento (kg ha^{-1}). La interacción entre el cultivar de soya y la época de siembra afectó el NG, PMG y el rendimiento. Los cultivares DM-6563-IPRO, TMG-7062-IPRO, 6505-B, NA-5909-RG, M-6410-IPRO, DM-6262-IPRO, SOJAPAR-R19, 6806-IPRO, 6205-B, M-5947-IPRO y SYN-1163-RR mostraron rendimientos más altos en las siembras tempranas y el cultivar NS-5959-IPRO en la siembra tardía. Los cultivares 5907-IPRO y DM-5958 mostraron rendimientos similares para las dos temporadas de siembra evaluadas. Los mayores rendimientos se obtuvieron a partir de una densidad de 266,600 plantas ha^{-1}. La interacción cultivar×temporada de siembra afectó el PMG siendo mayor en la siembra temprana que en la siembra tardía para la mayoría de los cultivares evaluados. El NVP fue afectado por la interacción cultivar, densidad y temporada de siembra. La combinación del NG y el PMG influyó significativamente en la generación de rendimiento en los cultivares de soya. Este estudio resalta la importancia de seleccionar genotipos teniendo en cuenta variaciones en la fecha de siembra y en la densidad de plantas. Esta información permitiría a agricultores paraguayos maximizar la producción en la misma área de cultivo optimizando los recursos disponibles.

Palabras clave: *Glycine max,* interacción genotipo-ambiente, densidad de plantas, fecha de siembra, cultivares de soya, aumento del rendimiento.

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Introduction

Soybean [*Glycine max* (L.) Merr.] is one of the most important crops globally due to its high-value grains used as a source of protein and oil for both animal and human consumption (Pagano & Miransari, 2016). Due to the increasing demand for this crop worldwide, the planting area needed is continuously expanding, and alternatives to improve productivity are very active research areas (Masuda & Goldsmith, 2009). According to the Paraguayan chamber of cereal and oilseed exporters (Cámara Paraguaya de Exportadores y Comercializadores de Cereales y Oleaginosas, 2020), Paraguay is the fifth-largest producer and fourth-largest soybean exporter in the world with a production of 10,000,000 t of grains in 3,500,000 ha with a yield of 2,857 kg ha^{-1}. Alto Parana, Canindeyu, Itapua, Caaguazu, and San Pedro are the most productive soybean areas in the country (Cohener & Aguayo, 2009).

The profitability of soybean allows many farmers to invest in improving their production either by increasing the growing area, improving land productivity, or investing in a second planting season (Teixeira *et al*., 2016). However, the increment of the production area is currently limited due to environmental protection laws restricting the expansion of the crop into new regions (Palau *et al*., 2012).

Therefore, increasing land productivity is a more practical alternative. This increase in productivity can be achieved with practices that preserve the soil's physical and chemical conditions and allow better pest, weed, and disease control (Gudelj *et al*., 2018). One of these practices is the use of improved and environmentally specific cultivars (Peluzio *et al*., 2005; Pires *et al*., 2005). Additionally, the use of specific cultivars and the adjustment of planting dates and densities can also improve the yield in small areas (Vega & Andrade, 2000).

Alternative planting seasons or a second planting performed in the middle of the summer (also known as "Zafrina") allow farmers to obtain higher income. The downside of this practice is that under these conditions, the photoperiod is lower and, consequently, yields are inferior because days start getting shorter as they approach autumn. Some cultivars may not perform well during the early planting season because average temperatures are lower as this planting coincides with the beginning of spring. The harvest results are conditioned by these phenomena that translate into inferior yields, reduced plant height, lower number of pods, and lower number of grains per plant (Marchiori *et al*., 1999; Teixeira *et al*., 2016).

Thanks to the success of soybean breeding programs, a high number of cultivars on the market are highly productive, resistant to pests and diseases, and adapted to various edaphoclimatic conditions (Sediyama, 2009). The growth of these cultivars is influenced by environmental factors like temperature, rain, relative humidity, soil humidity, and photoperiod. Consequently, the planting season has a decisive influence on the production's quantity and quality (Motta *et al*., 2000). Therefore, evaluating new cultivars must be a constant practice to provide valuable information for extension agents, consultants, and farmers (Vernetti & Vernetti Júnior, 2009).

Plant density is another factor that can be modified to obtain higher land productivity. Optimal plant density is defined as the minimum number of plants that allow the cultivar to achieve its maximum yield (Vega & Andrade, 2000). One of the main concerns among farmers is reducing the amount of seed used per ha to lower the cost of inputs. Thus, understanding the development of different soybean cultivars planted at different planting densities is fundamental for recommendations for the most appropriate guidelines to maximize yields.

Research on adapted cultivars is of fundamental importance to optimize soybean production. In Paraguay, few published works report adapted cultivars that allow optimizing land productivity. So, there is a need for updated information about the eco-physiological behavior and yield of the new varieties offered on the market. Besides, private companies own the existing data, and it is not publicly available. Therefore, the present study aims to establish a benchmark for an appropriate choice of soybean cultivars in Paraguay, evaluating the productive performance of 14 commercial cultivars planted at three plant densities and in two planting seasons in the Yguazu region during 2017-2018.

Materials and methods

Trials were conducted in the experimental field of the Centro Tecnológico Agropecuario del Paraguay (CETAPAR), located in Yguazu, Alto Parana, Paraguay (25°27'41.97" S, 55°02'26.66" W, and 258 m a.s.l.). We obtained data on rainfall and maximum, minimum, and mean daily temperatures during soybean cultivation from September 2017 to April 2018 from the meteorological station of CETAPAR. The water balance graph was constructed using the potential evapotranspiration (ET_c) data of the experimental area from the MOD16A2 MODIS/Terra net evapotranspiration database with a spatial resolution of 500 m (Fig. 1) (Running

et al., 2017). Mean temperatures of 24.9°C and total precipitation of 1,925 mm were recorded. The experimental area's soil was characterized as 69.0% clay, 2.2% organic matter, a pH of 5.9, and a base saturation of 70.3%.

The experiment was arranged using a completely randomized block design with a 14×3×2 factorial arrangement and three replicates. Factor A consisted of 14 soybean cultivars (Tab. 1); factor B consisted of three planting densities (177,700, 266,600 and 355,500 plants ha^{-1}), and factor C consisted of early (September 20, 2017) and late (November 20, 2017) planting seasons. Date selection follows soybean planting practices in Paraguay, which generally begin in September. However, when the weather is not appropriate (excessive rains or lack of rain), producers start sowing in mid-October and, exceptionally, in November. In Paraguay, it is common to carry out a second planting no later than February. Our experimental unit consisted of five rows 5 m long and a space between the rows of 0.45 m (a total of 2.25 m wide). The distance between experimental units within each block was 1 m and between blocks was 3 m. In each experimental unit, the useful plot was delimited at 4.05 m^2. To delineate the plots, 1 m was removed from the ends of each experimental plot, and a row from each edge was discarded. Sowing was carried out with a tractor/planter set (model SHP 249, Semeato Plantio Direto, Passo Fundo, RS, Brazil), using a zero-tillage system with a 0.45 cm space between the rows. Other cultural management and treatments followed standard agronomic recommendations for soybean cultivation (Díaz-Zorita & Duarte, 2004). Base fertilization dosage was 150 kg ha^{-1} of a N-P-K fertilizer (04-30-10) at the time of planting on both planting dates (early and late planting), in order to meet the nutritional needs of the crop.

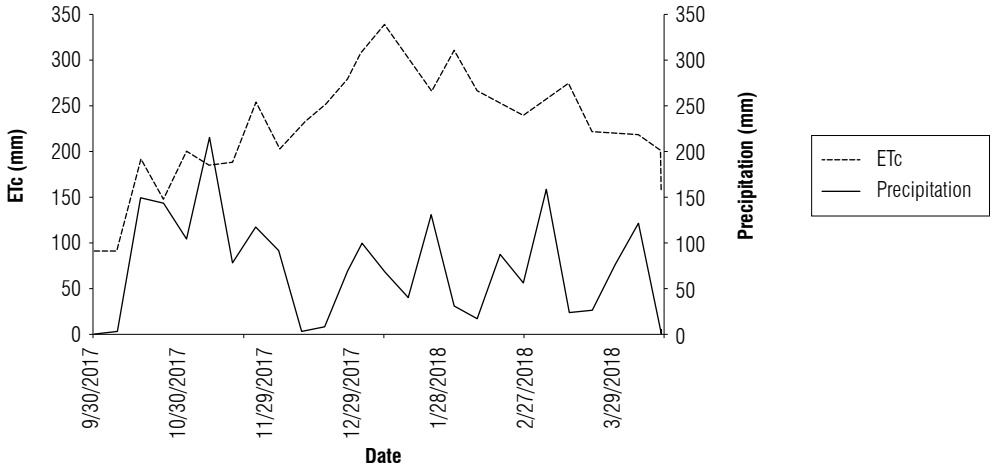

FIGURE 1. Water balance during the experimental period 2017/2018. ET_c - potential evapotranspiration.

TABLE 1. Characteristics of the soybean cultivars evaluated.

Cultivar	Maturity group (MG)	Precocity	Breeder
DM-6563-IPRO	VI	Semi-early	Semillas Don Mario
TMG-7062-IPRO	VI	Semi-early	Tropical Melhoramento & Genética
5907-IPRO	Late V	Semi-early	BASF Paraguaya S. A.
6505-B	VI	Semi-early	BASF Paraguaya S. A.
NS-5959-IPRO	Late V	Semi-early	Semillas Nidera
NA-5909-RG	Late V	Semi-early	Semillas Nidera
M-6410-IPRO	VI	Semi-early	Monsanto Paraguay S. A.
DM-6262-IPRO	VI	Semi-early	Semillas Don Mario
SOJAPAR-R19	VI	Semi-early	Instituto Paraguayo de Tecnología Agraria
6806-IPRO	VI	Semi-early	BASF Paraguaya S. A.
6205-B	VI	Semi-early	BASF Paraguaya S. A.
DM-5958	Late V	Semi-early	Semillas Don Mario
M-5947-IPRO	Late V	Semi-early	Monsanto Paraguay S. A.
SYN-1163-RR	VI	Semi-early	Syngenta Paraguay S. A.

When the crop reached the phenological stage of harvest maturity (R8) (Fehr *et al*., 1971), manual harvesting was carried out from the useful plot of each experimental unit. The number of pods per plant (NPP) was quantified by taking 10 plants per experimental unit, and the average NPP was calculated for each experimental unit. To obtain yield and 1000-grain weight (TGW) data, plants of the useful area were threshed. Weight was determined on an electronic balance (AJ150, Mettler Toledo, Columbus, Ohio, USA), and the value was divided by the harvested area and later extrapolated to kg ha^{-1}. Subsequently, the TGW was determined by quantifying four subsamples of 1000-grains for each experimental unit with a seed counter (KC-10, Fujiwara®, Seisakusho, Japan), and the seeds were weighted with a digital precision balance (JA2003, Hongzuan, Shangai, China). The TGW and yield were adjusted to 13% humidity. The number of grains per m^{-2} (NG) was estimated from the ratio of yield to seed weight.

Data analysis

The effect of cultivars, plant densities, planting season and their interaction on the yield, NG, TGW, and NPP were studied. For statistical analysis, the SAS (version 9.4) and Infostat (version 2017) software were used. The variance analysis (ANOVA) was performed following the instructions described in SAS for completely randomized block designs. The Tukey's significance test with a family-wise error rate of 5% was used for the comparison of treatment means. Equation 1 describes the model used:

$$y_{ijkl} = V_i + B_j + D_k + (VD)_{ik} + E_l + (VE)_{il} + (DE)_{kl} + (VDE)_{ikl} + \varepsilon_{ijkl} \quad (1)$$

where y_{ijkl} corresponds to the variable response of the *i*-th cultivar, *k*-th plant density, *l*-th planting season in the *j*-th block. This is the general mean of the response variable. V_i is the effect of the *i*-th cultivar; B_j is the effect of the *j*-th block; D_k is the effect of *k*-th plant density; $(VD)_{ik}$ is the effect of the *ik*-th interaction; E_l is the effect of the *il*-th planting season; $(VE)_{il}$ is the effect of the *ill*-th interaction; $(DE)_{kl}$ is the effect of the *kl*-th interaction; $(VDE)_{ikl}$ is the effect of *ikl*-th interaction; and ε_{ijkl} is the experimental error.

A principal component analysis (PCA) of the agronomic variables of soybean cultivars was performed using data from the variables NG, TGW, NPP and the yield. Results from the PCA are shown as a biplot to illustrate the correlation between the variables.

Results and discussion

The effects of experimental factors on the response variables evaluated are summarized in Table 2. The effect of the blocks was not significant for any of the response variables. There was statistical evidence of second-order interactions only for the effects of the cultivar×planting season interaction on the variables yield, NG, and TGW ($P<0.0001$). A significant third-order interaction was observed for NPP on the effects of the cultivar×density×planting season interaction ($P<0.0001$). The simple result of the density was only effective for the variable yield ($P = 0.0002$).

Yield

No significant interaction was observed between the effects of the factors cultivar, density, and planting season ($P = 0.4286$), not even when considering the interactions between density×planting season ($P = 0.1645$) or cultivar×density ($P = 0.8937$). However, the interaction between cultivar×planting season was highly significant ($P<0.0001$). This interaction implies that the yield of some cultivars is not affected by the planting season, while in others, the yield increases or decreases significantly depending on the planting season. A similar yield was observed during both planting seasons for cultivars 5907-IPRO, NA-5909-RG, and DM-5958. On the other hand, the yields of cultivars DM-6563-IPRO, TMG-7062-IPRO, 6505-B,

TABLE 2. Summary of probability values for all fixed factors related to the agronomic variables yield, number of grains m^{-2} (NG), 1000-grain weight (TGW), and number of pods per plant (NPP).

Factor	Yield	NG	TGW	NPP
Cultivar	<0.0001	<0.0001	<0.0001	<0.0001
Density	0.0002	0.6642	0.9116	<0.0001
Planting season	<0.0001	<0.0001	<0.0001	<0.0001
Cultivar×density	0.8937	0.1078	0.4297	<0.0001
Cultivar×planting season	<0.0001	<0.0001	<0.0001	<0.0001
Density×planting season	0.1645	0.8608	0.2756	0.3274
Cultivar×density×planting season	0.4286	0.3688	0.3535	<0.0001
Coefficient of variation (CV)	21.92%	25.75%	8.541%	11.90%

The factor cultivar consisted of 14 soybean cultivars (Tab. 1), density consisted of three planting densities (177,700; 266,600, and 355,500 plants ha^{-1}), and planting season consisted of early (September 20, 2017) and late (November 20, 2017) planting seasons.

NA-5909, M-6410-IPRO, DM-6262-IPRO, SOJAPAR-R19, 6806-IPRO, 6205-B, M-5947-IPRO, and SYN-1163-RR were significantly higher for the early planting season compared to the late planting season (Fig. 2). Therefore, we can infer that water scarcity affected yields since there was a more significant water deficit in the last season than during the first (Fig. 1). Low yields may be the result of water stress at a critical phenological time. The adverse effects of the lack of water are particularly evident during flowering, seed formation, and seed filling. Lack of available water can reduce yield by reducing the number of pods, the number of seeds, and the mass of seeds that corresponds well with our data (Desclaux *et al*., 2000).

Because the plant density factor did not interact with any other experimental factor, its simple effect on yield was analyzed ($P = 0.0002$). The plant density that achieved the highest yield was 266,666 plants ha^{-1}. On the other hand, the lower seeding density produced significantly lower yields than the densities of 266,600 and 355,500 plants ha^{-1}. However, the yield for these last two densities was not statistically different (Fig. 3).

Similarly to the results obtained in this research, the reduction of soybean yield due to late plantings has been reported in previous studies (Girón *et al*., 2014; Martignone *et al*., 2016; Teixeira *et al*., 2016). The planting delay harms yield due to the influence of a smaller number of daily light hours, lower precipitation, and the high temperatures to which the plants are subjected during their initial phase (Martignone *et al*., 2016). These factors lead to a shorter duration of the vegetative stage, a lower number of nodes per

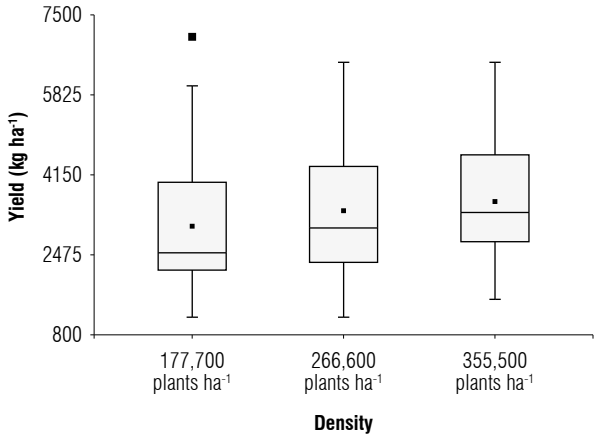

FIGURE 3. Effect of plant density on the yield (kg ha^{-1}) of soybean cultivars. Each box represents the distribution (25th to the 75th percentile) of the yield (kg ha^{-1}) for each treatment. Black dashes inside the boxes represent the medians, and black dots the means. Whiskers represent the maximum and minimum values. Black squares outside the boxes represent extreme values deviating from the expected distribution.

plant and leaf area index, and less dry matter accumulation. Also, the canopy's delayed and inefficient closure causes a more significant loss of water by evaporation (Toledo, 2019).

The soybean response to plant density variations depends on the genotype, soil water conditions, and geographic location (Gaso, 2018). In most soybean cultivars, the response to higher plant density is hindered due to the ability to compensate for gaps between plants, generating longer branches and reducing the energy use for grain filling (Cox & Cherney, 2011). However, different authors mention that soybean yields do not increase significantly when plant

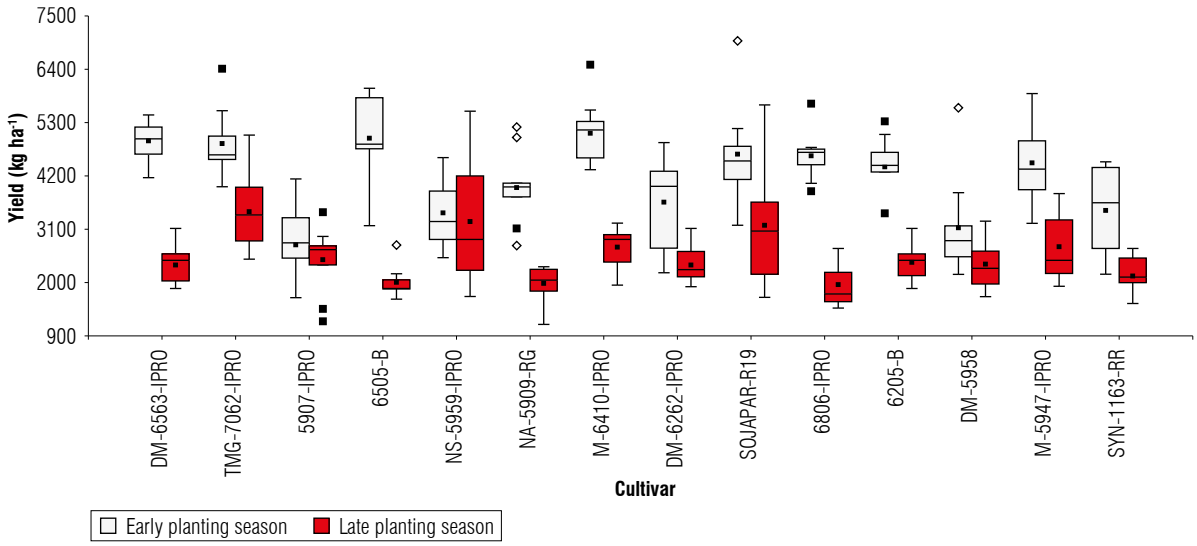

FIGURE 2. Yield (kg ha^{-1}) for different soybean cultivars sown in two different planting seasons. Each box represents the distribution (25th to the 75th percentile) of the yield (kg ha^{-1}) for each treatment. Black dashes inside the boxes represent the medians, and black dots the means. Whiskers represent the maximum and minimum values. Black squares outside the boxes represent extreme values deviating from the expected distribution.

densities range from 100,000 to 600,000 plants ha^{-1} (Lee *et al*., 2008; Thompson *et al*., 2015).

According to Rodríguez *et al*. (2015), at densities lower than 200,000 plants ha^{-1}, there is no competition between plants and the number of branches and pods per plant increases; but the tradeoff fails to compensate for the lower number of plants. Therefore, yield is reduced, a situation that was observed at a density of 177,700 plants ha^{-1}. Gaso (2018) found that a significant increase in yield is observed using a density of 300,000 plants ha$^{-1,}$ and, above this density, yields do not increase. In this research, we observed that yields did not increase significantly above 266,600 plants ha^{-1}.

Increasing plant density may be beneficial to mitigate the adverse effects of planting delay that would allow a better use of resources through maximum soil coverage and minimal water loss (Toledo, 2019). Higher plant densities compensate for spaces not covered by the canopy, increasing the number of nodes per m^{-2} (Martignone *et al*., 2016).

Number of grains

For the number of grains per m^{-2}, no statistical evidence of significant interaction was observed between the cultivar, density, and planting season effects (P = 0.3688) (Tab. 2). No interaction was observed between the density and planting season factors (P = 0.8608) or between the cultivar and density (P = 0.8937). However, the interaction between cultivar and planting season was highly significant (P<0.0001); this implies that the NG that can be produced depends on combining a specific cultivar and the planting season. The cultivars that obtained the same NG in both planting seasons were TMG-7062-IPRO, 5907-IPRO, NS-5959-IPRO, NA-5909-RG, NA-5909, 6262-IPRO, 6205-B, NS-5959-IPRO, M-5947-IPRO, and SYN-1163-RR. On the other hand, the cultivars DM-6563-IPRO, 6505-B, M-6410-IPRO, SOJAPAR-R19, and 6806-IPRO obtained a higher NG in the early planting season compared to the late one (Fig. 4).

The NG was the component that best explains crop productivity variations (Toledo, 2018). The increase of this component is directly proportional to the duration of the period between the emergency and the start of grain filling (R5). The NG is strongly associated with canopy photosynthesis and also the growth rate of the crop during flowering and pod development (growth stage R1–R5) (Egli, 2013). Besides, a particular relationship is implied between the number of nodes per area and the NG. The greater the number of nodes, the greater the NG. This characteristic is related to the cultivar, environment, and management. Thus, to maximize soybean yields, genotypes with a higher number of plant nodes and rapid soil coverage must be selected since they intercept more than 90% of radiation by R5 (Martignone *et al*., 2016). The decrease in NG observed in most cultivars in the second season may be because the delay in planting shortened the plant cycle, causing a lower rate of photosynthesis, less growth and, therefore, a reduction in the production of nodes and grains per m^{-2}. In this research, the density of plants did not influence the NG.

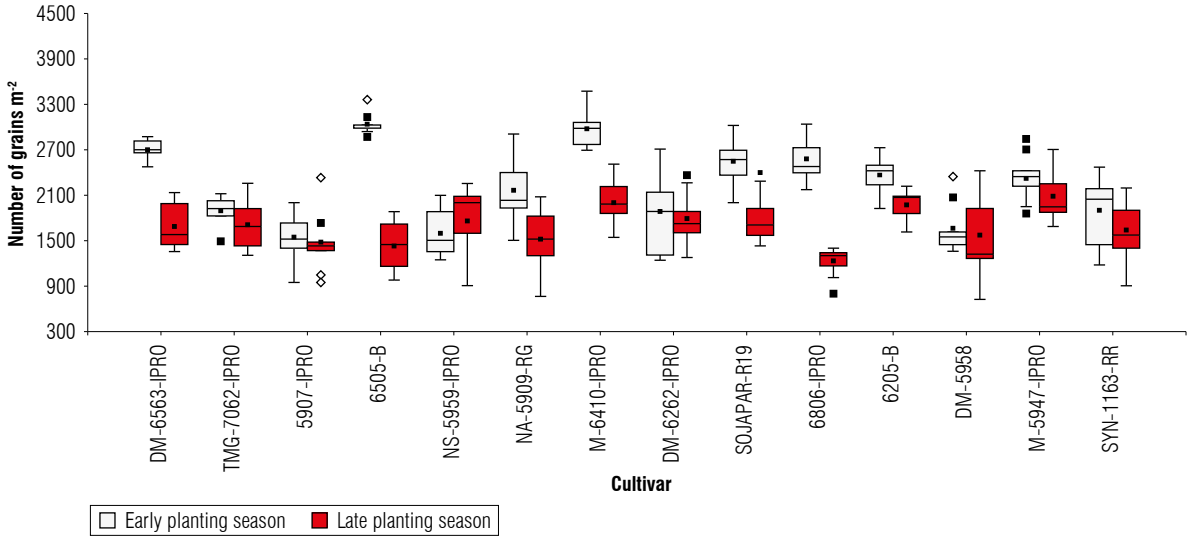

FIGURE 4. Number of grains m^{-2} for different soybean cultivars during two different planting seasons. Each box represents the distribution (25th to the 75th percentile) of the number of grains m^{-2} for each treatment. Black dashes inside the boxes represent the medians, and black dots the means. Whiskers represent the maximum and minimum values. Black squares outside the boxes represent extreme values deviating from the expected distribution.

However, it can be expected that increasing plant density will typically maximize the NG due to the increase in the number of nodes per m^{-2} (Gan *et al*., 2002).

1000-grain weight

The interaction between the cultivar and planting season was highly significant ($P<0.0001$). The cultivar that obtained a similar weight of 1000 grains in both planting seasons was 6806-IPRO (Fig. 5). The rest of the cultivars had a significantly higher TGW during the early planting season than during the late planting season.

Grain weight is the second component that best explains soybean yield and is an inherent characteristic of the cultivar (Toledo, 2018). The water deficit during November (Fig. 1) could have influenced the decrease in TWG in the second sowing season. Moreover, delayed planting causes a lower daily accumulation of dry matter during the reproductive stage. The TGW tends to be lower as temperature and solar radiation decrease, resulting in the interruption of grain filling as autumn approaches (Martignone *et al*., 2016; Teixeira *et al*., 2016).

Number of pods per plant

The interaction between the effects of the factors cultivar, density, and planting time affected the number of pods per plant ($P<0.0001$). This implies that the number of pods that the soybean plant can produce will depend on the cultivar, planting density, and planting season. The cultivars that had a NPP higher than 80 were SOJAPAR-R19, SYN-1163-RR, 6806-IPRO, M-6410-IPRO, DM-6262-IPRO, 6505-B, NA-5909, DM-5958. However, due to the interaction, the effect on the NPP is complex; for example, at a density of 355,500 plants ha^{-1} cultivar 6505-B produced an average of 30 pods per plant during the late planting season, while in the early planting season the average was 40 pods per plant. The same cultivar with a density of 266,600 plants ha^{-1} during the late planting season produced an average of 45 pods per plant, while during the early planting season, the average was 86 pods per plant. Similarly, with a density of 177,700 plants, the average number of pods per plant was 58, but with the same density during the early planting season, the average was 80 pods per plant (Fig. 6). In contrast, other cultivars such as M-5947-PRO did not significantly increase NPP regardless of the density and planting time. In general, low densities during the first planting season allowed obtaining a higher NPP with the SOJAPAR-R19 cultivar at a density of 177,700 plants during the early planting season, showing the highest NPP (121 pods per plant).

The formation of pods begins in the phenological phase R3 and ends in R6. Pod development is delayed at temperatures below 22°C and tend to fall from the plant with long photoperiods and temperatures greater than 32°C (Toledo, 2018). The formation of pods is susceptible to various types of stress, such as water deficit or the presence of pests and diseases (Toledo, 2019). Moreover, the quality and quantity of solar radiation that reaches the lower layers of the canopy stimulate the establishment of reproductive structures in soybeans (Quijano & Morandi, 2011). The delay of planting causes the canopy to take longer to close, explaining the higher NPP in some genotypes in the second planting season.

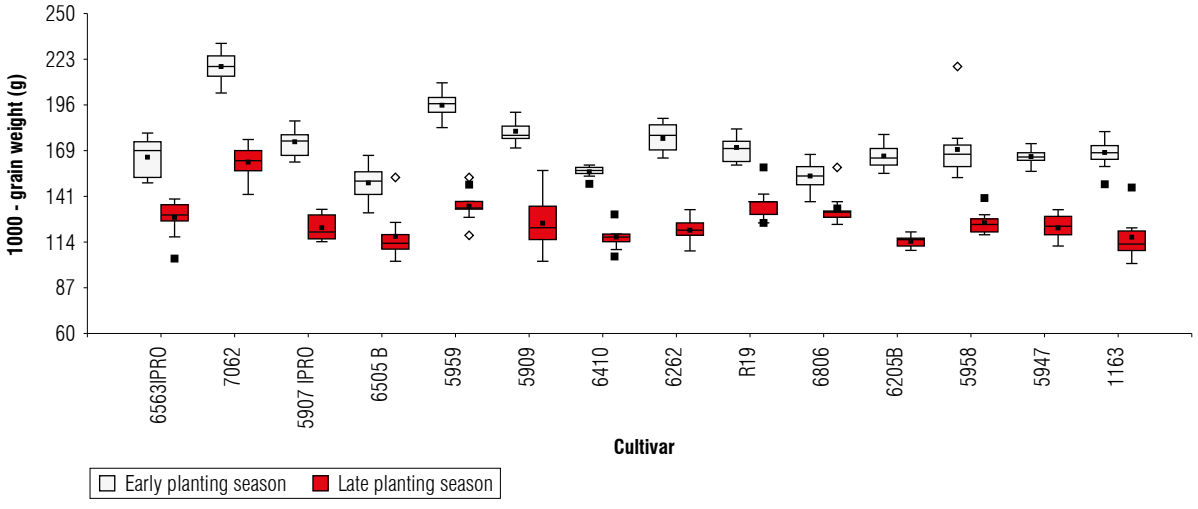

FIGURE 5. 1000-grain weight for different soy cultivars sown in two different planting seasons. Each box represents the distribution (25th to the 75th percentile) of 1000-grain weight for each treatment. Black dashes inside the boxes represent the medians, and black dots the means. Whiskers represent the maximum and minimum values. Black squares outside the boxes represent extreme values deviating from the expected distribution.

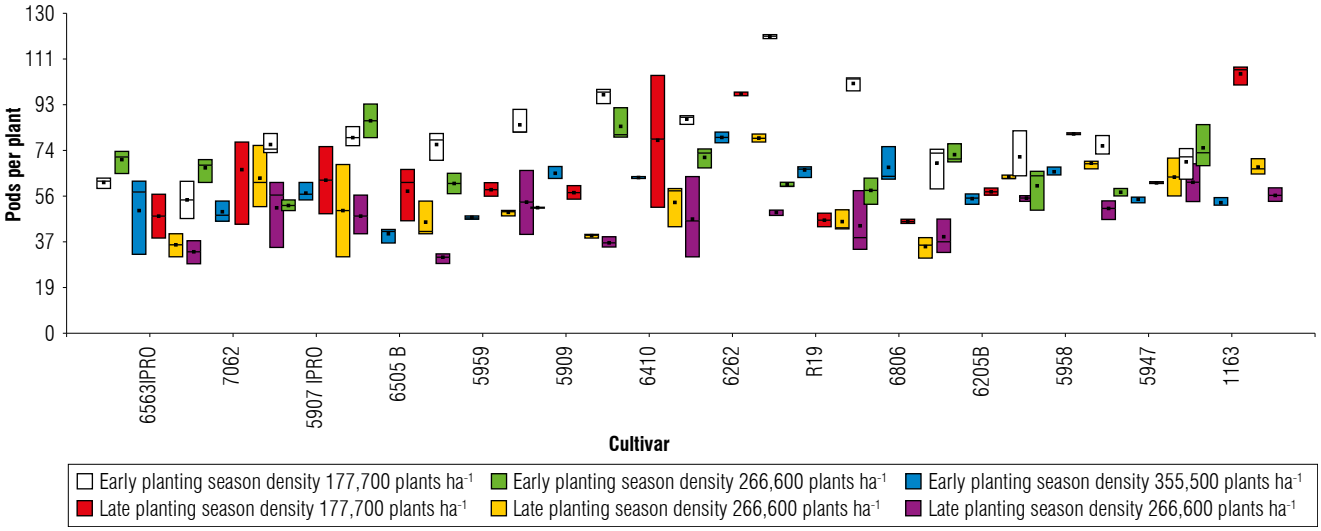

FIGURE 6. Number of pods per plant for different soybean cultivars sown at three densities in two different planting seasons. Each box represents the distribution (25th to the 75th percentile) of pods per plant for each treatment. Black dashes inside the boxes represent the medians and black dots the means. Whiskers represent the maximum and minimum values. Extreme values deviating from the expected distribution are represented by black squares outside the boxes.

The NPP tends to increase with lower plant densities (Toledo, 2019). The number of pods of branches and stems and the number of branches per plant are strongly associated with phenotypic plasticity of indeterminate soybean cultivars. Soybean growth then compensates a lower plant density by showing higher branch emission, higher branch growth and stems, and higher NPP (Balbinot Júnior *et al.*, 2018). Therefore, a higher NPP was observed at a lower plant density.

Principal component analysis

The regions in the biplot contained groups of soybean cultivars with similar characteristics. Cultivars that were closely clustered in one region of the plot represent cultivars that have similar performance patterns. Vectors pointing roughly in the same direction represented yield components that have positive correlations. Yield was slightly correlated with NPP (r = 0.23, $P = 0.0002$), while the response variables NG (r = 0.60, $P<0.0001$) and TGW (r = 0.56, $P<0.0001$) showed a higher correlation with yield. The TGW was not correlated with NG (r = 0.03; $P = 0.598$) and was weakly correlated with NPP (r = 0.17, $P = 0.003$), while NG was weakly correlated with NPP (r = 0.22; $P = 0.0005$). The principal component (PC1) accounted for 43.4% of the variance. The second component (PC2) accounted for 35.7% of the variance. Together, these components accounted for 79.1% of the variance during experiments. In the biplot, the cultivars that obtained the highest average yield are to the graph's right, while cultivars with the lowest yield are to the left (Fig. 7).

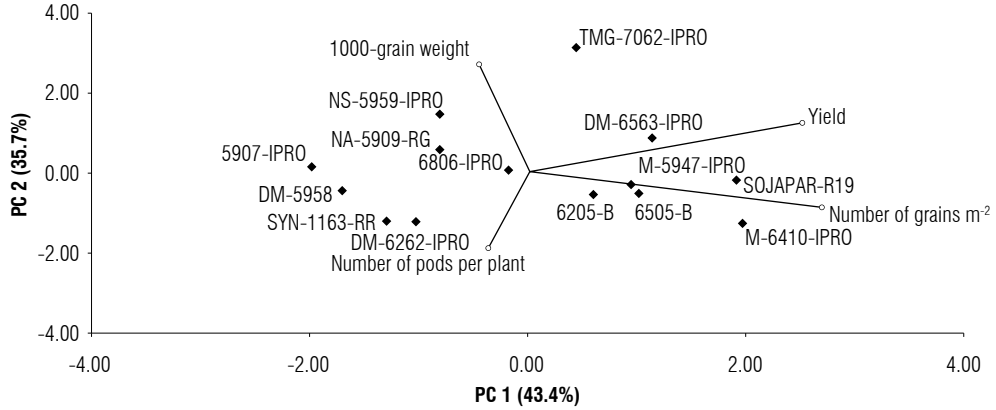

FIGURE 7. Biplot of the principal component analysis of the agronomical variables of soybean cultivars yield (kg ha^{-1}), number of grains m^{-2}, 1000-grain weight, and number pods per plant, during two planting seasons and at three plant densities. The X-axis represents projections of the first principal component (PC1) and the Y-axis the second component (PC2); the two components represent 79.1% of the variance during the field experiments.

This research provided valuable information on the productivity obtained from the interaction between genotype and environment for Paraguayan conditions. It is essential to consider that the meteorological conditions are different each year and that the performance of these genotypes could vary in different locations.

Because of the abundant supply of soybean cultivars in Paraguay, we suggest that subsequent experiments evaluate the impact of climatic factors during the cycle on the yield and its components for cultivars of different maturation groups at different planting dates. Correspondingly, these experiments should include other soybean growth components, such as the leaf area index and the number of nodes per m^{-2}.

Conclusions

This study highlighted the importance of selecting soybean genotypes according to their response to variations in planting date and plant density to increase crop production in the same area. The interaction between the soybean cultivar and planting season affected soybean yield components. Therefore, specific cultivars should be chosen for the early planting season. Cultivars DM-6563-IPRO, TMG-7062-IPRO, 6505-B, NA-5909-RG, M-6410-IPRO, DM-6262-IPRO, SOJAPAR-R19, 6806-IPRO, 6205-B, M-5947-IPRO and SYN-1163-RR can be recommended for early plantings because they show the highest yields, while for late plantings, we recommend cultivating NS-5959-IPRO. Cultivars 5907-IPRO and DM-5958 can be planted in both seasons because they show similar yields for the two planting seasons. Significantly higher yields were obtained starting from a density of 266,600 plants ha^{-1}. The cultivar×planting season interaction affects the weight of 1000-grains that determines the quality of the grain. Therefore, the early planting season provided a greater 1000-grain weight than the late planting season. The number of pods per plant depended on the cultivar, density, and planting season. Still, since it correlated poorly with yield, this characteristic could be less important when selecting a cultivar. The combination of the number of grains and the 1000-grain weight had a more significant influence on the generation of yield in the soybean cultivars evaluated. Therefore, it can work as a proxy of yield to select new cultivars.

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Author's contributions

GEM formulated the overarching research goals and aims, obtained the financial support for the project leading to this publication, designed the methodology, and carried out the manuscript's critical review. ASV carried out activities to annotate scrub data and maintain research data for initial use and later re-use, applied statistical techniques for data analysis, oversaw data presentation, and carried out the critical review of the manuscript. FFR conducted the research process, explicitly performing the experiments, and managed and coordinated the research activity planning and execution. JDN wrote the initial draft and carried out the commentaries of the manuscript. PFS provided the study materials and carried out the critical revision of the manuscript. WLP oversaw and led the research activity planning and execution and carried out the critical review of the manuscript.

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Productive characterization of the blueberry cultivar Bluegem in Brazil

Caracterización productiva del cultivar Bluegem de arándanos en Brasil

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ABSTRACT

Due to the increase in consumption and the possibilities of exportation and industrialization, blueberry cultivation has been expanding in Brazil. Since Rabbiteye is one of the more cultivated groups of blueberries in Brazil, this study aimed to assess the productive characterization of the blueberry cultivar Bluegem, group Rabbiteye. Assessments were conducted with plants in full production during two harvest seasons. We collected data on the following variables: flower and vegetative bud distribution, open flowers and formed fruit in two different shoot lengths, and number of floral primordia. The results showed a greater number of vegetative buds and the intercalation between flower and vegetative buds in a proportion of 78% and 48% in long and short shoots, respectively. The number of total buds and the number of flower and vegetative buds were influenced by the harvest season and the shoot length. The number of floral primordia was influenced by the bud position on the shoot, but not by the harvest season. The number of flowers and fruits, in general, was greater in apical buds and in long shoots.

Key words: flowering, growth, bud position, Rabbiteye, fructification, *Vaccinium* spp.

RESUMEN

Debido al aumento del consumo y las posibilidades de exportación e industrialización, el cultivo de arándanos se ha expandido en Brasil. Dado que Rabbiteye es uno de los grupos de arándanos más cultivados en Brasil, este estudio tuvo como objetivo evaluar la caracterización productiva del cultivar Bluegem de arándano, grupo Rabbiteye. Las evaluaciones se realizaron con plantas en plena producción durante dos temporadas de cosecha. Se recolectaron datos para las siguientes variables: distribución de yemas florales y vegetativas, flores abiertas y frutos formados en ramas de dos longitudes diferentes, y número de primordios florales. Los resultados mostraron mayor cantidad de yemas vegetativas y la intercalación entre yemas florales y vegetativas en una proporción de 78% y 48% en brotes largos y cortos, respectivamente. El número total de yemas y el número de yemas florales y vegetativas estuvieron influenciados por la temporada de cosecha y la longitud de los brotes. El número de primordios florales estuvo influenciado por la posición de la yema en el brote, pero no por la temporada de cosecha. El número de flores y frutos, en general, fue mayor en yemas apicales y en brotes largos.

Palabras clave: floración, crecimiento, posición de la yema, Rabbiteye, fructificación, *Vaccinium* spp.

Introduction

Blueberries (*Vaccinium* spp.) have stood out among the small fruits cultivated in Brazil due to their flavor, economic value, and nutraceutical properties (Peña *et al*., 2012). Cantuarias-Avilés *et al.* (2014) and Radünz *et al.* (2016) have shown their great nutraceutical and nutritional values, besides the plant's easy adaptation to small areas of cultivation. These characteristics make blueberries an attractive species for diversifying the productive capacity of families (Radünz *et al*., 2014).

Rabbiteye (*Vaccinium ashei* Reade) is among the world's most commercially cultivated species (Strik, 2007; Cantuarias-Avilés *et al*., 2014), mainly because this group has plants with high yields, vigor, longevity, resistance to heat and drought, tolerance to fungus-related diseases, and low chilling requirements (Ehlenfeldt, 2007). In southern Brazil, a low number of chill hours is very common, a fact that increases the importance of this group for fruit farming in temperate zones.

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Genetic and climatic factors may influence the productive characteristics of blueberry plants (Antunes *et al*., 2008) as well as the quality of the harvested fruits (Rodrigues *et al*., 2011; Gündüz *et al*., 2015). For temperate regions of Brazil, there is a need for studies regarding blueberry species and management practices to better adapt these plants to the local edaphoclimatic conditions and, therefore, to obtain higher productivity and fruit quality (Fachinello *et al*., 2011).

Originally, all the buds in blueberry plants are vegetative (Pescie & López, 2007), and in order for the flower buds to become differentiated, the presence of low temperatures is necessary to meet the required chilling hours of the particular cultivar (Coletti *et al*., 2011). The number of vegetative buds differing from flower buds might vary depending on the cultivar, day length, temperature (Spann *et al*., 2003; Pescie & López, 2007; Williamson *et al*., 2015), and the phytosanitary status of the plants (Williamson *et al*., 2015). Therefore, there might be a variation in the number of flower buds, flowers, and fruits for the same cultivar, depending on the climatic conditions of the place where the plants are grown.

Thus, in order to obtain increased knowledge on the bioclimatology of blueberry plants cultivated under the climatic conditions of southern Brazil, especially in relation to their fruiting habit and their production linked to environmental conditions, it is important to characterize the crops in this region. The characterization will allow the proper use of management practices and obtain greater yields and better fruit quality. Since studies regarding the characterization of blueberries grown under climatic conditions of Brazil are scarce, this research focused on characterizing the blueberry (*Vaccinium* spp.) cv. Bluegem group Rabbiteye (*Vaccinium ashei* Reade) grown under southern Brazilian climatic conditions.

Materials and methods

The study was carried out during two harvest seasons, 2012/2013 and 2013/2014, in a commercial orchard located in Morro Redondo, RS, in Brazil (31°32' S, 52°34' W, 150 m a.s.l.). The average temperatures during the experiment were 17.1°C and 18.2°C for the 2012/13 and 2013/14 harvests, respectively. Climatological normal data for the period 1971 to 2000 demonstrate that the region has a mean annual rainfall of 1366.9 mm, mean annual temperature of 17.8°C and a mean temperature of the hottest month of 23.2°C in January (Embrapa, 2016).

Eight-year-old plants of the cv. Bluegem belonging to the Rabbiteye (*Vaccinium ashei* Reade) group and in full production were chosen. For this region of the country under the conditions of the experiment, pruning was carried out in mid-July. This cultivar showed vegetative growth for approximately 40 d during the months of July and August. The period of floral initiation and formation of flower buds occurred for approximately 15 d during the months of August and September. The period that comprises the formation and maturation of fruits took place for approximately 140 d during the months of September, October, November, December, and January. The fruit harvest started in January.

A totally randomized experimental design was used and conducted under two factorial arrangements. A 2×2 factorial arrangement (shoot length × harvest season) was used to check the distribution of vegetative and flower buds on the shoot. A 2×3×2 factorial arrangement (shoot length × bud position on the shoot × harvest season) was used to check the number of floral primordia, open flowers, and developed fruits in the plants.

Four groups of plants were randomized for each variable. Each group consisted of four plants. Two central plants in each group were selected and a total of ten long shoots (31 to 50 cm) and ten short shoots (15 to 30 cm) were randomly selected for each harvest season. The shoots evaluated corresponded to the secondary shoots (secondary branches originated on the main stem). Shoots were grouped into long and short to obtain more accurate results, since shoots of different sizes in the plant may have a different number of buds. For the selected shoots, counts of flower and vegetative buds, the position of these buds on the shoots, the base and top diameter of the shoots, and their lengths were assessed. Weekly observations in marked and defined buds were carried out to determine the number of open flowers during the flowering period and the number of developed fruits until the end of the harvest season (Tab. 1).

TABLE 1. Morphological characterization of the shoots of blueberry, cv. Bluegem, harvest seasons 2012/2013 and 2013/2014.

Harvest season	Shoot length	Shoot length (cm)	Base diameter of shoot (mm)	Top diameter of shoot (mm)
	Long	39.4	4.7	1.7
13/14	Long	34.9	4.2	1.8
12/13	Short	25.0	3.5	1.6
13/14	Short	23.5	3.3	1.6
12/13	Long shoot mean	37.2	4.5	1.8
13/14	Short shoot mean	24.3	3.4	1.6

In order to evaluate the number of primordia, shoots were collected from plants on five different dates between April and August for each harvest season, covering the period of leaf senescence until the opening of flowers. In each of the assessment dates, a total of ten short and ten long shoots were collected from each group of bushes and were taken to the Fruit Laboratory of the Federal University of Pelotas for the dissection of apical, medial and basal buds, and the counting of floral primordia. At the end of each counting, a mean number of floral primordia was obtained for the apical, medial and basal portions of the shoots.

The shoot diameter was measured with a digital caliper. Measurements were performed twice at the base and at the top of each shoot and the values were averaged. The shoot length was measured with a measuring tape, from the base to the top of the shoot.

The total number of flower and vegetative buds in long and short shoots were counted and weighted in relation to the number of shoots in which the buds were present, for the different levels of shoots. The levels were characterized by the number of times that they showed flower and vegetative buds intercalated, as shown in Figure 1. This method of weighting was performed to avoid overrating the total number of buds. Thus, the flower and vegetative buds that occupied the first level, i.e., the apical part of the shoot, were multiplied by 1, since they could be found in all evaluated shoots. The flower and vegetative buds from the second level were multiplied by the respective number of shoots in which they were found; for example, when they were found in six shoots, they were multiplied by 0.6. The same was done for the other levels (Radünz *et al.*, 2018).

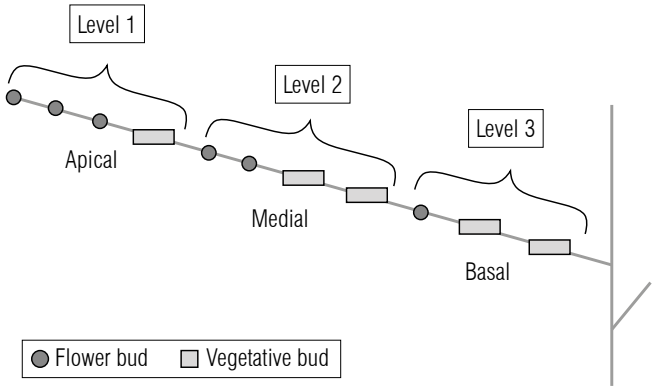

FIGURE 1. Representation of the levels used to characterize the fruiting habit on the secondary shoots of blueberry cv. Bluegem.

Data on the total number of flower and vegetative buds as a function of the shoot length and the harvest season, and the number of floral primordia, flowers, and fruits as a function of the shoot length, bud position, and harvest season were subjected to an ANOVA and, when statistically different, the Tukey's HSD test ($P \leq 0.05$) was used to compare the means of the treatments.

Results

The results of the ANOVA ($P \leq 0.05$) for the number of flower and vegetative buds, floral primordia, number of flowers, and number of fruits are shown in Table 2.

The interaction between the factors harvest season and shoot length did not significantly affect the total number of flower and vegetative buds. However, the single effect of these variables influenced the total amount of flower and vegetative buds in the plants (Tab. 2). A greater number of

TABLE 2. Results of the analysis of variance (ANOVA) for the number of flower (FB) and vegetative (VB) buds, floral primordia (FP), number of flowers (NFW) and number of fruits (NFT) of the blueberry cv. Bluegem.

Factor	DF	FB	VB
Shoot length	1	*	**
Harvest season	1	**	**
Shoot length × harvest season	1	NS	NS

Factor	DF	FP	NFW	NFT
Shoot length	1	**	**	**
Bud position	2	**	**	**
Harvest season	1	**	NS	NS
Shoot length × bud position	2	**	NS	**
Shoot length × harvest season	1	NS	NS	NS
Bud position × harvest season	2	NS	NS	NS
Shoot length × bud position × harvest season	2	NS	**	NS

*, **, *** significant at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively; NS - not significant. DF - Degrees of freedom.

flower and vegetative buds (Fig. 2) was observed for the 2012/13 and 2013/14 harvest seasons, respectively. When analyzing the number of flower and vegetative buds as linked to the shoot length, a statistical difference was found between short and long shoots. Long shoots showed a greater number of flower and vegetative buds, having on average 53% more buds than short shoots (Fig. 2; Tab. 3). For the proportion between the number of vegetative and flower buds, 1.43, and 1.41 vegetative buds were observed for each flower bud in long and short shoots, respectively (Tab. 3).

Analyzing the levels in which flower buds were intercalated with vegetative ones for every ten evaluated shoots on average, 78% and 48% showed intercalated buds in long and short shoots, respectively. In the third level, the intercalation was of 17% in long shoots and 2% in short shoots, while for the fourth level an intercalation of 5% was observed only in long shoots (Tab. 3). The highest number of flower buds was found in long and short shoots of the first level (Tab. 3), which, on average, showed 8.2 and 5.8 flower buds, accounting for 82% and 88% of all the buds found on these shoots, respectively.

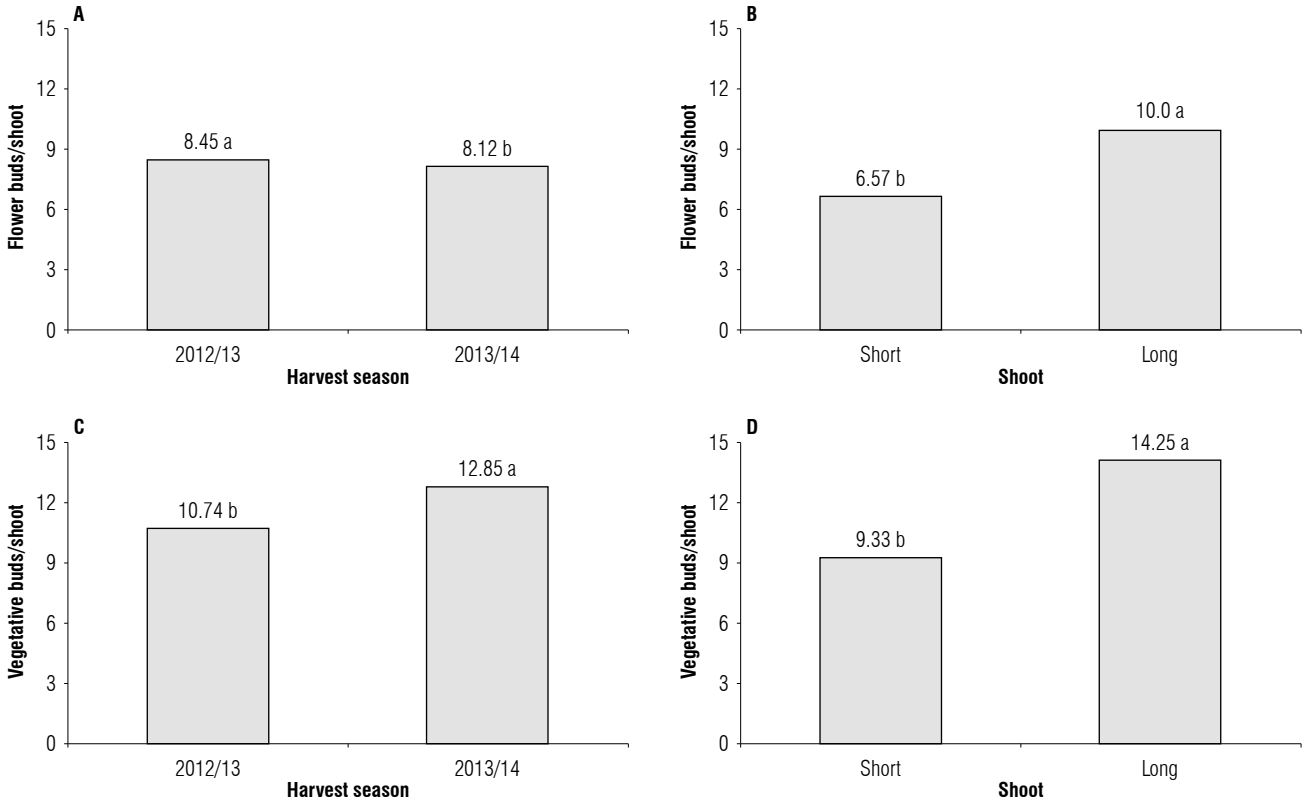

FIGURE 2. Total mean number of A and B) flower and C and D) vegetative buds as a function of the harvest season and secondary shoot length (short shoot: 15 to 30 cm and long shoot: 31 to 50 cm) for blueberry cv. Bluegem. Means followed by the same letter in the figure are not significantly different according to the Tukey's HSD test ($P \leq 0.05$).

TABLE 3. Bud distribution in levels and total buds (TB) for blueberry cv. Bluegem as a function to the harvest season (H) and shoot length (S).

H	S	Level 1			Level 2			Level 3			Level 4			Total		TB
		NS	F	V	NS	F	V	NS	F	V	NS	F	V	F	V	
12/13	Long	10	8.7	4.2	8.0	1.6	9.9	1	1	10.3	-	-	-	10	13.1	23.2
13/14		10	7.7	5.2	7.7	2.4	8.8	2.3	1.1	8.5	1.0	0.8	9.7	9.9	15.4	25.3
12/13	Short	10	6.2	5.3	4.0	1.5	7.0	0.3	0.3	2.0	-	-	-	6.8	8.3	15.1
13/14		10	5.5	6.1	5.7	1.6	7.5	-	-	-	-	-	-	6.3	10.3	16.6
Long shoot mean		10	8.2	4.7	7.8	2.0	9.3	1.7	1.1	9.4	0.5	0.4	4.8	10	14.3	24.3
Short shoot mean		10	5.8	5.7	4.8	1.5	7.2	0.2	0.2	1.0	-	-	-	6.6	9.3	15.9

Mean of three plants (30 shoots). NS - Number of shoots in which levels are present; F - number of flower buds in the level; V - number of vegetative buds in the level.

Regarding the total distribution of buds on the shoots as a function of shoot length (Tab. 3), we verified that for every 1.53 cm (0.65 buds cm^{-1}) there was a bud in both short- and long shoots. The similarity in the values indicated that the total number of buds per shoot was directly associated with the shoot length, and so was the proportion between flower and vegetative buds.

A distinct statistical tendency was identified for the number of primordia, flowers, and fruits. An interaction between the shoot length and the bud position was observed for the number of primordia and fruit, as well as the single effect of the harvest season on the number of primordia. The number of flowers was affected by the three-way interaction shoot length × bud position × harvest season.

When analyzing the interaction between the position of the bud (apical, medial, and basal) and the shoot length (short and long) for the number of floral primordia (Tab. 2), we found differences between the shoot lengths only in relation to the medial and basal positions, since long shoots showed the greatest number of floral primordia. When analyzing the interaction of the short shoot as a function of the bud position, we observed that buds were found in a greater number in the apical portion, followed by the basal and the medial ones. This behavior was different from that observed in the long shoot, in which the medial portion stood out (Tab. 4). We observed no significant differences for the number of primordia as a function of the harvest season (mean 7.98), showing that the results referring to the number of floral primordia were stable and were not influenced by the edaphoclimatic characteristics of the region.

TABLE 4. Number of floral primordia of blueberry cv. Bluegem as a function of the shoot length and bud position.

Shoot length	Bud position		
	Apical	Medial	Basal
Short	8.10 aA	7.63 bC	7.74 bB
Long	8.17 aB	8.39 aA	7.83 aC
CV (%) = 1.51			

Means followed by the same letters (lowercase in the column and uppercase in the same line) are not significantly different according to theTukey's HSD test ($P \leq 0.05$). CV - coefficient of variation.

The number of flowers was significantly influenced by all the factors involved in the research (Tab. 2). However, it did not differ for the apical bud position for the two harvest seasons assessed. We observed a similar behavior for the medial and basal bud positions in the two harvest seasons, with a greater number of flowers in long shoots (Tab. 5). When analyzing the interaction for short shoots, only the position of the buds affected the number of flowers, as the apical position was on average 17.2% greater than the basal one (Tab. 5). The behavior for the long shoot was similar, with a difference between the apical and basal bud position, although we found no differences between the apical and medial positions, and the medial and basal positions, except for the medial position in the 2013/14 harvest season (Tab. 5).

The relationship between the shoot length (S) and the bud position (P) as a function of the harvest season can be observed in Table 5. No statistical difference was observed between the harvest seasons; however, the interaction between the bud position and the shoot length had a similar behavior. In both cases, we found the highest number of flowers on the long shoot - apical bud position (mean 7.30), which did not differ from the long shoot - medial bud position (mean 7.15) and the short shoot - apical bud position (mean 7.15). Nevertheless, the lowest number of buds was found on the short shoot - basal bud position, which did not differ statistically from the short shoot - medial bud position for both harvest seasons, and from the long shoot - basal bud position in the 2013/14 harvest (Tab. 5).

The number of flowers for the interaction between shoot length (S) and harvest season (H) as a function of the bud position (P) can be seen in Table 5. The interaction between the harvest season and the shoot length did not influence the number of flowers in the apical bud position of the shoot. However, this interaction had a similar behavior in the medial and basal positions, considering that in both cases (medial and basal positions) the greatest number of flowers was observed in long shoots, regardless of the harvest season. This number of flowers was similar to that observed in short shoots from the harvest 2013/14 (Tab. 5). When analyzing the number of flowers as a function of the bud position on the shoot, in all cases the lowest number of flowers was found in the basal position whereas the highest number of flowers was found in the apical one. However, we observed no differences between the apical and the medial positions for long shoots (Tab. 5).

Regarding the number of fruits for the interaction between the position of the bud and the shoot length (Tab. 2), the apical bud position was not influenced by the shoot length and produced on average 7.10 fruits. For the medial and basal position of buds on the shoots, the behavior was similar. The short shoot showed a greater number of fruits, on average 12.3% and 11.0% greater than the long shoot, respectively (Tab. 6).

TABLE 5. Number of blueberry cv. Bluegem flowers as a function of the interaction between the bud position and the harvest season on the shoot length.

Shoot length	Bud position (P) × harvest season (H)					
	PA × H1	PA × H2	PM × H1	PM × H2	PB × H1	PB × H2
Short (SS)	7.1 aA	7.2 aA	6.5 bBC	6.7 bAB	6.0 bC	6.2 bBC
Long (LS)	7.3 aA	7.3 aA	7.1 aABC	7.2 aAB	6.6 aC	6.7 aBC

Shoot length (S) × Bud position (P)	Harvest season	
	H1 (2012/13)	H2 (2013/14)
SS × PA	7.1 abA	7.2 abA
SS × PM	6.5 cdA	6.7 bcA
SS × PB	6.0 dA	6.2 cA
LS × PA	7.3 aA	7.3 aA
LS × PM	7.1 abA	7.2 abA
LS × PB	6.6 bcA	6.7 bcA

Shoot length (S) × Harvest season (H)	Bud position		
	PA	PM	PB
SS × H1	7.1 aA	6.5 bB	6.0 bC
SS × H2	7.2 aA	6.7 abB	6.2 abC
LS × H1	7.3 aA	7.1 aA	6.6 aB
LS × H2	7.3 aA	7.2 aA	6.7 aB
CV (%) = 6.29			

Means followed by the same letters (lowercase in the column and uppercase in the same line) are not significantly different according to the Tukey's HSD test ($P \leq 0.05$). PA - apical; PM - medial; PB - basal. H1 - 2012/13; H2 - 2013/14. CV- coefficient of variation.

TABLE 6. Number of blueberry cv. Bluegem fruits as a function of the bud length in the bud position.

Shoot length	Bud position		
	Apical	Medial	Basal
Short	7.00 aA	5.30 aB	4.55 aC
Long	7.20 aA	4.65 bB	4.05 bC
CV (%) = 8.73			

Means followed by the same letters (lowercase in the column and uppercase in the same line) are not significantly different according to the Tukey's HSD test ($P \leq 0.05$). CV - coefficient of variation.

When analyzing the same shoot length, we saw that the behavior was the same regardless of the length; in both cases, the values of the apical position of the buds were higher than those of the medial position, which in turn were higher than those of the basal position. We observed for the short shoot that the medial and basal bud positions showed 24.3% and 35.0% fewer fruits than the apical one, respectively. For the long shoot, the apical position of the buds showed 35.4% and 43.8% more fruits than the medial and basal positions, respectively (Tab. 6).

Discussion

The observed behavior for the number of buds is of extreme importance for fruit production and quality, as vegetative buds are those that will support the production of photoassimilates (Radünz *et al.*, 2016). In Georgia, USA, the maximum number of flower buds formed in each shoot piece of 20 cm is 14 for the cultivar Premier belonging to the Rabbiteye group (Ojiambo *et al.*, 2006).

In blueberry bushes, flower buds are found in the upper part of the shoot while the vegetative buds are found in the basal part (Karimi *et al.*, 2017). The results from this work can be attributed to the climatic conditions found in the farming region, especially those in southern Brazil, where mild winters are frequent (Fachinello *et al.*, 2011). Originally, all the buds of blueberry bushes are vegetative and, depending on the day length and temperature, they can turn into flower buds (Song & Walworth, 2018). The existence of intercalated buds in the shoots might be related to the adjustment of the cultivar to the region since it is an exotic species for the climatic conditions of Brazil.

When analyzing the total number of buds per shoot, similarities were seen, leading to the belief that the total number of buds per shoot is directly associated with the shoot length and so is the proportion between flower and vegetative buds. So, it is possible to estimate the number of buds for a given shoot length. There might be a correlation between the shoot thickness and the fruit size, as it

represents a natural evolution of the species where larger and thicker shoots may provide better conditions to support higher yields (Braha & Zajmi, 2015).

The apical portion of the shoots showed a greater number of flowers when compared to the basal portions of the shoots. These results are associated with the greater capacity of solar radiation interception in the external portions of the plant canopy, considering that the architecture of the plant is a factor that influences its functions and characteristics (Retamal-Salgado *et al*., 2017). The vegetative morphology of the plant is correlated with its reproductive characteristics. Because of its evolutionary traits, the development of flowers happens in places where they can be better found by pollinators (Stournaras & Schaefer, 2017). Plant morphology also influences the number of flowers as basal flowers are located in the canopy of the plants and play a role in the attractiveness and arrival of pollinators (Stournaras & Schaefer, 2017). Floral induction takes place through environmental stimuli such as temperature and photoperiod that, in turn, might influence the number of differentiated primordia (Wilkie *et al*., 2008).

Conclusion

The harvest season and the shoot length influenced the total number of buds and the number of flower and vegetative buds, since a greater number of vegetative buds were observed. The bud position on the shoot influenced the number of floral primordia but was not influenced by the harvest season. The number of flowers and fruits, in general, was greater in apical buds and in long shoots, compared to the other positions and shoot lengths.

These results can be used by farmers as a decision-making tool to define the moments in which cultural practices are carried out. However, further studies are necessary for the detailed evaluation of floral differentiation and the reasons why it occurs in an intercalated form. Additionally, research that addresses the formation of flowers and fruit set and their relationship with the climatic characteristics of the region is also suggested here.

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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

ALR and FGH designed the experiments, LLR contributed to the data analysis, ALR, MR, MAS, VNS, STT, IKG, ARB and RDBD wrote the article. All authors reviewed the manuscript.

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Effect of a mix of oligogalacturonides on symbiotic nitrogen fixation in common bean

Efecto de una mezcla de oligogalacturónidos en la fijación simbiótica del nitrógeno en frijol común

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ABSTRACT

The objective of this study was to evaluate the effect of a mix of pectic oligosaccharides in the common bean-rhizobia symbiosis. Cuba Cueto-25-9-N bean seeds were inoculated with *Rhizobium tropici* CIAT 899 and treated with a mix of oligogalacturonides, either by application to the seed (10 mg L^{-1}, 1 ml per seed) at sowing and inoculation or by foliar spray (5 or 100 mg L^{-1}, 1.5 ml per plant) to plants with two trefoils. Plant growth, nodulation, nitrogenase activity, and the gene expression of glutamine synthetase and glutamate synthase in nodules were evaluated in inoculated plants and oligogalacturonide-treated inoculated plants at 18 d post-inoculation. The oligogalacturonide-treated plants showed increases in shoot and root growth and number of nodules. Regarding nodule function, the oligogalacturonides increased the nitrogenase activity and the expression level of the gene coding for NADH-dependent glutamate synthase. The positive effects of oligogalacturonides resulted in higher effectiveness of the symbiotic nitrogen fixation. The application of oligogalacturonides can offer an alternative to increasing the symbiotic nitrogen fixation in common bean plants.

Key words: oligosaccharides, symbiosis, *Phaseolus vulgaris* L., *Rhizobium*.

RESUMEN

El objetivo de este estudio fue evaluar el efecto de una mezcla de oligosacáridos pécticos en la simbiosis frijol común-rizobios. Las semillas de frijol Cuba Cueto-25-9-N se inocularon con *Rhizobium tropici* CIAT 899 y se trataron con una mezcla de oligogalacturónidos por aplicación a la semilla (10 mg L^{-1}, 1 ml por semilla) en el momento de la siembra e inoculación o por aspersión foliar (5 o 100 mg L^{-1}, 1.5 ml por planta) a plantas con dos trifoliolos. A los 18 d después de la inoculación, se evaluó el crecimiento de las plantas, la nodulación, la actividad nitrogenasa y la expresión génica de la glutamina sintetasa y la glutamato sintasa en nódulos de plantas inoculadas y en plantas inoculadas y tratadas con oligogalacturónidos. Las plantas tratadas con oligogalacturónidos mostraron aumentos en el crecimiento del vástago y raíces y en el número de nódulos. En cuanto a la función de los nódulos, los oligogalacturónidos aumentaron la actividad nitrogenasa y el nivel de expresión del gen que codifica la glutamato sintasa dependiente de NADH. Los efectos positivos de los oligogalacturónidos resultaron en una mayor efectividad de la fijación simbiótica del nitrógeno. La aplicación de oligogalacturónidos puede ofrecer una alternativa para incrementar la fijación simbiótica del nitrógeno en plantas de frijol común.

Palabras clave: oligosacáridos, simbiosis, *Phaseolus vulgaris* L., *Rhizobium*.

Introduction

Nitrogen (N), an essential element for all living organisms, is the primary nutrient limiting crop production (Xin *et al*., 2014). Legume crops are less affected by N deficiency in soils due to their capacity for establishing symbiosis with N_2-fixing bacteria collectively known as rhizobia that directly fix the atmospheric N_2 in forms that can be assimilated by the plant (Kuypers *et al*., 2018). Symbiotic N_2-fixation (SNF) capacity varies among legume species. Compared to other legumes, the common bean (*Phaseolus vulgaris* L.) has lower SNF capacity (Polania *et al*., 2016).

The common bean is the most important legume crop for human consumption and the main source of protein for people in African and Central and South American countries (Broughton *et al*., 2003). Strategies to improve SNF capacity of the common bean include the selection of improved SNF common bean varieties as well as rhizobia strains efficient for different soil and environmental

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conditions (Farid *et al*., 2017; Oliveira *et al*., 2017), co-inoculation with rhizobia plus growth promoting bacteria (Hungria *et al*., 2013; Jesus *et al*., 2018), and the improvement of inoculants by the addition of biostimulating compounds (Lara-Acosta *et al*., 2019).

Oligosaccharins are biostimulating compounds widely used in agriculture; these compounds include chitin, chitosans, and oligogalacturonides (OGs). The OGs are linear oligosaccharin molecules of about 2 to 20 α-1,4-D-galacturonic acid residues that are released upon fragmentation of pectic polysaccharides from the plant primary cell wall (Ridley *et al*., 2001). Oligogalacturonides are considered plant endogenous elicitors or bioregulators that may regulate the amount and action of plant hormones, thus modulating different developmental processes (Savatin *et al*., 2011). The positive effect of OGs has been reported for different crops (Camejo *et al*., 2011; Cabrera *et al*., 2013), but their effect on SNF-legume crops remains poorly analyzed. Recent work from our group aimed to analyze the effect of an OGs mix in *Rhizobium*-inoculated common bean plants (Lara-Acosta *et al*., 2019). This work demonstrated that the application of a mix of OGs, either onto the seeds when planting or by foliar spray in inoculated plants promoted nodulation and plant growth. For this reason, we hypothesized that an OGs mix may have a positive effect on the SNF process of common bean plants. Therefore, the objective of this work was to evaluate the effect of the application of an OGs mix on the common bean-rhizobia symbiosis. The *Rhizobium tropici* CIAT 899 strain used as inoculum is broadly used in commercial inoculants for application on the common bean in South America and Africa because it displays intrinsic resistance to abiotic and biotic stresses (Ormeño-Orrillo *et al*., 2012).

Materials and methods

The common bean Cuban (Mesoamerican) black-seeded Cuba Cueto-25-9-N cultivar was used in this research. The seeds, supplied by the Instituto de Investigaciones Fundamentales en Agricultura Tropical "Alejandro de Humboldt", Cuba (INIFAT), were surface sterilized (Ramírez *et al*., 2013). Subsequently, seeds were germinated over moistened sterile paper towels at 30°C for 2-3 d in darkness. The strain *Rhizobium tropici* CIAT 899, initially isolated from a common-bean nodule in Colombia (Martínez-Romero *et al*., 1991), was used as inoculum. The seedlings were planted in pots with sterile vermiculite and inoculated with 1 ml per plant of *Rhizobium tropici* CIAT 899 (1×10^{10} colony-forming units (CFU) ml^{-1}) saturated liquid culture. Inoculated seedlings were subjected to OGs treatment that consisted of a mixture of OGs with a degree of polymerization of 2 to 8 galacturonic acid residues obtained by enzymatic digestion with Pectinex enzyme (Sigma-Aldrich, Mexico City, Mexico) of commercial citric pectin (Cabrera *et al*., 2003) and characterized by MS analysis (MALDI) in a Q-Tof Premier mass spectrometer (Waters Corporation, MA, USA). An OGs mix was applied either to the seed (10 mg L^{-1}, 1 ml per seed) at the time of sowing and inoculation or by foliar spray at a low (5 mg L^{-1}, 1.5 ml per plant) or high (100 mg L^{-1}, 1.5 ml per plant) concentrations onto plants with two trefoils. The OGs mix concentrations per treatment were selected based on data from Lara-Acosta *et al*. (2019) since these concentrations obtained the best results on increased root growth and nodulation from those tested in seed applications and foliar sprays.

Control and OGs mix-treated inoculated plants were grown in growth chambers under controlled environmental conditions (26-29°C, 16 h photoperiod, 4,800 LUX, and 60% relative humidity) and were watered with a N-free nutrient solution as reported by Summerfield *et al*. (1977). For the fertilized (non-symbiotic) condition, a full nutrient Summerfield solution was used for watering. Control and OGs mix-treated SNF common bean plants were harvested at 18 d post-inoculation (DPI) for comparative phenotypic analysis. The different parameters were determined from 10 plants (biological replicates) from the control and each of the OGs mix treatments.

Shoot and root lengths (cm) were determined from freshly harvested plants. Excess vermiculite was carefully detached from the roots by rinsing with tap water to avoid breakage of the root. Nodules were detached from the root and counted. Subsequently, roots, shoots, and nodules from each plant were dried in an oven (Laboratory Equipment BG, Mexico City, Mexico) at 60°C for 3 d and then weighed on an analytical scale (Sartorius GmbH, Göttingen, Germany) to calculate the dry weight (DW, g). Nitrogenase activity was determined by the acetylene reduction assay (ARA) (Hardy *et al*., 1968) in detached nodulated roots. Specific activity of nitrogenase was expressed as μmol C_2H_4 h^{-1} g^{-1} nodule DW per plant.

Nodule transcript levels of the genes coding for ammonium assimilation enzymes glutamine synthetase (GS) and NADH-dependent glutamate synthase (NADH-GOGAT) were analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Total RNA was isolated from 200 mg of frozen nodules using Trizol reagent (Life Technologies Corporation, Frederic, MD, USA) following

the manufacturer's instructions. Three replicate RNA samples were isolated for each treatment. cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) from 1.5 mg total RNA, according to manufacturer's instructions. Resulting cDNAs were then diluted and used to perform qRT-PCR assays using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). Reactions were analyzed in a real-time thermocycler (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) with settings of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. The primers used for qRT-PCR amplification of GS1 (Phvul.001G229500.1) and GOGAT (Phvul.001G076400.19) cDNAs were designed from the coding sequence of each gene obtained from the *Phaseolus vulgaris* v2.1 genome sequence available in the Phytozome data base (https://phytosome.jgi.doe.gov/pz/portal.html). The sequences of primers were the following: for NADH-GOGAT: Forward 5'-ACCAGGAGGTTGTGGATTTT-3' and Reverse 5'-TTTTTGCTTTCCTTCCTTCG-3', and for GS Forward 5'-GGAGCATATCGCTGCTTATGG-3' and Reverse 5'-AGTTTCGTGTCGTCCTGTCAGA-3'. The relative expression level for each sample was calculated using the comparative C_t method and normalized with the geometrical mean of three housekeeping genes: ubiquitin (*UBC,* Phvul.006G110100.1), malate dehydrogenase (*MDH,* Phvul.007G273500.1) and heat-shock protein (*HSP*, Phvul.001G039700.1) (Hernández *et al*., 2007).

The SNF effectiveness for inoculated plants (control or each OGs treatment) was calculated using the following formula:

$$\text{SNF effectiveness} = \frac{\text{DW inoculated per plant}}{\text{DW fertilized per plant}} \times 100 \qquad (1)$$

The plant DW was determined by adding the weights of dried roots, shoots, and nodules from each plant. Ten inoculated plants from each treatment and 10 fertilized (full nutrient) plants were analyzed.

Statistical analysis for parameters growth, nodulation, nitrogenase activity and the gene expression was performed using the Student's *t*-test ($P<0.05$). The SNF effectiveness data were analyzed by ANOVA using SPSS v22 (IBM Corp., USA). Differences between means were evaluated using the Tukey's honestly significant difference test ($P<0.05$).

The experiment was carried out under a completely randomized design with 10 replicates and was repeated on two occasions. In the article, only the results of one experimental repetition are shown given the similarity in the behavior of the results.

Results and discussion

Figure 1 shows data of growth and nodulation from 18 DPI inoculated common bean plants subjected to the different OGs mix treatments compared to untreated control plants. The values for shoot/root growth showed positive effects on inoculated plants subjected to foliar sprays of an OGs mix. Shoot length increased 37% and 35% and root length increased 25% and 15%, with foliar sprays of an OGs mix at low or high concentrations, respectively. However, shoot/root growth in plants from OGs mix-treated seeds was similar to that from control plants (Fig. 1A-B). The OGs mix-treated plants showed similar shoot biomass compared to control plants (Fig. 1C). Regarding root biomass, a positive effect was observed in plants subjected to the two types of OGs mix treatments, showing increases of 54%, 45% and 55% in root DW after seed or foliar sprays of an OGs mix at low or high concentrations, respectively (Fig. 1D).

As observed in previous studies (Lara-Acosta *et al*., 2019), the OGs mix-treated plants showed an increase in nodule number of 68%, 73% and 29% in plants subjected to seed or foliar sprays of an OGs mix at low or high concentrations, respectively (Fig. 1E). However, OGs-treated plants showed similar nodule biomass compared to control plants (Fig. 1F).

The observed effects of the OGs mix in root and shoot growth of common bean plants may be correlated with altered auxin synthesis and concentration or location in common bean plants (Izquierdo *et al*., 2016), although the mechanism underlaying this effect has not been described yet. Auxin is the major hormone governing lateral root formation and primary root growth in plants (Bensmihen, 2015). The increased nodule number determined for SNF common bean plants treated with an OGs mix could be related to auxin signaling. The bacterial infection and nodule organogenesis in the *Rhizobium*-legume symbiosis is finely regulated by different phytohormones, including auxin (Bensmihen, 2015). The role of auxins in nodule initiation is due to its effects on both lateral/hairy root and nodule development (Kohlen *et al*., 2018). Barraza *et al*. (2018) reported that common bean plants with increased synthesis and concentration of indole-3-acetic acid have increased the number of nodules per plant.

Bacteroid nitrogen fixation and nodule cell ammonia assimilation are important aspects of nodule function. Thus, we assessed nodule function of OGs mix-treated plants by evaluating the nitrogenase activity and the expression level of genes coding for the GS and NADH-GOGAT enzymes

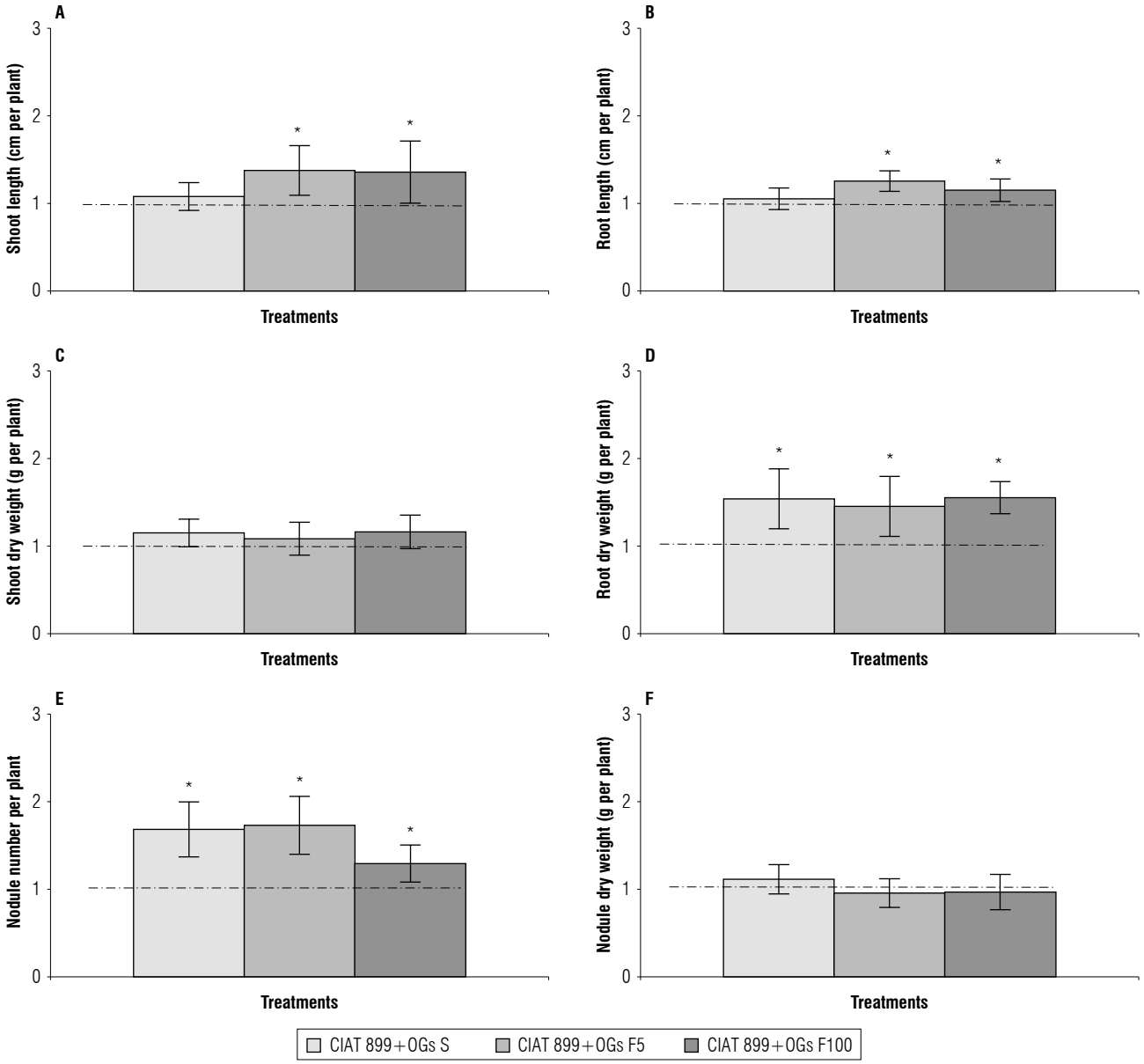

FIGURE 1. Effect of a mix of oligogalacturonides (OGs) on A) shoot length, B) root length, C) shoot dry weight, D) root dry weight, E) nodule number, and F) nodule dry weight of Cuba Cueto-25-9-N-common bean plants. The OGs mix treatments applied to *Rhizobium tropici* CIAT 899-inoculated plants were: CIAT 899+OGs S: seed application (10 mg L^{-1}), CIAT 899+OGs F5: foliar spray at 5 mg L^{-1}, and CIAT 899+OGs F100: foliar spray at 100 mg L^{-1}. Parameters were evaluated in 18 d post-inoculation plants. Values were normalized to the value from the control condition (inoculated plants without OGs mix addition) that was set to 1 as indicated by a dashed line. Values represent the average ± SD from 10 different plants per treatment. Student's *t*-test was used to analyze the difference in each parameter compared to the control treatment. Columns marked with an asterisk (*****) represent significantly different means according to the statistical analysis ($P<0.05$).

that catalyze initial ammonia assimilation (Temple *et al*., 1998). The highest values of nitrogenase activity were observed in the plants from seeds treated with an OGs mix and plants with foliar sprays of an OGs mix at a high concentration with an increase of 19% and 27%, respectively (Fig. 2A).

Although a higher nitrogenase activity could be related to increased nodulation, it can also be related to the positive effect of the OGs mix on photosynthetic activity since sucrose derived from photosynthesis is the main carbon source for nodule function (Roy *et al*., 2020). OGs treatments increase stomatal density in banana leaves, thus

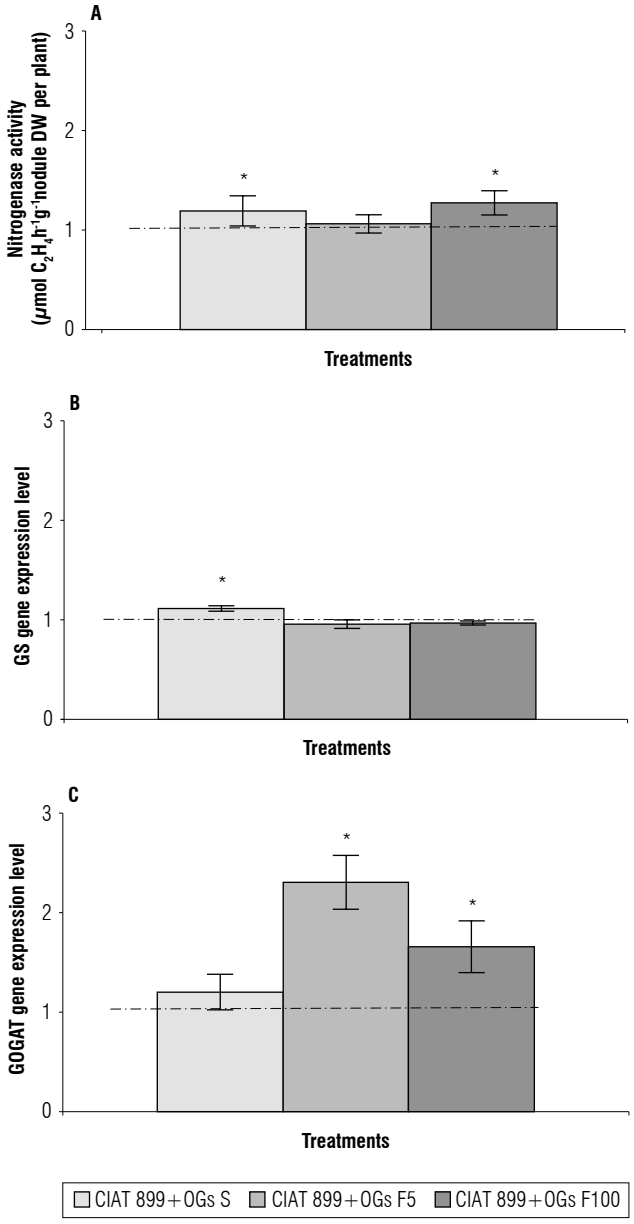

FIGURE 2. Effect of a mix of oligogalacturonides (OGs) on A) nitrogenase activity, B) GS gene expression level, and C) NADH-GOGAT gene expression level of symbiotic N_2-fixation (SNF) Cuba Cueto-25-9-N-common bean plants. The OGs mix treatments applied to *Rhizobium tropici* CIAT 899-inoculated plants were: CIAT 899+OGs S: seed application (10 mg L^{-1}), CIAT 899+OGs F5: foliar spray at 5 mg L^{-1}, and CIAT 899+OGs F100: foliar spray at 100 mg L^{-1}. Parameters were evaluated from 18 d post-inoculation plants. GS - glutamine synthetase. NADH-GOGAT - NADH-dependent glutamate synthase. Values were normalized to the value from the control condition (inoculated plants without OGs mix addition), that was set to 1 as indicated by a dashed line. Values represent the average ± SD from 10 different plants per treatment. Student's *t*-test was used to analyze the difference in each parameter as compared to the control treatment. Columns marked with an asterisk (*****) represent significantly different means according to the statistical analysis ($P<0.05$).

favoring photosynthetic activity through improving gas exchange and decreasing water loss by transpiration (Izquierdo *et al*., 2016).

The GS gene expression level was only increased (11%) in plants from OGs mix-treated seeds (Fig. 2B). However, NADH-GOGAT gene expression level showed increases of 130% and 65% in plants with foliar sprays of the OGs mix at low and high concentrations, respectively (Fig. 2C). Nodule NADH-GOGAT is considered the limiting step of the GS/NADH-GOGAT ammonia assimilation cycle (Temple *et al*., 1998; Roy *et al*., 2020). Nodules from inoculated alfalfa plants silenced for NADH-GOGAT gene expression show a strikingly altered symbiotic phenotype including reduced nitrogenase activity (Cordoba *et al*., 2003).

Based on the data from Figure 1, which indicate the positive effects of an OGs mix on *Rhizobium*-inoculated common bean plants, we evaluated their SNF effectiveness by comparing plant biomass of N-fertilized plants (DW = 0.998 ± 0.08 g) with biomass of inoculated plants treated with an OGs mix and control-plants. While inoculated common bean plants showed 50% SNF effectiveness, the OGs mix-treated plants showed increased SNF effectiveness to 60% (Fig. 3).

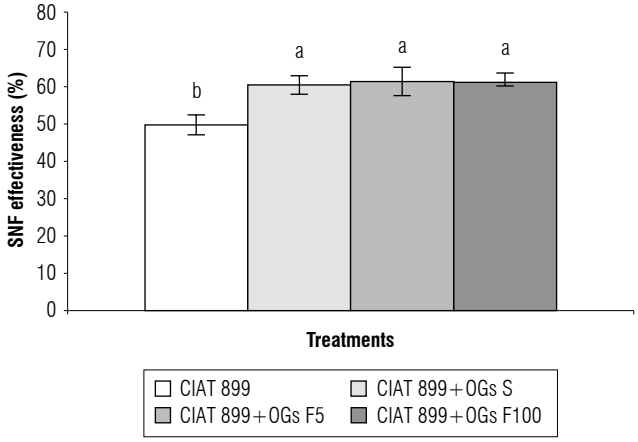

FIGURE 3. Effect of a mix of oligogalacturonides (OGs) on symbiotic N_2-fixation (SNF) effectiveness of Cuba Cueto-25-9-N-common bean plants inoculated with *Rhizobium tropici* CIAT 899. The SNF effectiveness, or the dry weight (DW) from inoculated plants compared to DW from fertilized plants, was determined at 18 d post-inoculation. CIAT 899: control *R. tropici*-inoculated plants. The OGs mix treatments applied to *Rhizobium tropici* CIAT 899-inoculated plants were: CIAT 899+OGs S: seed application (10 mg L^{-1}), CIAT 899+OGs F5: foliar spray at 5 mg L^{-1} and CIAT 899+OGs F100: foliar spray at 100 mg L^{-1}. Values represent the average ± SD from 10 different plants per treatment. Different lowercase letters indicate statistically different groups according to the Tukey test ($P<0.05$).

The increase in effectiveness of SNF in inoculated common bean plants and treated with an OGs mix is interpreted as the result of the positive effects of OGs on nodulation, nitrogen fixation, and nodule function. A greater number of nodules guarantees a greater number of sites to fix atmospheric nitrogen (Haag *et al*., 2013). In addition, the higher nitrogenase activity increases the availability of NH_4^+ for the synthesis of glutamate due to the action of the enzymes glutamine synthetase and glutamate synthase (Chalk *et al*., 2017), which will be used by the plant for its growth.

Conclusions

The application of a mix of OGs with a polymerization degree from 2 to 8 galacturonic acid residues shows a positive effect on *Rhizobium tropici* CIAT 899-inoculated common bean plants. The OGs treatment can constitute an ecologically and economically suitable alternative for improving SNF effectiveness in the common bean. The positive effect of an OGs mix on root growth, nodulation and nodule function could guarantee an adequate growth and development that would translate into grain yield increase of this legume.

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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

GH, DL, MCN and ABFR designed the experiments. DL, MR and AL performed the experiments. DC, MCN, ABFR, DL and GH analyzed the data. GH and DL wrote the paper. All authors read and approved the final manuscript.

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Effects of swine manure biochar on sorption equilibrium of cadmium and zinc in sandy soils

Efectos del biocarbón hecho a base de estiércol porcino en el equilibrio de sorción de cadmio y zinc en suelos arenosos

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ABSTRACT

Swine manure is an agricultural waste that can increase soil fertility. However, this residue has a high content of heavy metals, particularly zinc (Zn) and cadmium (Cd), that are not only toxic to plants and soil organisms but they also pose a great threat to human health due to the potential accumulation of these metals through the food chain. Transforming swine manure into biochar and adding it to soils can improve the soil's capacity to retain heavy metals. The main objective of this research was to study the capacity of sandy soils mixed with different doses of swine manure biochar (SMB) to retain Cd and Zn as well as to evaluate the sorption equilibrium of these metals. Sorption essays were performed by adding solutions of Zn ($ZnCl_2$) or Cd ($CdCl_2$) at different concentrations (0, 2.5, 5, 10, 50 and 100 mg L^{-1}) to soil samples mixed with different doses of SMB (0, 0.25, 0.75, 1.5, and 3.0 % (w/w)). The data were modelled using both Langmuir and Freundlich adsorption isotherm models to describe the adsorption processes. The data were best represented by the Langmuir model (R^2>0.97), indicating a mono-layer sorption to the surface. Results showed that sorption capacity of Zn and Cd increased with the dose of SMB, improving metal retention. The Langmuir constant (K_L) for soil without SMB for Cd and Zn were 0.01 L mg^{-1} and 0.05 L mg^{-1}, respectively. With the highest dose of SMB, K_L increased to 9.86 L mg^{-1} and 1.26 L mg^{-1} for Cd and Zn, respectively. Results suggest that SMB has the potential to mitigate Zn and Cd contamination of sandy soils.

Key words: pyrolized carbon, agricultural wastes, sorption, heavy metals.

RESUMEN

El estiércol de cerdo es un residuo agrícola que puede aumentar la fertilidad del suelo. Sin embargo, este residuo tiene un alto contenido de metales pesados, principalmente zinc (Zn) y cadmio (Cd), los cuales no sólo son tóxicos para las plantas y los organismos del suelo, sino que también representan una gran amenaza para la salud humana por su potencial acumulación en la cadena alimentaria. Transformar el estiércol en biocarbón y agregarlo al suelo puede mejorar la capacidad del suelo para retener metales pesados. El objetivo principal de este trabajo fue estudiar la capacidad de suelos arenosos mezclados con diferentes dosis de biocarbón de estiércol porcino (BEP) para retener Cd y Zn, y evaluar el equilibrio de sorción de estos metales. Las pruebas de sorción se realizaron agregando soluciones de Zn ($ZnCl_2$) o Cd ($CdCl_2$) en diferentes concentraciones (0, 2.5, 5, 10, 50 y 100 mg L^{-1}) a muestras de suelo mezcladas con diversas dosis de BEP (0, 0.25, 0.75, 1.5, y 3.0% (w/w). Los datos fueron modelados de acuerdo con las isotermas de Freundlich y Langmuir para describir los procesos de adsorción. El modelo de Langmuir representó mejor los datos (R^2>0.97), lo que indica una absorción de monocapa a la superficie. Los resultados mostraron que la capacidad de sorción de Zn y Cd aumentó con la dosis de BEP, mejorando la retención de metales. La constante de Langmuir (K_L) para el suelo sin BEP para Cd y Zn fue 0.01 L mg^{-1} y 0.05 L mg^{-1}, respectivamente. Con las dosis más altas de BEP, K_L aumentó a 9.86 L mg^{-1} y 1.26 L mg^{-1} para Cd y Zn, respectivamente. Los resultados sugieren que el BEP tiene el potencial de mitigar la contaminación por Zn y Cd en suelos arenosos.

Palabras clave: carbono pirolizado, residuos agropecuarios, sorción, metales pesados.

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Introduction

Technological advances in agricultural practices and production systems allow for the expansion of agriculture into areas with sandy soils. Nowadays, agricultural production in this type of soil is extensive, particularly in the state of Mato Grosso in Brazil (IBGE, 2017) that is now considered a hub for agribusiness. Grain production in the area also stimulated the growth of animal husbandry, particularly pig and cattle farming. Integration of crop and animal production systems allows farmers to use animal manure as fertilizer. This practice reduces costs and increases soil nutrient content and water retention but, at the same time, it can have detrimental consequences if animal manure is applied in excess (De Vrieze *et al*., 2019) since it has a high content of heavy metals such as copper (Cu), zinc (Zn), and cadmium (Cd).

Intensive application of swine manure can promote the accumulation of heavy metals in soils and the contamination of waterways through runoff and leaching (Zhang *et al*., 2003; He *et al*., 2005). This contamination can pose a risk to human health due to accumulation through the food chain (Scherer *et al*., 2010). Soil remediation is based on the use of chelating agents to immobilize metals and avoid uptake by plant roots, as well as leaching. This process is usually expensive and time- and resource-consuming (Wuana & Okieimen, 2011; Yao *et al*., 2012; Paz-Ferreiro *et al*., 2014).

The organic matter of agricultural residues has the potential to act as a biosorbent, and, thus, it can help mitigate contamination from heavy metals in soils and water streams (Sud *et al*., 2008; Liu *et al*., 2018; Purakayastha *et al*., 2019). Biosorbents can act through complex processes, such as ion exchange, adsorption on surface and into pores, complexation, chelation, etc. (Puls & Bohn, 1988; Sud *et al*., 2008; Chen *et al*., 2019). The management of this type of residues in tropical regions can be very challenging due to environmental conditions that increase the rate of soil organic matter mineralization and decomposition.

A potential solution for residue stabilization in these areas is the use of biochar. Pyrolysis provides a promising method for the treatment of animal manure with the advantages of destroying pathogens, breaking down antibiotics, producing value-added energy and biochar products, immobilizing heavy metals, and reducing the volume of waste stream (Tian *et al*., 2019; Xu *et al*., 2019).

When applied to soils, biochar can have several beneficial properties. It can increase the amount of recalcitrant organic matter in soils, improve nutrient and water retention, decrease greenhouse gas emissions, and increase the soil capacity for heavy metal retention (Woolf & Lehmann, 2012; Bartoli *et al*., 2020). Transforming agricultural residues into biochar can be useful for improving soil chemical and physical properties and mitigating soil contamination by heavy metals. However, more research is still needed in the use of biochar for heavy metal immobilization. Biochar properties vary depending on pyrolysis temperature and time, the biomass used to produce the biochar, and the dose and the type of soil that biochar is applied to (Zheng *et al*., 2013; Abujabhah *et al*., 2016). For example, Shen *et al*. (2020) indicate that the characteristics and speciation of heavy metals in the biochar produced from pig manure are affected by the pyrolysis temperature. Between 500°C and 700°C is the ideal temperature for improving the characteristics of the biochar structure. In addition, the pyrolysis process can immobilize heavy metals, transforming unstable fractions into more stable fractions, and reducing environmental risk.

This study focused on the use of biochar from swine manure, one of the main residues produced in agricultural systems in the state of Mato Grosso. Swine manure is heavily used as fertilizer in sandy soils as a potential mitigation strategy for zinc (Zn) and cadmium (Cd) contamination in this type of soil. Thus, this study aimed to evaluate the capacity of an Ustoxic Quartzipsamment soil and the mixture of soil with swine manure biochar to adsorb Zn and Cd, and the chemical changes conferred to the soil after adding the biochar.

Materials and methods

Soil collection and biochar production

Soil classified as an Ustoxic Quartzipsamment (91% sand, 4% silt, and 5% clay) (Soil Survey Staff, 2014) was collected from the 0-0.2 m layer from an agricultural area located in the city of Campo Verde, Mato Grosso, Brazil (15°13'55.2" S, 54°57'43.4" W). The soil was dried and sieved (2 mm) and its chemical properties were analyzed according to the methodology described by MAPA (2017) and Teixeira *et al*. (2017). Soil pH was measured with a pH meter in a soil solution of 1:2.5 distilled water. To determine the contents of sodium (Na^+) and potassium (K^+), the soil was extracted with a $NH_4CH_3CO_2$ buffer (pH = 7). Na^+ and K^+ were determined using a flame photometer (model Pegassvs II, Tecnow, Maringa, Brazil).

To determine the contents of manganese (Mn) and phosphorus (P), the soil was extracted with a 1:1 solution of

H_2SO_4. Mn was determined by measuring the solution's absorbance at 550 nm, and P was determined by measuring the absorbance at 660 nm. The contents of calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe^{3+}), zinc (Zn^{2+}), cadmium (Cd^{+2}) and copper (Cu^{2+}) were determined by acid digestion of the soil with HCl and metal concentrations were measured using an atomic adsorption spectrometer (SpectrAA 50 Varian, Mulgrave, Australia).

Swine manure biochar (SMB) was commercially produced (SPPT, Mogi Morim, São Paulo, Brazil) via slow pyrolysis at 400ºC for 17 min. After pyrolysis, the biochar was sieved and homogenized to obtain a particle size of 0.5 mm. The chemical properties of SMB were determined following the methodology described above (MAPA, 2017; Teixeira *et al*., 2017).

Soil density and porosity were analyzed according to the method described by Teixeira *et al*. (2017). For soil bulk density (ρ_d), 35 ml of dried soil was measured in a graduated cylinder, and Equation 1 was applied:

$$\rho_d = \frac{m}{V} f \quad (1)$$

where ρ_d is the soil bulk density (kg dm^{-3}), m is the mass of dried soil added to the cylinder (g), V is the volume of soil in the cylinder (cm^{-3}), and f is a correction factor for soil humidity.

Soil porosity was obtained using Equation 2:

$$P = 1 - \frac{\rho_d}{\rho_p} f \quad (2)$$

where P is the soil porosity, ρ_p is the difference between total porosity and volumetric humidity at field capacity (θcc), and ρ_d is the soil bulk density.

Soil-biochar mix characterization

Soil samples were incubated with different doses of SMB (0, 0.25, 0.75, 1.5 and 3% (w/w)) for 30 d. Soil moisture was kept constant at 60% of the soil water holding capacity, and temperature was maintained at 25°C (Souza *et al*., 2007).

After the incubation period, the chemical properties of the mixture were determined using the methodology previously described by MAPA (2017) and Teixeira *et al*. (2017). Total organic carbon (TOC) was determined by wet combustion, following the methodology of Yeomans and Bremner (1988). Organic matter from soil was oxidized with a mixture of 10 ml of 0.167 potassium dichromate ($K_2Cr_2O_7$) and 20 ml of concentrated sulfuric acid (H_2SO_4) while stirring it to ensure good mixing of the soil with the reagents. The excess $K_2Cr_2O_7$ was then titrated with $(NH_4)_2Fe(SO_4)_2$ and TOC was estimated as the equivalent of $K_2Cr_2O_7$ that reacted with the soil. Labile carbon (LC) was determined using the method proposed by Blair *et al*. (1995) and adapted by Shang and Tiessen (1997) that consisted of the partial oxidation of soil carbon with $KMnO_4$ 0.033 M. The amount of oxidized C was calculated and considered as the labile component of soil organic C. Non-labile carbon (NLC) was determined as the difference between TOC and LC.

Sorption essays

Sorption essays were performed separately for each metal. Twenty ml of aqueous solutions of Cd ($CdCl_2$) and Zn ($ZnCl_2$) at different concentrations (0, 2.5, 5, 10, 50 and 100 mg L^{-1}) were added to 50 ml falcon tubes containing 2 g of soil or soil-biochar mixture. The samples were then agitated for 24 h at 120 rpm and subsequently centrifuged for 15 min at 9000 rpm and filtered. The temperature was kept constant at 25°C. Metal concentration in the supernatant was measured using an atomic absorption spectrophotometer (SpectrAA 50 Varian, Agilent technologies, CA, USA). Characterization tests and analyses were performed in four replicates within a completely randomized design.

The concentration of metals adsorbed in soil was calculated as the difference between the initial concentration of metals added and the concentration of metals that remained in the solution (Harter & Naidu, 2001), according to Equation 3.

$$Q_e = (C_o - C_e) \frac{Vol}{m} \quad (3)$$

where Q_e is the concentration of metals in soil (mg kg^{-1}); C_o and C_e are the initial concentration of metals and concentration of metals in the solution (in equilibrium), respectively (mg L^{-1}); Vol is the volume of the metal aqueous solution added to the falcon tube (L), and m is the amount of soil added to the falcon tube (kg).

Sorption efficiency was calculated using Equation 4.

$$Sorption\ efficiency = \frac{C_o - C_e}{C_o} \times 100\% \quad (4)$$

The Langmuir and Freundlich isotherms were then used to understand the equilibrium behind metal sorption to the surface. The equations are summarized in Table 1.

Although linear and non-linear models are mathematically equivalent, linearization often can introduce bias and increase the error distribution (Bolster & Hornberger, 2007). To avoid these errors, we fitted the Langmuir and Freundlich non-linear models using the R package 'PUPAIM' version 0.2.0 that provides a collection of isotherm models, and the software R version 4.0.3 and RStudio version 1.3.1093.

TABLE 1. Langmuir and Freundlich equations.

Isotherm model	Non-linear equation	Linear equation	
Langmuir	$q_{e} = \frac{q_{max} K_L C_e}{1 + K_L C_e}$	$\frac{C_e}{Q_e} = \frac{1}{K_L q_{max}} + \frac{C_e}{q_{max}}$	(5)
Freundlich	$q_e = K_F C_e^{1/n}$	$lnQ_e = \frac{1}{n} lnQ_e + lnK_F$	(6)

Q_e - concentration of sorbate adsorbed by the surface in the equilibrium (mg kg^{-1}); q_{max} - maximum sorption capacity (mg kg^{-1}); K_L - Langmuir distribution constant (L mg^{-1}); K_F - Freundlich distribution constant (mg kg^{-1}); C_e - sorbate concentration in the equilibrium solution (mg L^{-1}), and n - heterogeneity parameter.

Results and discussion

Soil-biochar chemical and physical properties

Biochar application had a significant effect on soil chemical and physical properties (Tab. 2). SMB addition increased soil pH. Soil without biochar had a pH of 5.3, and it increased to 7.9 with the highest SMB dose. Previous studies (Rondon *et al.*, 2006; Houben *et al.*, 2013) show that these changes can be attributed to the high ash content in biochar. Ash is mainly composed of alkaline metal oxides that can interact with the soil solution and release bases and other ions, increasing the pH (Steenari *et al.*, 1999; Glaser *et al.*, 2015). Another factor that may be related to the increase in pH is the alkalinity of the biochar caused by the presence of organic (-OH, -CO, -C=O, -COOH, -COH) and inorganic ($-PO_4^{3-}$, -Si-O-Si) functional groups, and inorganic halogens (KCl and $CaCl_2$). These groups belong to negatively charged surfaces (-COO- and -O-) that can capture protons from the solution (Tsai *et al.*, 2012; Martinsen *et al.*, 2014; Ding *et al.*, 2016). This may also explain the decrease in H^+ with the increase in the dose of biochar shown in TOC, LC and NLC also increased with the addition of SMB. As SMB is a substance with high carbon content (Wu *et al.*, 2012; Speratti *et al.*, 2017), the addition of biochar to the soil provides the necessary carbon sources for carbon cycling. Likewise, biochar can provide the soil with complex aromatic structures that increase NLC (Woolf & Lehmann, 2012) or labile compounds, such as aliphatic carbon compounds, increasing the LC content in the soil mixture.

Additionally, after SMB application there was a significant increase in the content of macro and micronutrients (P, Zn^{+2}, Cu^{+2}, Mn^{+2}, Na^+, K^+, Ca^{+2}, Mg^{+2}) (Tab. 2). This is due to the type of organic matter used for biochar production. Swine manure usually has a high concentration of these nutrients, so when SMB is produced, it inherits these compounds (Tsai *et al.*, 2012; Ding *et al.*, 2016; Subedi *et al.*, 2016).

Although there are many studies that show that biochar can increase soil porosity and improve water holding capacity and nutrient retention (Kuzyakov *et al.*, 2014; Ajayi & Horn, 2017), soil porosity did not change significantly with SMB while soil density decreased slightly with the increases in SMB doses (Tab. 3). One possible explanation is that, although the number of pores did not change, its distribution on the surface did. Speratti *et al.* (2017), using the same biochar as in this study, performed a BET analysis to obtain the pore size and distribution of swine manure biochar and found that SMB had a total surface area of 7.2 $m^2 g^{-1}$ and a total pore volume of 0.0002 $cm^3 g^{-1}$ (micropore area was 0.7 $m^2 g^{-1}$ and mesopore area was 4.1 $m^2 g^{-1}$). Considering this, biochar particles could have changed pore characteristics like size, shape, connectivity,

TABLE 2. Soil chemical properties before and after swine manure biochar (SMB) addition.

SMB dose	pH	P	Na^+	K^+	Ca^{2+}	Mg^{2+}	H^+	TOC	LC	NLC	Fe	Zn	Cd	Mn	Cu
%w/w	H_2O	mg dm^{-3}			$cmol_c$ dm^{-3}			g kg^{-1}			mg dm^{-3}				
0 (soil)	5.3	2.0	1.0	8.0	1.5	0.8	3.4	6.6	1.0	5.6	171.0	7.0	-	12.0	1.1
0.25	6.2	44.0	3.0	18.0	2.3	0.9	2.5	7.0	1.2	5.9	173.4	9.0	-	25.0	8.8
0.75	6.8	86.0	4.0	26.0	2.5	0.9	1.3	8.9	1.3	7.6	169.0	10.0	-	25.0	15.0
1.5	7.5	125.0	6.0	37.0	2.6	1.1	0.8	10.8	1.4	9.5	169.0	14.0	-	69.0	32.0
3.0	7.9	170.0	11..0	111.0	3.1	1.1	0.7	17.9	1.4	16.5	182.0	21.0	-	125.0	79.0
100 (SMB)	9.3	6536.0	52.0	421.0	137.4	46.3	-	32.2	3.0	29.2	230.0	136.0	-	9215.0	292.5

(-) Below the limit of detection. TOC - total organic carbon, LC - labile carbon, NLC - non-labile carbon.

and volume and, therefore, changed nutrient, water, and heavy metal retention. For example, Liu *et al.* (2006) report that small biochar particles could enter bigger soil particles, decreasing its size.

TABLE 3. Soil physical properties before and after swine manure biochar (SMB) addition.

SMB dose (% w/w)	Soil density (kg m^{-3})	Total porosity (m^3 m^{-3})
0 (Soil)	1.66	0.40
0.25	1.63	0.39
0.75	1.61	0.38
1.5	1.58	0.38
3	1.55	0.39
100 (SMB)	-	-

Cadmium and Zinc sorption

Sorption isotherms

Results from the sorption assays showed that the amount of adsorbed metal to the surface increased with biochar application (Fig. 1). This effect was particularly evident for a higher concentration of the metal solutions (>50 mg L^{-1}) and higher SMB doses. Considering these data, Freundlich and Langmuir isotherms were used to evaluate metal sorption to soils with and without SMB addition. Table 4 summarizes the results obtained for the sorption essays.

The Langmuir model is based on the hypothesis that, when molecules are adsorbed into the surface, they form a single, uniform layer that covers the whole surface. The Langmuir constant (K_L) is a parameter related to the affinity of metal ions to the sorption sites, and q_{max} is the maximum absorption capacity of the material. The Langmuir model showed a good fit for soil with and without SMB (R^2 varied between 0.97 and 0.99, for both Cd and Zn). K_L and q_{max} increased with the dose of SMB for all the cases, except for soil without biochar in which q_{max} for Cd was almost 20 times higher than q_{max} for a SMB dose of 3%. So, K_L of 3% SMB was larger than K_L of soil for Cd, but q_{max} was larger for soil without SMB. Since higher K_L values indicate that the interaction between adsorbate and adsorbent is stronger, and lower K_L values indicate a weaker interaction, this could mean that, although the soil has more sorption sites for Cd, biochar binds Cd stronger than soil. This could also indicate that SMB application favors chemisorption (stronger interactions, monolayer adsorption) over physisorption (weaker interactions, multilayer adsorption) that could be explained by the increase in functional groups that can bind the Cd to the surface (Ho & McKay, 1998; Kołodyńska *et al.*, 2012; Wang *et al.*, 2020).

The Freundlich isotherm is valid for heterogeneous surfaces and considers a multi-layer adsorption to explain the relationship between sorbate and adsorbent. This model had a good fit for both metals and all the concentrations of SMB used (R^2>0.95). In the Freundlich isotherm, K_F is related to the adsorption capacity of the surface, and n is a parameter related to the intensity of adsorption. If 1/n<1, adsorption is favorable; if n = 1, the partition between the two phases is independent of the concentration; finally, if 1/n>1, it means that adsorption is cooperative (Komkiene & Baltrenaite, 2016).

For Cd, K_F increased with the dose of SMB (Tab. 4). Initially, the soil without SMB had a K_F = 472.66 mg kg^{-1} and

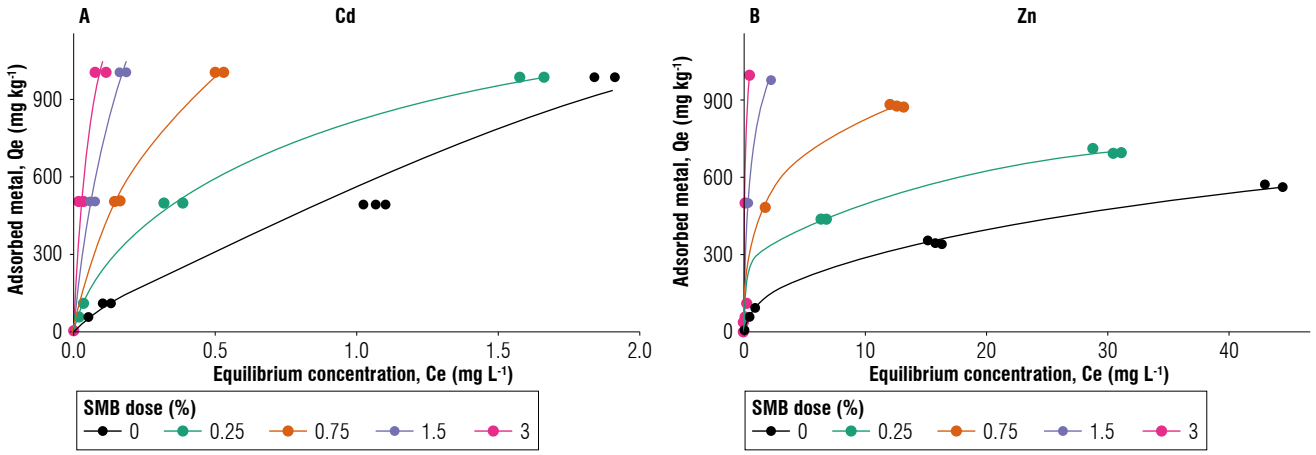

FIGURE 1. Equilibrium concentration (Ce, mg L^{-1}) vs. amount of adsorbed metal (Qe, mg kg^{-1}) for A) cadmium (Cd) and B) zinc (Zn) and for every dose of swine manure biochar (SMB) applied.

TABLE 4. Langmuir and Freundlich isotherm coefficients for Zn and Cd with and without swine manure biochar (SMB) addition to soil.

SMB dose (%) w/w	Langmuir			Freundlich		
	K_L (L mg^{-1})	q_{max} (mg kg^{-1})	R^2	K_F (mg kg^{-1})	1/n	R^2
			Cd			
0.00	0.01	46360	0.99	472.66	1.14	0.99
0.25	1.44	1393.29	0.99	762.24	0.53	0.99
0.75	2.78	1677.02	0.99	1450	0.58	0.99
1.50	4.44	2325.48	0.98	3597.56	0.72	0.98
3.00	9.86	2045.82	0.98	5376.32	0.72	0.97
			Zn			
0.00	0.05	788.59	0.99	86.04	0.49	0.99
0.25	0.17	829.66	0.99	195.92	0.38	0.99
0.75	0.56	990.14	0.99	313.81	0.41	0.98
1.50	1.29	1310.38	0.99	616.55	0.58	0.96
3.00	1.26	2373.70	0.97	1582.91	0.81	0.95

K_L - Langmuir distribution constant (L mg^{-1}), q_{max} - maximum sorption capacity (mg kg^{-1}), K_F - Freundlich constant (mg kg^{-1}), n - heterogeneity parameter, R^2 - goodness of fit measure for each regression.

increased to 5376.32 mg kg^{-1} with the highest dose of SMB; this was an 11-fold increase in K_F. In the case of Zn, the same trend was observed. Soil without SMB had an initial K_F of 86.04 mg kg^{-1} and increased to 1582.91 mg kg^{-1} with 3% SMB (18-fold increase). When comparing the K_F for both metals, K_F for Zn was lower than K_F for Cd for every SMB dose (values 3-6 times higher for Cd than for Zn). Park *et al.* (2015) and Chen *et al.* (2019) also find that biochar increases the soil's capacity to retain heavy metals, and that adsorption is up to 6 times higher for Cd than for Zn.

The intensity of adsorption (n) of the metals also changed with biochar addition. For Zn, 1/n was always lower than 1, suggesting that adsorption of the metals to the surface was favorable. In the case of Cd, 1/n was higher than 1 without biochar, indicating cooperative adsorption (i.e., the amount of metal adsorbed to the surface had an effect on the adsorption of new metal ions to the surface). With the addition of biochar, 1/n was lower than 1, indicating that the adsorption was favorable. That difference in 1/n between the soil and the soil with SMB for Cd was also an indication of different mechanisms of interaction between Cd and the surface when SMB is present.

Although both models were able to explain the data, the Langmuir isotherm was slightly better than the Freundlich isotherm, particularly for higher doses of SMB. This effect has also been observed in other studies with biochar and is attributed to the monolayer adsorption that occurs in the biochar surface (binding sites in the surface over electrostatic interactions) (Kołodyńska *et al.*, 2012; Park *et al.*, 2015; Wang *et al.*, 2020).

Sorption efficiency

The sorption efficiency was used to evaluate the capacity of soil (with and without biochar) to retain Cd and Zn (Fig. 2). Soil without SMB addition was very efficient in removing Cd from the solution, even for high doses of the metal (efficiency = 97%). Sorption efficiency increased with biochar application. Doses of 1.5 and 3% SMB showed a sorption efficiency of ~100% for all the studied Cd concentrations (0, 2.5, 5, 10, 50 and 100 mg L^{-1}).

Sorption efficiency for Zn without SMB application was high for low metal concentrations (~90% for Zn concentrations of 0, 2.5, 5 and 10 mg L^{-1}). However, for higher concentrations of Zn (>50 mg L^{-1}), sorption efficiency to the surface decreased to ~50%. Soil-SMB mixture had a sorption efficiency of ~100% for lower Zn doses (≤10 mg L^{-1}) and it varied between 70-100% for higher SMB doses (depending on Zn initial concentration).

This increase in sorption efficiency with SMB application was expected, as biochar characteristics favor metal retention (Mohamed *et al.*, 2018). Particularly, SMB has different functional groups (*e.g.*, COOH, phenolic, etc.) that are sorption sites for cations by forming metallic complexes (Wuana & Okieimen, 2011; Rees *et al.*, 2014).

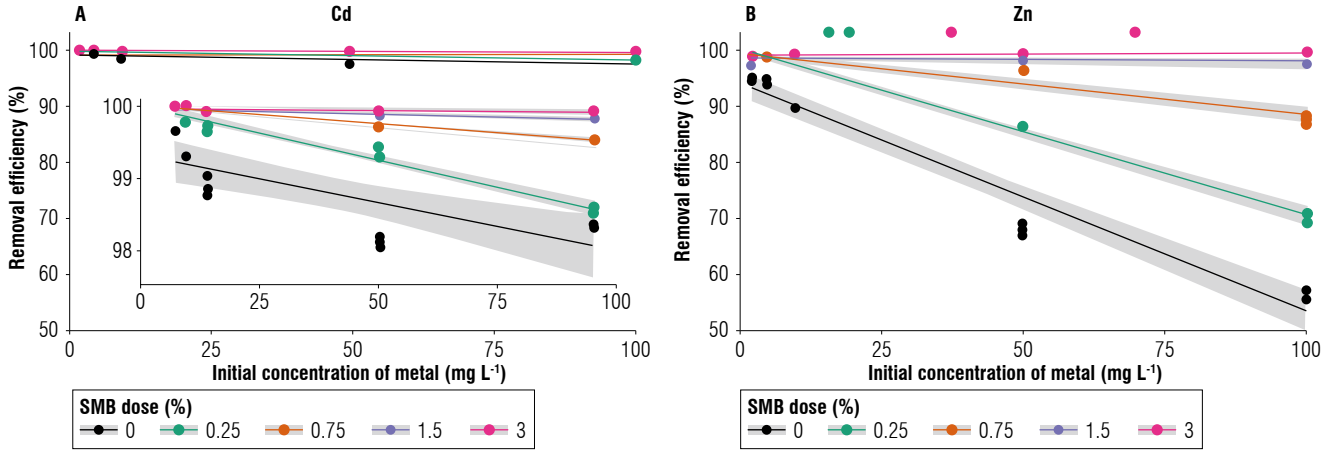

FIGURE 2. Sorption efficiency for A) cadmium (Cd) and B) zinc (Zn) for each dose of swine manure biochar (SMB) applied.

Figure 2 also shows that the relationship between SMB dose and sorption efficiency is different for both metals. Efficiency for Cd was higher than that for Zn for all SMB doses and even for soil without SMB. This difference could be related to the chemical properties of each metal, such as electronegativity and ionic radius. Zn and Cd show electronegativity values of 1.66 and 1.46 and ionic radius values of 0.074 and 0.098, respectively (Liu *et al.*, 2006). Puls and Bohn (1988) show that the retardation factor (*i.e.*, estimate of solute movement in the soil fraction) increases with the ionic radius and decreases with increasing electronegativity; this explains the highest retention for Cd over Zn. Another explanation could be related to the amount of Zn and Cd in the soil and in the biochar used for this study. The Cd concentration in the soil and in the biochar was below the limit of detection, whereas the initial concentration of Zn in the soil was 7 mg kg^{-1} and SMB had a Zn concentration of 136 mg kg^{-1} (Tab. 2).

As higher concentrations of Zn solution were added to the soil, the binding sites for Zn in the soil or soil-SMB surface become less available, and more Zn was left in the solution.

Additionally, as shown in Table 2, SMB increased soil pH. An alkaline pH can promote the precipitation of metal hydroxides and, therefore, can decrease the mobility of metal ions in the soil profile (Pierangeli *et al.*, 2009).

Conclusions and implications of the use of SMB to retain heavy metals in sandy soils

The results show the high capacity of SMB to help in Zn and Cd sorption in sandy soils, potentially avoiding problems with bioaccumulation, contamination, and toxicity via leaching and runoff.

The data adjusted best to the Langmuir isotherm, particularly for higher SMB doses, suggesting a monolayer sorption to the surface. Additionally, with SMB application a stronger adsorbate-adsorbent interaction was observed (higher K_L) that could mean that SMB favors chemisorption over physisorption. On the other hand, soil without SMB showed weaker interactions with the metals.

Both SMB and the studied Ustoxic Quartzipsamment had a higher affinity towards Cd than Zn. This was related to the initial Zn content in both soil and SMB (7 and 136.25 mg kg^{-1}, respectively), as well as higher electronegativity and ionic radius of Zn.

Despite the greater adsorptive capacity observed with 3% SMB, it is important to also consider the high cost of biochar application and look for the best cost/benefit relationship. In that regard, SMB applied at 0.75% proved to be effective for the retention of both metals with high efficiency. At this dose, the K_F was about three times higher for Zn and Cd compared to the soil without the addition of SMB.

SMB addition to the soil increased the content of P, Na, K, TOC, NLC, LC, Zn, Mn, Cu and increased pH, making the soil more alkaline and more suitable for agriculture.

Although the results of this study are promising for Cd and Zn retention in sandy soils, more research is needed in the area. Particularly, more studies are necessary that focus on competition between Cd, Zn, and other heavy metals for the binding sites of SMB, metal desorption from the SMB-soil surface, and availability of the adsorbed metals to plants in order to evaluate if SMB addition is a good strategy for minimizing the risks associated with heavy metal pollution in sandy soils.

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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

EGC, OLSW and EFGD designed the experiments. WSA carried out the field and laboratory experiments. ECN, BD, and WSA contributed to the data analysis, and ECN, BD and EGC wrote the article. All authors reviewed the manuscript.

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Development of soybean plants using a substrate based on green coconut fiber

Desarrollo de plantas de soya usando un sustrato con base en la fibra de coco verde

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ABSTRACT

This study aimed to evaluate the effect of different doses (0, 10, 20, 30, 40, and 50 t ha^{-1}) of green coconut fiber in growth substrate on the early development and physiology of two soybean cultivars (Pampeana 40RR and Pampeana 60RR). The experiment was carried out in a greenhouse using a completely randomized experimental design in a 2×6 factorial arrangement (two genotypes and six doses) with five replicates. Each replicate was made up of one plant, totaling 60 experimental units. Biometric variables (height, number of leaves, stem diameter, leaf area, and dry matter) and physiological variables (photosynthesis, stomatal conductance, transpiration, internal carbon, water use efficiency, and photosynthetic pigments) were evaluated. After obtaining the data 30 d after sowing, the means were subjected to an analysis of variance and, when significant for the F test, they were subjected to regression analysis and comparison of means by the Tukey's test. Through the regression analysis, the ideal minimum dose for each variable could be calculated. We observed an increase in plant height, stem diameter, number of leaves, leaf area, leaf dry mass, stem dry mass, and root dry mass of around 51.10%, 31.60%, 52.83%, 61.78%, 79.65%, 81.52%, and 6.06%, respectively, when we compared the values of the minimum doses with the maximum points found in each variable. Regarding the gas exchange, cultivar 60 RR was superior to cultivar 40 RR. In conclusion, the green coconut fiber compound had a positive influence on the growth and physiology of the cultivars, with the best response being obtained at the dose of 30 t ha^{-1}.

Key words: organic fertilization, biometrics, *Glycine max* (L.) Merrill, gas exchange.

RESUMEN

El objetivo de este estudio fue evaluar el efecto de diferentes dosis (0, 10, 20, 30, 40 y 50 t ha^{-1}) de fibra de coco verde en sustrato de crecimiento sobre el desarrollo temprano y la fisiología de dos cultivares de soya (Pampeana 40RR y Pampeana 60RR). El experimento se llevó a cabo en un invernadero utilizando un diseño experimental completamente al azar en un arreglo factorial de 2×6 (dos genotipos y seis dosis) con cinco repeticiones. Cada repetición estuvo formada por una planta, totalizando 60 unidades experimentales. Se evaluaron variables biométricas (altura, número de hojas, diámetro del tallo, área foliar y masa seca) y fisiológicas (fotosíntesis, conductancia estomática, transpiración, carbono interno, eficiencia en el uso del agua y pigmentos fotosintéticos). Después de obtener los datos, a los 30 d después de la siembra, las medias fueron sometidas a análisis de varianza y, cuando fueron significativas según la prueba F, fueron sometidas a análisis de regresión y comparación de medias por la prueba de Tukey. A través del análisis de regresión, fue posible calcular la dosis mínima ideal para cada variable. Se observó un aumento en la altura de la planta, el diámetro del tallo, el número de hojas, el área foliar, la masa seca de hojas, masa seca de tallo y masa seca de raíces de alrededor de 51.10%, 31.60%, 52.83 %, 61.78%, 79.65%, 81.52%, y 6.06%, respectivamente, cuando comparamos los valores de las dosis mínimas con los puntos máximos encontrados en cada variable. En cuanto al intercambio de gases, el cultivar 60 RR fue superior al cultivar 40 RR. En conclusión, el compuesto de fibra de coco verde influyó positivamente en el crecimiento y fisiología de los cultivares, obteniendo la mejor respuesta con la dosis de 30 t ha^{-1}.

Palabras clave: fertilización orgánica, biometría, *Glycine max* (L.) Merrill, intercambio de gases.

Introduction

Soybean (*Glycine max* (L.) Merrill) is a legume of great economic importance and nutritional value. It is part of a set of agricultural activities with greater global prominence and is the fourth most consumed and produced grain only after corn, wheat, and rice (Hirakuri & Lazzaroto, 2014). Soybean cultivation has expanded in Brazil as a promising crop, with prominence initially in the south and central-east and later in the north and northeast regions (Barrozo & Rosa, 2018; Machado *et al.*, 2018).

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Some factors are essential for the crop to show good biometric and physiological development, and consequently, high productivity and quality of grains. These factors include solar radiation, water availability, and fertilization, with the latter being a decisive factor for productive success (Sediyama *et al*., 2012; Taiz & Zeiger, 2013; Tejo *et al*., 2019). However, although mineral fertilization is indispensable, it has a high cost. Thus, the use of other sources for nutrient supply for plants is becoming increasingly common (Dourado Neto *et al*., 2012). Adeyeye *et al*. (2014), in a study with soybean, demonstrate that the application of maize stover compost and N fertilizer significantly increase plant growth (plant height, number of leaves, nodes, and branches). In addition, dry matter production and number of fruits per plant are also significantly affected by different levels of those compounds.

Because of these problems, the use of organic fertilizers appears as an economically viable alternative, besides being less harmful to the environment. Organic fertilizers are a source of mineral nutrients and contribute to improvements in the biological characteristics of the soil (Freitas *et al*., 2012). Among the main organic fertilizers used, we can highlight organic compounds originated from the composting process as viable alternatives for use in agriculture.

Among these compounds, we can find the green coconut fiber, which is a by-product of the industrialization of coconut water. The fiber is constantly discarded incorrectly, causing negative environmental impacts. Therefore, we can consider it a prominent environmental liability (Klein, 2015). When compost is applied to crops in appropriate doses, it adds organic matter, improves soil structure and water retention, reduces the need for fertilizers and the potential for soil erosion (Mattos *et al*., 2011), contributing to the full development of soybean. Scientific advances in technologies for soil management, techniques for the correction of acidity, and balanced fertilization with macro and micronutrients allow crops to express their potential under the different edaphoclimatic conditions in Brazil (Freitas, 2011).

Given the above, we hypothesized that the substrate compound based on green coconut fiber helps in the development of soybean plants. Therefore, this research aimed to evaluate the influence of the green coconut fiber applied at different doses to the substrate on the initial development and gas exchange of soybean plants.

Materials and methods

Characterization of the experimental area

This experiment was carried out from August to October 2019 in a greenhouse covered with 200-micron UV plastic that favors the spread of light within the environment. The greenhouse belonged to the Phytotechnics Department located at Campus do Pici, Federal University of Ceará (UFC), in Fortaleza, Brazil. The maximum temperature was 29.4°C, while the minimum was 16.4°C, and the average relative humidity was around 32%, according to data obtained through a data logger (model U12/012, HOBO®, Marleston, Adelaide, Australia) installed in the center of the greenhouse. According to Köppen, the local climate is of Aw type, *i.e*., very hot rainy tropical with predominant rains in the summer and autumn (Oliveira *et al*., 2013).

Experimental design and treatments

The experimental design was completely randomized in a 2×6 factorial arrangement, with two soybean cultivars (Pampeana 40RR and Pampeana 60RR), six doses of compost containing crushed green coconut considered as fiber (shell + mesocarp + endocarp) (0, 10, 20, 30, 40, and 50 t compost ha^{-1}), and five replicates. Each replicate was made up of one plant per pot.

Obtaining the compost

The compost used in the experiment was obtained by the action of aerobic microorganisms on green coconut fiber mixed with poultry manure, in the proportion of 3:1 (v/v) and arranged under layers in rows of 3 m long, 1 m wide, and 1 m deep, under an average temperature of 27.8°C. The windrows were turned over weekly to assist oxygenation and decomposition of the material by the microorganisms.

After 7 months, the compost was sieved in a 5 mm mesh and placed to dry for 48 h in an oven with forced air circulation (model MA033/1080, Marconi®, Piracicaba, SP, Brazil) at 45°C. Then, it was weighed according to the following treatments: control (0 t ha^{-1}), 50 g (10 t ha^{-1}), 100 g (20 t ha^{-1}), 150 g (30 t ha^{-1}), 200 g (40 t ha^{-1}), and 250 g (50 t ha^{-1}) and mixed with the soil (4 kg per pot). For this, the doses were estimated using a soil density of 1.25 and a depth of 0.20 cm. The chemical characteristics of the compound are shown in Table 1, according to Silva (2009).

TABLE 1. Chemical characteristics of the green coconut fiber-based compound.

Ca	Mg	K	P	S	Na	Zn	Fe	Mn	Cu	pH	EC
..... g L^{-1}				..... mg L^{-1}						-	dS m^{-1}
2.88	0.89	0.34	0.06	2.13	0.06	0.15	-	0.92	0.2	6.8	2.4

EC - Electric conductivity.

TABLE 2. Soil fertility analysis.

C	OM	pH	P	K	Ca	Mg	Na	Al	H+Al	BS	CEC	V	ESP	EC
g kg^{-1}			mg dm^3	mmolc dm^3								%		dS m^{-1}
7.39	12.75	6.6	102	1.24	20.7	6.6	0.33	-	8.3	28.8	37.1	78	1	0.35

OM - organic matter; BS - base saturation; CEC - cation exchange capacity; ESP - exchangeable sodium percentage; EC - electrical conductivity; V - base saturation.

Plant growth experiments

The soybean seeds were sown in pots coated with plastic polyethylene vases containing 4 kg of soil (Red-Yellow Argisol) as classified by Lima *et al*. (2002). The soil was homogenized with the compost containing the green coconut fiber in its respective doses. The chemical characteristics of soil fertility are shown in Table 2, according to Silva (2009). Thinning was carried out a week after planting, leaving only one plant per pot to avoid competition for space and light, since inoculation with *Bradyrizobium* sp. had not been carried out.

The irrigation was performed manually using the water retention capacity (WRC) as a reference that was previously determined in the laboratory. The WRC was determined as described by Souza *et al*. (2000), considering the difference between the weight of the wet soil after saturation and free drainage, and the weight of the dried soil.

Water retention capacity was maintained daily in all pots by gravimetry, weighing them and replacing the volume of water lost by evapotranspiration, using a scale (model3/0, Songhe Tools®, São José dos Pinhais, PR, Brazil). Manual removal of weed plants was carried out throughout the experiment.

Analyzed variables

Biometric variables

During the pre-flowering period 30 d after sowing (DAS), plant height (PH), stem diameter (SD), and the number of leaves (NL) were measured. We used a measuring tape graduated in centimeters to measure the height of the plant from the bottom of the stem until the last insertion of the leaf. A digital caliper was used to measure the SD at the bottom of the stem. The NL was determined based on the count of each fully developed leaf.

At the end of the experiment at 30 DAS, the collective destruction of the plants was carried out to obtain the dry mass of the organs (dry mass of leaves, stems, and roots). The leaf area was measured using a surface integrator (LI - 3100 Area Meter, Li-Cor Inc., Lincoln, Nebraska, USA).

Shoots and roots were collected, rinsed, packaged in paper bags, and transferred to a drying oven (model MA033/1080, Marconi®, Piracicaba, SP, Brazil) at 65°C until a constant mass was obtained. The vegetative organs were weighed using a precision scale (model AL200, Marte®, São Paulo, SP, Brazil) for obtaining the respective dry mass. The total dry mass of the plants was determined by adding the dry mass of the leaves, stem, and roots.

Physiological variables

The analyses of physiological variables were performed 30 DAS (pre-flowering period). SPAD was used to measure the relative chlorophyll index of the last three fully developed leaves located in the central leaflet. At the end of the experiment, photosynthetic pigments were also evaluated (contents of chlorophyll a, b, total, and carotenoids in leaves), following the methodology described by Wellburn (1994).

The values of chlorophyll a (Chl a), b (Chl b), total (Chl t), and carotenoids were estimated using the following equations:

$$\text{Chl a} = (12.47 \times A665) - (3.62 \times A649) \quad (1)$$

$$\text{Chl b} = (25.06 \times A649) - (6.5 \times A655) \quad (2)$$

$$\text{Chl t} = (7.15 \times A665) + (18.71 \times A649) \quad (3)$$

$$\text{Carotenoids} = \frac{(1000 \times A480 - 1.29 \times Ca - 53.78 \times Cb)}{220} \quad (4)$$

where A represents the absorbance at a respective wavelength with values obtained in μg ml^{-1} and expressed in mg g^{-1} of dry matter (DM).

The gas exchange analysis was carried out between 08:00 am and 11:00 am on the third fully expanded leaf using an infrared gas analyzer (model LCI, BioScientfic, Great Amwell, England). The liquid photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (*gs*, mol H_2O m^{-2} s^{-1}), internal CO_2 concentration (Ci, μmol CO_2 mol^{-1}), and transpiration (*E*, mmol m^{-2} s^{-1}) were estimated. From the A/*E* ratio, we calculated the water use efficiency (WUE).

Statistical analysis

The results of the evaluated variables were subjected to an analysis of variance (ANOVA) and to the Shapiro-Wilk normality and homogeneity tests. When the variables were significant for the F test, they were subjected to a regression analysis and comparison of means by the Tukey's test, using the computer program RStudio. We made the graphics using SigmaPlot version 11.0.

Results and discussion

Biometric variables

The results of the analysis of variance (Tab. 3) showed that for the cultivars tested, only the variables stem diameter (SD) and root dry mass (RDM) did not show significant responses, while the others showed a significance of 1% and 5% probability by the F test. Regarding the dose factor, all the variables tested were influenced at the level of 1% probability, showing a highly significant effect on the development of soybean plants. Regarding the interaction between factors, only the variables plant height (PH) and RDM showed significant responses at the 5% probability level by the F test.

Regarding the variable plant height (Fig. 1A), two quadratic equations were adjusted. We observed that the cultivar 1

TABLE 3. Summary of the analysis of variance for the variables plant height (PH), leaf area (LA), number of leaves (NL), stem diameter (SD), leaf dry mass (LDM), stem dry mass (SDM), and root dry mass (RDM) in two soybean cultivars grown under different doses of green coconut fiber organic compound.

		Medium square						
SV	**DF**	**PH**	**LA**	**NL**	**SD**	**LDM**	**SDM**	**RDM**
		(cm)	(cm^2)		(mm)	---------- (g) ----------		
Cultivar (C)	1	16833.8**	50518**	36.82**	0.20 ns	0.59*	1.22**	0.03 ns
Doses (D)	5	978.9**	86648**	8.86**	1.08**	2.16**	0.47**	0.45**
C x D	5	559.1*	2388 ns	0.37 ns	0.06 ns	0.032 ns	0.03 ns	0.11*
Residue	48	126.2	6103	1.4	0.17	0.09	0.03	0.04
Total	59	-	-	-	-	-	-	-
CV (%)		23.81	24.64	21.07	12.23	21.93	24.71	27.21

SV - source of variation; DF - degree of freedom; C x D - cultivar x doses interaction; CV - Coefficient of variation. *, ** Significant at 5% and 1% by the F test, respectively. ns - not significant.

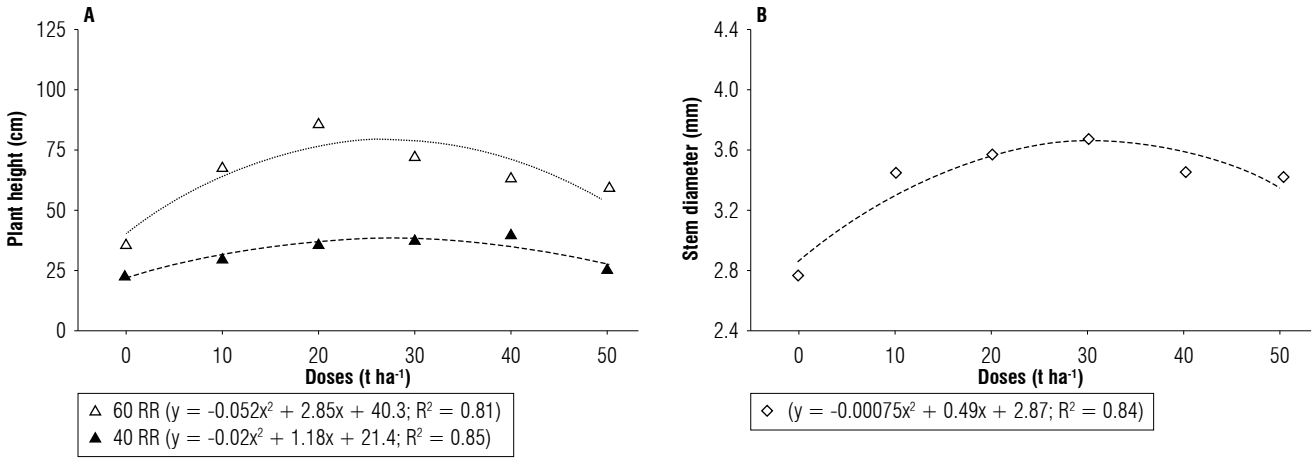

FIGURE 1. A) Plant height and B) stem diameter in two soybean cultivars (Pampeana 60RR and Pampeana 40RR) grown at different doses of compost containing green coconut fiber.

(Pampeana 60RR) showed superiority at all tested doses compared to cultivar 2 (Pampeana 40RR). The maximum height for the cultivar Pampeana 60RR was 79.35 cm when the plants were fertilized with a dose of 27.40 t ha^{-1}, whereas the cultivar Pampeana 40RR was 36.33 cm under a dose of 29.50 t ha^{-1}. After these doses, there was a reduction in the height of the plants, possibly due to a toxic effect, or an imbalance of a certain nutrient provided by the compound. Making a comparison between the maximum points of the two cultivars, we observed an increase of more than 100% of the cultivar Pampeana 60RR compared to the cultivar Pampeana 40RR. Hence, for this variable, 'Pampeana 60 RR' was highly responsive to the use of a coconut fiber-based compound.

Regarding the effect of the doses for increasing the stem diameter (Fig. 1B), a quadratic equation for the data was adjusted, finding a maximum point of 3.67 mm when the plant was fertilized with a dose of 32.66 t ha^{-1}. When making a comparison with dose 0 that had a maximum point of 2.51 mm, there was an increase of 31.60%.

Analyzing these results, we could observe the influence of the doses of the compound on plant growth and the differentiation of meristematic cells of the vascular cambium and the bark. Oliveira *et al.* (2018) found similar results when working with cherry tomato plants under organic fertilization in different cultivation environments. They verified an increase in plant height depending on the application of fertilizers and the evaluation periods. According to Camargo (2012), the efficient use of organic fertilizers provides improvements in the physical, chemical, and biological properties of the soil that can assist in the proper growth of plants and, consequently, promote better crop yields. Araújo *et al.* (2012) working with sources of organic matter in castor bean BRS Energia, report significant results with the use of sources of organic matter for the stem diameter.

In a study conducted by Castro *et al.* (2016), the authors find that for capim-palisade there is a linear increase in plant height depending on the application of cattle manure. Therefore, the higher results for plant height found in our research are associated with the availability of nutrients provided by the compound used in the experiment, especially Ca and Mg (growth-related nutrients) (Tab. 1). However, higher levels caused physiological disorders that hinder the development of the plants, especially for the cultivar Pampeana 40 RR.

For leaf area, the cultivar 60RR showed an average of 346.08 cm^2, significantly higher than the cultivar 40RR that showed an average value of 288.04 cm^2. Comparing these averages, we observed an increase of 16.78% for the cultivar 60RR compared to cultivar 40RR (Fig. 2A). Regarding the effect of doses on the increase of leaf area, a quadratic equation for the data was adjusted, finding a maximum point of 406.16 cm^2 when plants were fertilized with a dose of 32.7 t ha^{-1} (Fig. 2B). When we compared the dose 0 that showed a value of 155.22 cm^2, there was an increase of 61.78% in the maximum point. This result confirmed the values of plant height since there was possible toxicity. Therefore, there was a reduction in plant growth for the number of leaves emerging, influenced by the application of higher doses of the compound.

The increase in leaf area improves a plant's capacity to harness solar energy to carry out photosynthesis. Therefore, a higher leaf area will probably increase productivity

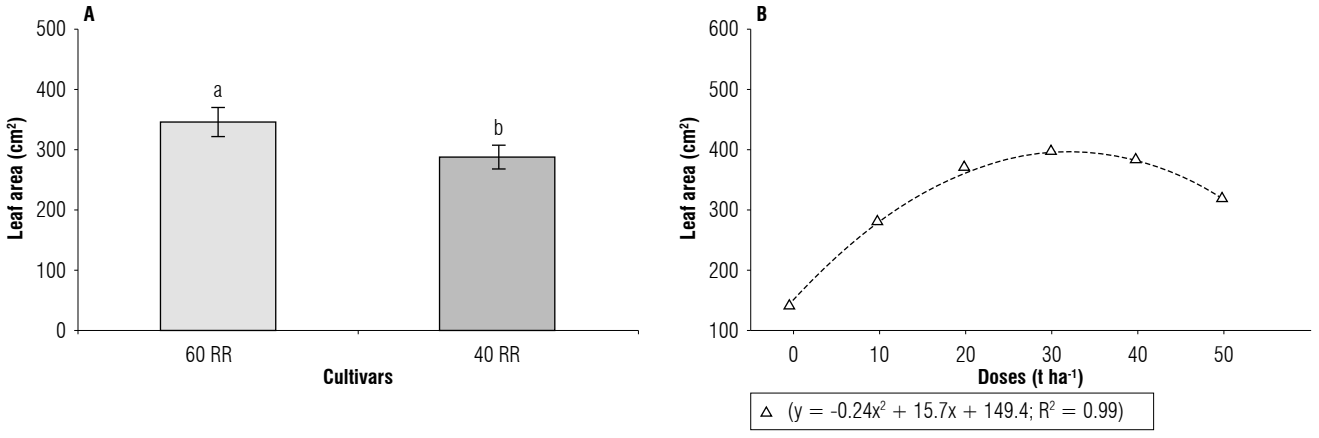

FIGURE 2. A) Leaf area of soybean cultivars Pampeana 60RR and Pampeana 40RR B) grown under different doses of compost containing green coconut fiber. Values are means of five plants (± standard error). Means followed by the same letters do not differ according to the Tukey's test at 5% probability.

since it is directly associated with photosynthetic rates, and this, in turn, is directly related to production. In that sense, the cultivar Pampeana 60RR again showed increased results. It is noteworthy that, although highly responsive to fertilization with the compost from green coconut fiber, the cultivars demonstrated toxic effects at the two highest levels of fertilization. This toxicity may be associated with a greater amount of Mn and Cu (Tab. 1).

The dry mass of leaves of the cultivar 60RR showed an average of 1.50 g, being superior to cultivar 40RR that showed an average of 1.30 g (Fig. 3A). Comparing these averages, an increase of 13.34% was seen for the effect of doses on the increase of the dry mass of leaves. A quadratic equation was adjusted to the data, and we found a maximum point of 1.72 g when the plant was fertilized with a dose of 35.00 t ha^{-1} (Fig. 3B). When making a comparison with dose 0 (0.351 g), there was an increase of 79.65%.

Lima *et al.* (2001) observe a higher dry mass of leaves that increase on average 0.3 and 1.1 g per plant. These results are directly related to the height of the plant since plants may generally have a higher number of leaves to carry out photosynthesis and meet the demand for assimilates.

For the dry mass of the stem, we found that the cultivar 60RR showed an average of 0.87 g, superior to the cultivar 40RR that showed an average of 0.58 g, representing an increase of 33.34% (Fig. 4A). Concerning the effect of doses on the increase of the dry matter of the stem, a quadratic equation was adjusted to the data resulting in a peak of 0.99 g when plants were fertilized with a dose of 32.50 t ha^{-1} (Fig. 4B). When comparing with the dose 0 (0.170 g) at the maximum point, there was an increase of 82.82%.

We can associate the reduction of several of the variables analyzed, such as stem dry mass, leaf dry mass, and leaf

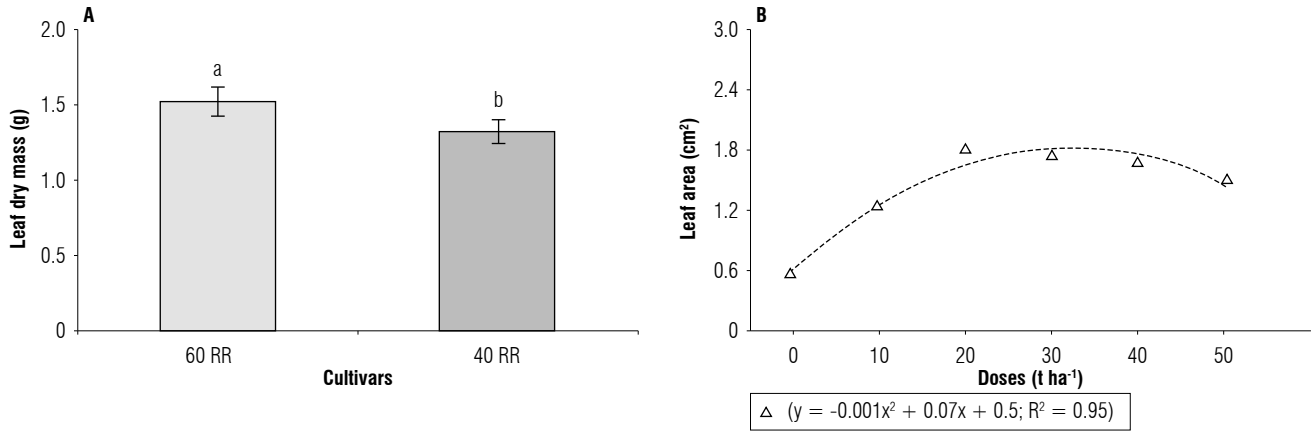

FIGURE 3. A) Leaf dry mass of soybean cultivars Pampeana 60RR and Pampeana 40RR B) under different doses of the organic compound of green coconut fiber. Values are the means of five plants (± standard error). Means followed by the same letters do not differ according to the Tukey's test at 5% probability.

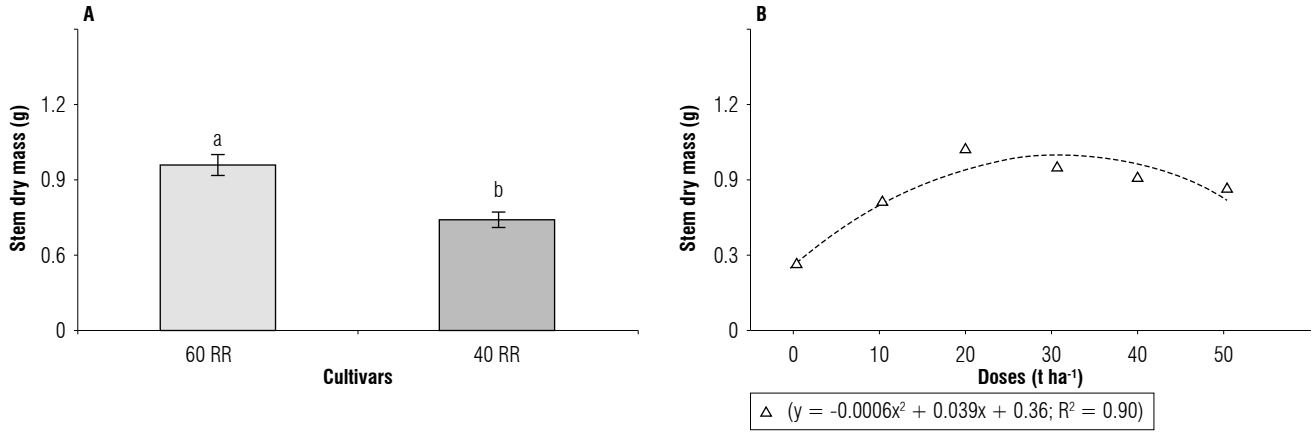

FIGURE 4. A) Stem dry mass of the of soybean cultivars Pampeana 60RR and Pampeana 40RR B) under different doses of the organic compound of green coconut fiber. Values are the means of five plants (± standard error). Means followed by the same letters do not differ according to the Tukey's test at 5% probability.

area, with the increase of the doses by the values of Zn (Tab. 1). Zinc is required in very low amounts by the plants (Marschner, 2012), so with the increase in the doses of the coconut fiber compound, this amount rose to values that caused toxicity in soybean plants that culminated in the reduction of plant development, as a whole.

The dry mass distribution in the plant is a variable that allows a discussion about the process of product translocation resulting from the photosynthetic process, facilitating the understanding of the plant response in terms of productivity. According to Lima *et al.* (2010), when evaluating the growth of coconut (*Cocos nucifera*) plants as a function of organic fertilization, the dry mass of the stem was significantly influenced by the application of organic matter combined with mineral fertilizers P and K.

Therefore, regarding the dry mass of the shoot (dry mass of leaves + dry mass of the stem), we observed that the cultivar 60RR is more responsive to fertilization, demonstrating that the organic compound is a material with great potential in comparison to chemical fertilization. The fact that it showed a higher dry mass of the shoot suggests a better mechanism for translocation of photo-assimilates and greater efficiency of nutrient absorption. The doses close to 30 t ha^{-1} were the ones that provided the highest values for this and other growth variables, such as stem diameter, leaf dry mass, and leaf area, indicating that in this dose, the supply of nutrients, as well as the balance between them, is the most appropriate.

Regarding the dry mass of roots (Fig. 5), two quadratic equations were adjusted. We observed that the cultivar Pampeana 40RR showed higher values up to the dose of 30 t ha^{-1} compared to the cultivar Pampeana 60RR.

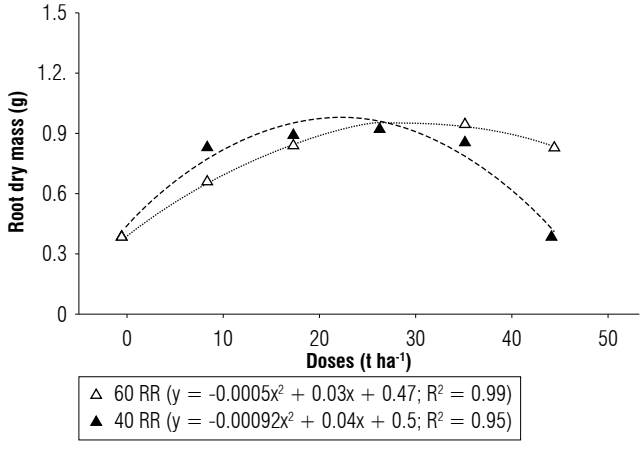

FIGURE 5. Root dry mass of soybean cultivars Pampeana 60RR and Pampeana 40RR grown under different doses of compost including green coconut fiber.

The maximum point of the dry mass of roots found for the cultivar 60RR was 0.92 g when the plants were fertilized with a dose of 30 t ha^{-1}, while the cultivar 40RR showed an average of 0.93 g with the dose of 21.74 t ha^{-1}.

The ideal root system is the one that explores the largest volume of soil, allowing the plant to explore a larger soil area (Parfitt *et al.*, 2017). The growth of the root system of plants is essential for the absorption of nutrients and better performance in translocating them to the vegetative organs. This can be seen by the results of the growth and accumulation of dry matter in the root, especially in the cultivar Pampeana 60 RR.

A possible explanation for this decrease in plant growth when subjected to higher doses (40 or 50 t ha^{-1}) of compost based on green coconut fiber is the number of nutrients present in the soil after adding the compost. In practical terms, nutrients play important roles in plants; however, the necessary amount, especially of micronutrients in the soil, is small due to the low requirement of this nutrient by crops (Marschner, 2012). So, this greater number of micronutrients in the soil with doses from 30 t ha^{-1} when the treatments were applied could have caused the opposite effect that culminated in a reduction in most of the growth variables evaluated.

Physiological variables

The analysis of variance for the values of chlorophyll a, b and total, and carotenoids is shown in Table 4. Only the variable carotenoids showed no significant response, while the others showed a 1% significance probability by the F test. Regarding the doses factor, only chlorophyll b showed no significant response, and the other variables tested were influenced at the level of 1% probability. Concerning the interaction between factors, only the variable chlorophyll a showed a significant response at the level of 1% probability by the F test.

For chlorophyll a, two quadratic equations were adjusted, finding a maximum point of 4.90 when plants were fertilized with a dose of 41.6 t ha^{-1} for cultivar 60RR, and a maximum point of 6.8 when plants were fertilized with a dose of 40.60 t ha^{-1} for cultivar 40RR (Fig. 6A). For chlorophyll b, the cultivar 60RR showed an average value of 6.62 which is higher than the cultivar 40RR that showed an average value of 4.07. There was an increase of 38.51% when comparing these averages (Fig. 6B).

Regarding total chlorophyll, we found that the cultivar 60RR showed an average of 11.10. This cultivar obtained

TABLE 4. Summary of analysis of variance for the variables chlorophyll a (Chl a), chlorophyll b (Ch b), total chlorophyll (Chl t), and carotenoids in two soybean cultivars grown under different doses of compost containing green coconut fiber.

SV	DF	Medium square			
		Chl a	Chl b	Chl t	Carotenoids
Cultivar(C)	1	40.739**	77.927**	136.688**	0.00138 ns
Doses (D)	5	36.947**	27.07 ns	132.32 ns	1.78**
C x D	5	6.999**	49.6 ns	4.786 ns	0.12 ns
Residue	48	1.358	5.982	4.418	0.16836
Total	59	-	-	-	-
CV (%)		25.35	45.72	22.31	29.55

SV - source of variation; DF - degree of freedom; C x D - cultivar x doses interaction; CV - coefficient of variation. *, ** Significant at 5% and 1% by the F test, respectively. ns - not significant.

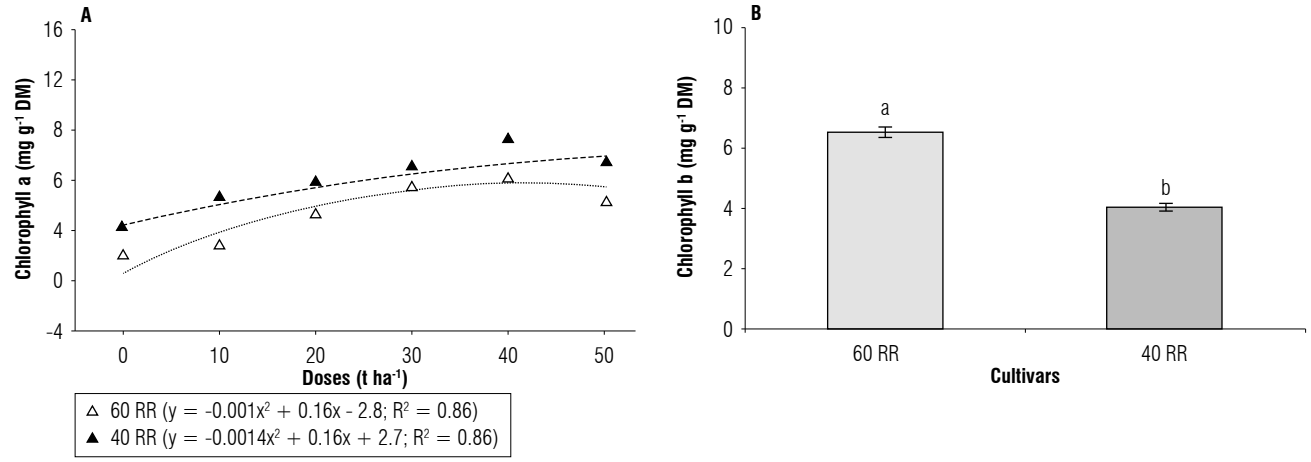

FIGURE 6. A) Chlorophyll a and B) chlorophyll b from soybean cultivars Pampeana 60RR and Pampeana 40RR grown under different doses of compost containing green coconut fiber. Values are the means of five plants (± standard error). Means followed by the same letters do not differ according to the Tukey's test at 5% probability.

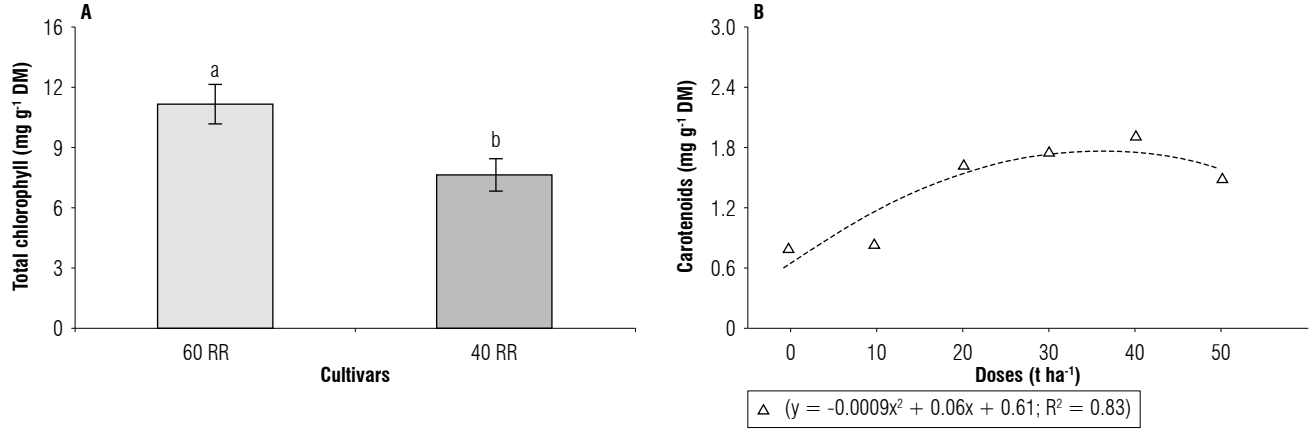

FIGURE 7. A) Total chlorophyll and B) carotenoids of soybean cultivars Pampeana 60RR and Pampeana 40RR grown under different doses of compost containing green coconut fiber. Values are the means of five plants (± standard error). Means followed by the same letters do not differ according to the Tukey's test at 5% probability.

higher values compared to the cultivar 40RR that showed an average of 7.73. An increase of 30.36% was observed when comparing these averages (Fig. 7A). Regarding the variable carotenoids, we found that there was no significant difference between cultivars. Concerning the effect of doses, a quadratic equation for the data was adjusted, and we found a maximum point of 1.60 when the plants were fertilized with a dose of 33.34 t ha^{-1}. When comparing the dose 0 (0.40) with the maximum point, there was an increase of 78.02% (Fig. 7B).

Chlorophyll a is the pigment used to perform photochemistry (the first stage of the photosynthetic process), while the other pigments assist in the absorption of light and the transfer of radiant energy to the reaction centers. Thus, they are called accessory pigments. The main accessory pigments also include other types of chlorophylls, such as chlorophyll b that is present in higher plants, green algae, and some bacteria (Taiz & Zeiger, 2013).

Chlorophyll is one of the main factors related to the photosynthetic efficiency of plants and, consequently, to growth and adaptability to different environments and adverse conditions caused by different types of stress. The apparent differences in the color of the plants are due to the presence and distribution of other associated pigments, such as carotenoids and anthocyanins that always accompany the chlorophylls. In plants, carotenoids are located in the plastids where they are synthesized and have a non-enzymatic antioxidant function. Anthocyanins are flavonoid compounds with various functions in plants, like defense against abiotic stresses. Therefore, these compounds have a fundamental role in combating reactive oxygen species (ROS) (Barbosa *et al*., 2014).

Based on the results obtained, we concluded that for the photosynthetic analysis, the cultivar 60RR was more responsive to the assimilation of photons that reflected its photosynthetic metabolism and the greater production of photoassimilates and made it metabolically more efficient than the cultivar 40RR. The essential role of fertilization with residues of green coconut fiber provided nutrients and good physical, chemical, and biological conditions so that soybean plants could increase the levels of photosynthetic pigments when compared to the treatment without fertilization. However, as has been shown, higher doses caused negative effects on the physiological development of the soybean plant.

In the analysis of variance shown in Table 5, we saw that for the cultivar factor, only the variables photosynthesis (A) and water use efficiency (WUE) did not show significant responses, while the others showed a significance of 1% and 5% probability by the F test. Regarding the dose factor, only the variable stomatal conductance (*gs*) did not show a significant response. Regarding the interaction between factors, only the variables A, *gs*, and transpiration (*E*) showed significant responses at the level of 1% probability according to the F test.

When evaluating the photosynthesis (Fig. 8A) two regression models were adjusted. For the cultivar 60RR, a quadratic model was adjusted, while for the cultivar 40RR a linear model was adjusted. The maximum point found for cultivar 60RR was 10.08 when plants were fertilized with a dose of 32 t ha^{-1}. For cultivar 40RR, there was an increment of 1.83 for each 1 t ha^{-1} of green coconut fiber that was added. When evaluating stomatal conductance (Fig. 8B), we adjusted a quadratic equation for cultivar 60RR and found a maximum point of 0.28 when the plants were fertilized with a dose of 23.13 t ha^{-1}. For the cultivar 40 RR, there was an increase of 0.0035 for each 1 t ha^{-1} added.

For transpiration, we adjusted a quadratic equation and found a maximum point of 2.86 when the plants were fertilized with a dose of 26.76 t ha^{-1}. For the cultivar 40RR, we observed an increment of 0.023 for each increase of 1 t ha^{-1}. We observed the highest average at the dose of 50 t ha^{-1} with a value of 2.8 (Fig. 9A). Regarding the effect of doses for internal carbon, a quadratic equation for the data was adjusted, finding a minimum point of 276.50 when the plants were fertilized with a dose of 39.52 t ha^{-1}. When we compared dose 0 (337) with the minimum point, there was a reduction of 18.5% (Fig. 9B).

When analyzing the water use efficiency that was adjusted to a quadratic equation to the data, we found a peak

TABLE 5. Summary of the analysis of variance for the variables photosynthesis (A), stomatal conductance (*gs*), transpiration (*E*), internal carbon (C_i), and water use efficiency (WUE) in soybean plants grown under different doses of compost containing green coconut fiber.

SV	DF	Medium Square				
		A	*gs*	*E*	C_i	WUE
Cultivar (C)	1	5.624 ns	0.111**	4.177**	2080.5*	0.37 ns
Doses (D)	5	68.241**	0.015 ns	0.595*	6361.9**	8.502**
C x D	5	19.979**	0.036**	1.019**	920.8 ns	0.849 ns
Residue	48	2.225	0.007253	0.2128	408.3	0.428
Total	59					
CV (%)		20.33	42.19	20.43	6.86	20.67

SV - source of variation; DF - degree of freedom; C x D - cultivar x doses interaction; CV - coefficient of variation. *, ** Significant at 5% and 1% by the F test, respectively. ns - not significant.

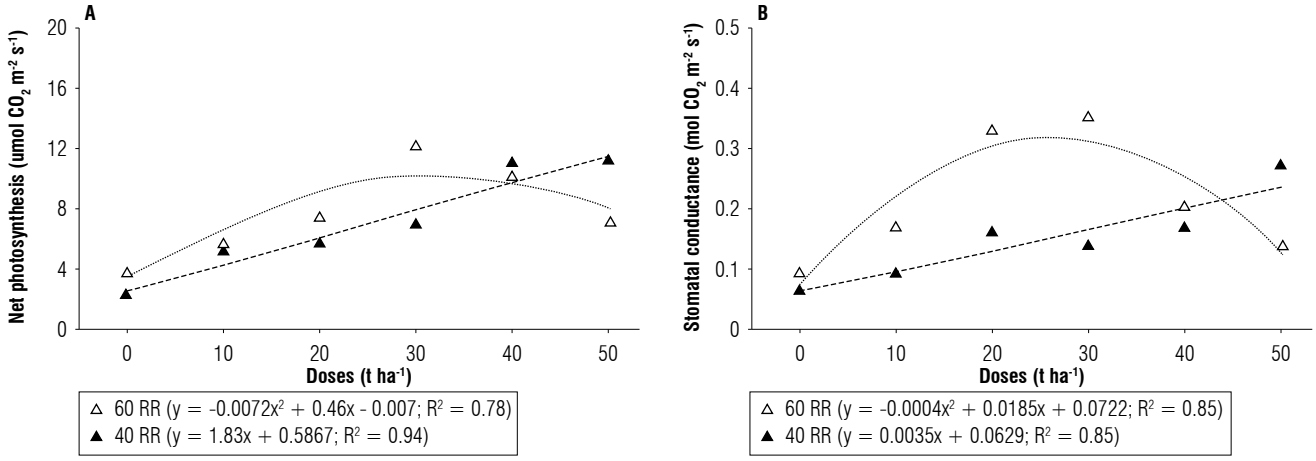

FIGURE 8. A) Net photosynthesis B) and stomatal conductance of soybean cultivars Pampeana 60RR and Pampeana 40RR grown under different doses of compost containing green coconut fiber.

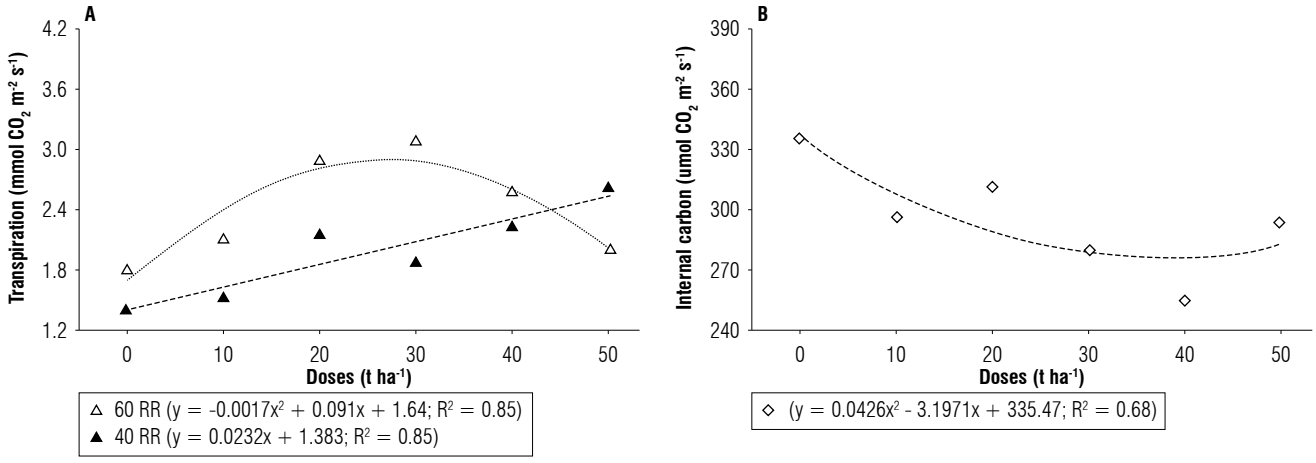

FIGURE 9. A) Transpiration and B) internal carbon of soybean cultivars Pampeana 60RR and Pampeana 40RR grown under different doses of compost containing green coconut fiber.

of 3.83 when plants were fertilized with a dose of 39.09 t ha^{-1}. When this dose was compared to dose 0 that showed a value of 1.47, there was an increase of 61.61 % (Fig. 10).

Gondim *et al.* (2015) find higher values of stomatal conductance in the presence of mineral fertilization when evaluating the effects of doses of manure in the presence and absence of mineral fertilization on gas exchange in beet plants. These results might be explained by the presence of potassium. The rate of net CO_2 assimilation, transpiration, stomatal conductance, and intercellular CO_2 concentration are correlated parameters that serve to diagnose physiological changes in plants when subjected to adverse conditions such as low and high amounts of nutrients (Gondim *et al.*, 2015).

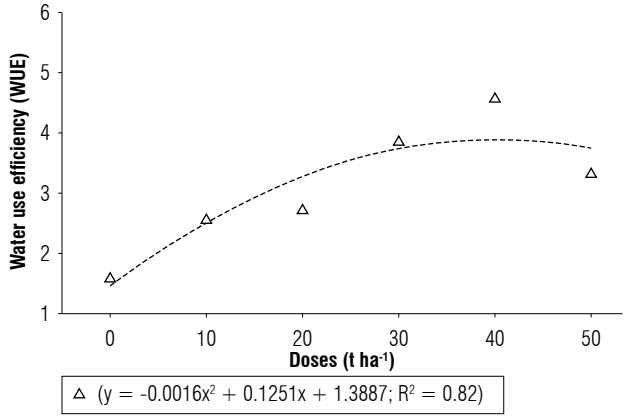

FIGURE 10. Water use efficiency (WUE) in soybean cultivars Pampeana 60RR and Pampeana 40RR grown under different doses of compost containing green coconut fiber.

In general, gas exchange data suggest the superiority of cultivar 60RR when compared to cultivar 40RR. For net photosynthesis rates, the highest values can be correlated with the fact that this cultivar had a larger leaf area and a higher number of leaves and plant height; all these values contribute to better photosynthetic performance. We also observed that this same material had a more efficient stomatal regulation, a fact observed in Figure 8B for the higher values of the stomatal conductance. Therefore, high levels of carbon dioxide influx provided higher photosynthetic rates.

For the internal concentration of CO_2, the cultivar 40RR showed lower values. This fact indicated that there was greater assimilation of CO_2. However, it did not influence higher rates of photosynthesis. The cultivar 60RR showed higher accumulations of internal carbon, differing from the other genetic material. Nevertheless, due to its better physiological responses, it showed greater assimilation of carbon. For water use efficiency, the responses to the significant effect of doses demonstrated that there was an increase in these rates through fertilization with the compost.

Miyake *et al*. (2017), when evaluating the substrates and nitrogen fertilization in the production of yellow passion fruit seedlings, find that the coconut fiber-based substrate favored the relative chlorophyll index when compared to the commercial substrate Vivatto®, especially from the dose of 300 mg dm^{-3}. These results are similar to what was found in our study since the use of doses from 10 t ha^{-1} increased this parameter for both soybean cultivars tested. Chlorophyll a is responsible for photon capture, while chlorophyll b constitutes accessory pigments in the photosynthetic apparatus, so they are closely related to the photosynthetic process and gas exchange itself.

Therefore, a possible explanation for the increase, not only in the amount of chlorophyll a, but also in gas exchange (A, *gs,* and *E*) may be associated with an increase in nutrients, especially N and Mg when the plants were subjected to higher doses of the green coconut fiber compound. It is worth mentioning that cultivar 60RR showed a positive response up to a certain dose of organic compost, while cultivar 40RR showed linear increases in gas exchange due to the increment in the added doses, thus, proving to be more nutritionally demanding.

Conclusions

The composition of green coconut fiber positively influenced the biometric and physiological characteristics of the soybean cultivars studied, and the recommended dose of the compound is 30 t ha^{-1}.

The cultivar Pampeana 60RR is more responsive to fertilization as it showed higher values of height, leaf area, number of leaves, diameter of the stem, dry mass of the leaves, and dry mass of the stem.

In general, the gas exchange of cultivar Pampeana 60RR was higher than the cultivar 40RR, indicating that it shows greater adaptability to the climatic conditions of the Brazilian northeast region.

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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

JSS and ROM designed the experiment. JOM conducted the experiment in the field. RSC and ARFO performed the analyses in the laboratory. MMB provided resources for soil and laboratory analysis. JSS and RSC wrote and edited the manuscript. ROM and MMB were responsible for supervising the experiment. Finally, JSS and ARFO performed the statistical analysis, and JOM was responsible for making the graphs. All authors have reviewed the manuscript.

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Content and distribution of micronutrients in calendula (*Calendula officinalis*) grown in Valle del Cauca, Colombia

Contenido y distribución de micronutrientes en caléndula (*Calendula officinalis*) cultivada en el Valle del Cauca, Colombia

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ABSTRACT

Calendula productivity depends on the nutritional demands of the crop. Knowledge of the nutritional requirements of calendula at its different phenological stages will allow the producer to improve fertilization programs. The objective of this research was to evaluate the content and distribution of essential micronutrients and beneficial elements in different organs and stages of development of calendula under field conditions. An experiment was set up in the municipality of Yumbo, in the Department of Valle del Cauca in Colombia, using a completely randomized design with five treatments related to the stage of development of the plants (2, 3, 5, 6 and 7 months after transplanting). Each treatment had three replicates. Leaves, stems, flowers and seeds were collected every month after transplanting to analyze the concentrations of Mn, Fe, Zn, Cu, B, and Na. The concentration of nutrients differed significantly between organs and stages of development. Calendula accumulated nutrients in descending order (from highest to lowest) as follows: Na>Fe>Mn>B>Zn>Cu. The highest concentrations of Na, Mn, Zn, B and Cu were found in the leaves and of Fe in the flowers. Among the phenological stages, the highest values of Na were recorded in the second and sixth months, B and Fe in the fifth month, Cu and Zn in the third month, and Mn in the third and fifth months. Thus, the stages and organs on the plant with the greatest nutrient accumulation were identified.

Key words: *Calendula officinalis*, medicinal plants, nutrients, Asteraceae, mineral nutrition.

RESUMEN

La productividad de la caléndula depende de la demanda de nutrientes por parte del cultivo. El conocimiento de los requerimientos nutricionales de la caléndula en sus diferentes estados fenológicos permitirá al productor mejorar los programas de fertilización. El objetivo de esta investigación fue evaluar el contenido y distribución de micronutrientes esenciales y elementos benéficos en diferentes órganos y etapas de desarrollo de la caléndula en condiciones de campo. Un experimento se llevó a cabo en el municipio de Yumbo, en el departamento de Valle del Cauca en Colombia, utilizando un diseño completamente al azar con cinco tratamientos relacionados con el estado de desarrollo de las plantas (2, 3, 5, 6 y 7 meses después del trasplante). Cada tratamiento tuvo tres repeticiones. Se recolectaron las hojas, tallos, flores y semillas en cada mes después del trasplante para analizar las concentraciones de Mn, Fe, Zn, Cu, B y Na. La concentración de nutrientes difirió significativamente entre los órganos y etapas de desarrollo. La caléndula acumuló nutrientes en orden descendente (de mayor a menor) de la siguiente forma: Na>Fe>Mn>B>Zn>Cu. Las mayores concentraciones de Na, Mn, Zn, B y Cu se encontraron en las hojas y de Fe en las flores. Entre las etapas fenológicas, los mayores valores de Na se registraron en el segundo y sexto mes, de B y Fe en el quinto, de Cu y Zn en el tercero, y de Mn en el tercer y quinto mes. De esta forma se identificaron las etapas y órganos en la planta con mayor acumulación de nutrientes.

Palabras clave: *Calendula officinalis*, plantas medicinales, nutrientes, Asteraceae, nutrición mineral.

Introduction

Medicinal plants constitute the basis of traditional medicine and continue to provide new molecules for the synthesis of new drugs (Majeed, 2017). In Colombia, their use is ancestral, and more than 50% of the herbs are collected (Cardona & Barrientos, 2011) including basil, thyme, chamomile, calendula, rosemary, etc. Calendula (*Calendula officinalis* L.) is one of the most used herbs among 156 medicinal, aromatic and condiment species that are marketed

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(Zamudio *et al*., 2006) and the second in the number of marketing authorizations (Guevara *et al*., 2010).

Calendula belongs to the Asteraceae family (Pérez Arbeláez, 1956) and its flower heads have different culinary and pharmacological uses such as antiphlogistic, healing, and anti-inflammatory properties (Acosta de la Luz *et al*., 2001; Franzen *et al*., 2019).

Knowledge about the functions of nutrients in plants is required during the management of soil and fertilization in agricultural agroecosystems since they allow carrying out vital processes related to the absorption, transport, and use of assimilates (Castro & Gómez, 2010). The cultural management of medicinal plants is a factor that strongly influences the synthesis of secondary metabolites, with fertilization being essential to achieve good productivity and satisfactory levels of bioactive compounds (Marques, 2019).

There are several studies on the effect of fertilization on the nutrition of calendula. In Iran, several authors measured the effect of N, P, K, vermicomposting, and gibberellic acid on growth, flowering, and availability of nutrients in the post-harvest soil (Tiwari *et al*., 2018). In Brazil, the effect of the application of N, P and bovine manure on the concentration of secondary metabolites was evaluated (Fernandes *et al*., 2013). Additionally, some authors determine that nitrogen fertilization increases the total content of flavonoids (Paim *et al*., 2010). Under the conditions of Poland, agronomic responses such as number of flowers, fresh and dry weight of flowers, and total flavonoids (1.14%) have been monitored (Król, 2011; Bielski & Szwejkowska, 2013). In Iran, it is observed that humic acids promote the increase of weight of fresh leaves and flowers (Mohammadipour *et al*., 2012). Ganjali *et al*. (2010) also show that the sowing dates, sowing density, and fertilizer dose significantly regulate the yield and essence of calendula.

Researchers at the international level have made contributions in the use of calendula in the pharmacological sector, but studies in terms of plant nutrition are very scarce. Colombia is not an exception, since producers are unaware of the phenological stages of greatest nutritional demand, the absorption of nutrients by vegetative organs and especially flowers, and the fertilization programs under field conditions. For these reasons, this pioneering research was carried out with the aim of evaluating the content and distribution of essential micronutrients (Mn, Fe, B, Cu, Zn) and beneficial elements (Na) in the different organs (stems, leaves, flowers, and seeds) and stages of development of calendula under field conditions.

Materials and methods

Characterization of the experimental area

The research was carried out in the municipality of Yumbo, Valle del Cauca, located at 3°35'0" N, 76°28'0" W, at an altitude between 1650 and 2000 m a.s.l., with 50% relative humidity in the dry season and 70% in the rainy season. The soil in this municipality was classified as Typic Dystrudepts (IGAC-CVC, 2004) in a low montane humid forest life zone bh-PM (Holdridge System) (CVC, 2017).

Samples of the soil where the calendula (*C. officinalis* L.) crop was established were collected at a depth of 0 cm to 20 cm (Tab. 1). The physical characteristics of the soil were determined in the laboratories of the Universidad Nacional de Colombia, Palmira campus, with the following methodologies.

TABLE 1. Physical characteristics of the soil in the study area.

Physical Characteristics	Unit	Methods	Reference
Texture	---	Robinson's pipette method	Jaramillo, 2002
Bulk density	(g cm^3)	Beveled cylinder method	
Real density		Pycnometer method	Montenegro & Malagón, 1990
Total porosity	%	Mathematical formula	Jaramillo, 2002

The analysis determined that the soil had a silt loam texture, 0.9 and 2.53 g cm^3 for apparent and real density, respectively, and 63.5% of total porosity. These characteristics provided the optimal conditions of workability, development of root system, and circulation of water and air within the soil.

The samples for analysis of the chemical properties (Tab. 2) were transferred to the Analytical services laboratory of the International Center for Tropical Agriculture (CIAT). The results were interpreted following several parameters at the national level (Castro, 2004; Zamudio *et al*., 2006).

The soil had a neutral pH with high availability of nutrients (S, B, P, Mn, Ca, Mg, Na and Zn) and nutrient exchange due to the levels of organic carbon and cation exchange capacity (CEC). Additionally, possible adverse effects on the plant due to low levels of Fe and Cu could be observed. In general, the soil had the capacity to provide sufficient nutrients during the calendula developmental stages.

TABLE 2. Chemical characteristics of the soil in the study area.

Parameter	Unit	Value	Level
pH	Global	6.92	Neutral
CO	g cm^{-3}	58.24	High
K		7.9	High
Ca		201.7	High
Mg	$Cmol_c$ Kg^{-1}	33.5	High
Na		2.2	High
CEC		298	High
P (Bray II)		275.4	High
S		60.03	High
B		1.37	High
Fe	mg kg^{-1}	1.21	Low
Mn		78.34	High
Cu		0.35	Low
Zn		17.36	High

CO - organic carbon; CEC - cation exchange capacity.

Experimental description of the research

The sampling unit was a plot of 1000 m^2 planted with calendula. The farmers of the area managed the crop with two fertilizations, the first based on a broadcast application of composted chicken manure that was removed along with the soil after four days during its preparation for sowing. The second fertilization consisted of mineral fertilization, using 5 to 8 g of urea (46% N) + triple 15 (15% of N, P and K). The amounts varied according to the empiricism of each product, which was applied in a crescent shape around the plant 1 month after sowing.

A completely randomized design was used, considering five phenological stages of the crop (2, 3, 5, 6 and 7 months after transplantation) as treatments and three replicates. The content of micronutrients (Mn, Fe, Cu, B, Zn) and beneficial element (Na) were evaluated at each stage in the organs of the plant shoot (leaves, stems, flowers, and seeds).

Tissues were collected in the flowering (2 to 7 months) and fruiting (5 months onwards) stages. The phenological stages can be shortened or prolonged according to the agronomic management of the producer and the agroclimatic conditions of the area.

At each stage, 10 complete plants were randomly harvested. The samples of leaves, stems and the floral head (flowers and seeds) were washed with distilled water. Each organ was dried at 60°C until obtaining a constant weight and then chopped with a tungsten knife mill (IKA LABORTECHNIK M20, Sao Paulo, Brazil). Samples were sieved (using a 2 mm sieve/net), marked, packed, and finally transferred to the laboratory. Nutrient determinations in leaves, stems, and floral head (flowers and seeds) were carried out following the CIAT laboratory methodologies (CIAT, 2006).

Analyses of variance (P<0.05) and Duncan mean tests were performed using the statistical package SAS (Statistical Analysis System) version 9.13. (SAS Institute Inc., 2002).

Results and discussion

Nutrient distribution in organs of calendula

The analysis of variance (Tab. 3) found statistically significant differences for some of the nutrients (Na, B, Mn and Zn) monitored in the different vegetative organs of calendula.

TABLE 3. Analysis of variance for the concentration of nutrients in vegetative organs of calendula.

Nutrient	R^2	C.V. %	Pr > F
Na	0.4602	4.31	0.0402 *
B	0.5773	41.4	0.0090 **
Cu	0.1678	56.56	0.4794 ns
Fe	0.1396	118.99	0.5667 ns
Mn	0.4998	74.83	0.0256 *
Zn	0.5409	44.36	0.0149 *

*significant, **highly significant, ns - not significant. C.V. - Coefficient of variation.

For manganese (Mn) and zinc (Zn), the ANOVA found significant differences (P<0.05) between the organs analyzed throughout the development stages (Fig. 1). The Mn dynamics were different, since the highest concentration was located in the leaf (161.84 mg kg^{-1}), followed by the stems (63.85 mg kg^{-1}), flowers (44.48 mg kg^{-1}) and, in the seeds (19.36 mg kg^{-1}) to a lesser extent.

Among organs, the greatest fluctuation of Mn occurred in the leaves with a perfect parabola; the greatest accumulation was recorded in the fifth month. One of the most notable functions of this element is its participation in photosynthetic activity, where oxygen is produced from water (Salisbury & Ross, 1994; Marschner, 2003). When there are deficiencies in the soil, symptoms are associated with interveinal chlorosis in the youngest leaves, depending on the species and its growth (Taiz & Zeiger, 2006).

Statistical differences were not found between plant organs for iron concentrations. The most elevated concentration was found in the flowers (1061.12 mg kg^{-1}), followed by the leaves (976.15 mg kg^{-1}), seeds (382.19 mg kg^{-1}) and finally

the stems (377.4 mg kg^{-1}). These values exceed by far the ranges from 50 to 250 mg kg^{-1} registered in other crops (Navarro & Navarro, 2003).

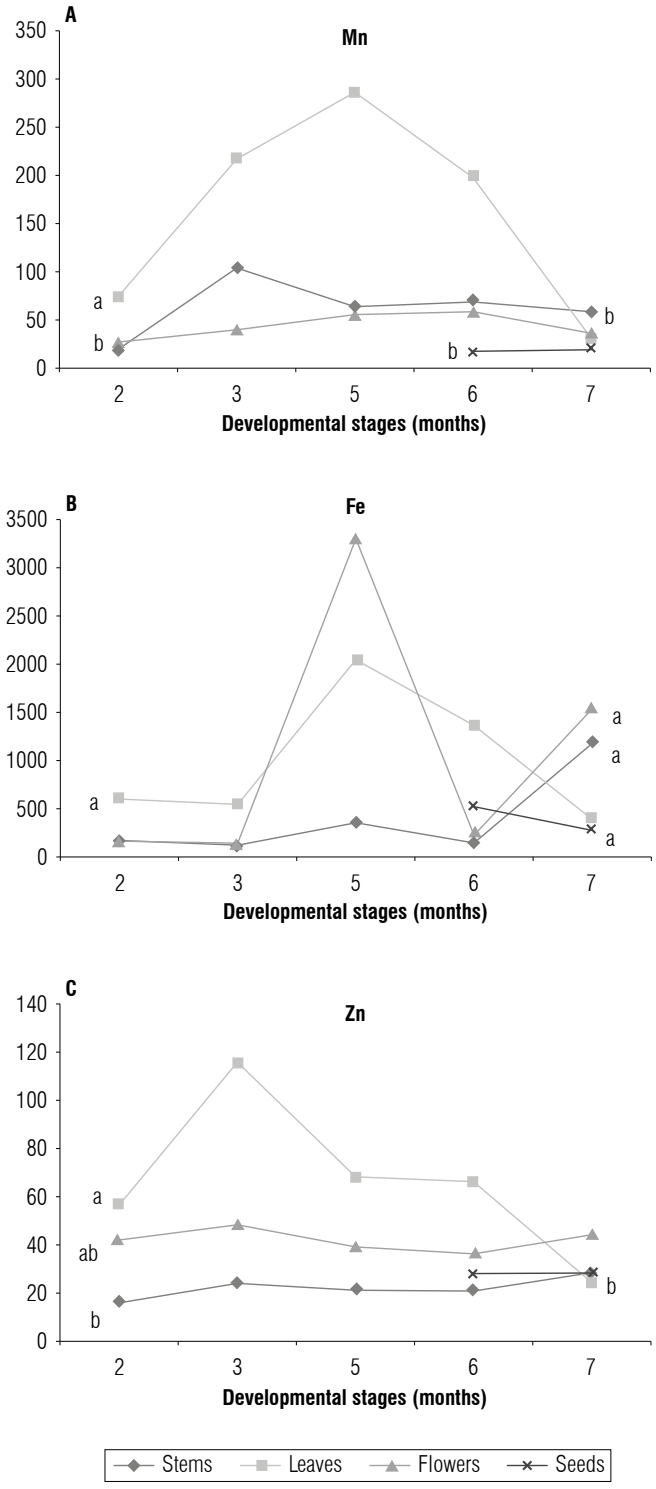

FIGURE 1. A) Manganese (Mn), B) iron (Fe), and C) zinc (Zn) contents (mg kg^{-1}) in leaves, stems, flowers, and seeds of the calendula plant at different developmental stages (months). Means with the same letter do not differ significantly according to the Duncan test ($P<0.05$).

Iron has low mobility in plant tissues due to the high concentration of P and Mn (López, 1998). Low iron mobility was observed in the crop due to P concentrations ranging from 3.51 to 5.38 g kg^{-1} (García Vivas *et al*., 2015).

Zinc showed a significantly higher accumulation in the leaf (66.33 mg kg^{-1}) with a tendency to decrease its concentration as the plant grew during the months in production. A dilution effect could be observed due to an increase in the leaf mass or the translocation towards the other organs (Malavolta *et al*., 1997). The flowers (42.18 mg kg^{-1}) were also valuable reserve tissues, followed by the seeds (28.41 mg kg^{-1}) and stems (22.31 mg kg^{-1}). However, in these last two organs, the concentrations were more constant throughout the crop cycle. The concentration of Zn in tissues ranged from 20 to 150 mg kg^{-1}, with some of the most important functions being the biosynthesis of indoleacetic acid, chlorophyll formation, and nitrogen metabolism (Azcón-Bieto & Talón, 2013).

The ANOVA detected significant differences ($P<0.05$) for sodium (Na) and boron (B) between plant organs, but not for copper (Cu) (Fig. 2). Although the leaves (19.39 mg kg^{-1}) are the organs of greater Cu accumulation, they did not vary much from the stems (14.02 mg kg^{-1}), flowers (13.16 mg kg^{-1}) and seeds (9.25 mg kg^{-1}), except in the third month, where the leaves accumulated more Cu and then homogenized their behavior with the other organs.

The highest concentrations of B, Mn and Zn were found in the leaves (81.66 mg kg^{-1}), where the highest photosynthetic activity occurs, and the lowest accumulation of these microelements was observed in the flowers (59.47 mg kg^{-1}), stems (28.37 mg kg^{-1}) and, finally, in seeds (28.02 mg kg^{-1}).

Boron shows little mobility in the plant, with the leaves and reproductive organs having the highest concentrations of this nutrient. Young plants absorb more boron than old ones (Navarro & Navarro, 2003), which acts on the apical growth points of the genetic material and facilitates the transport of carbohydrates throughout the internal membranes.

Figure 2C shows the behavior of Na between the plant organs, with the leaves (10556.57 mg kg^{-1}) being the largest accumulators of this nutrient, followed by the stems (5951.65 mg kg^{-1}), flowers (2772.87 mg kg^{-1}) and seeds (1000.69 mg kg^{-1}) where the concentration is lowest. Although the nutrient content fluctuates in each organ, it coincides to the months with the highest and lowest levels.

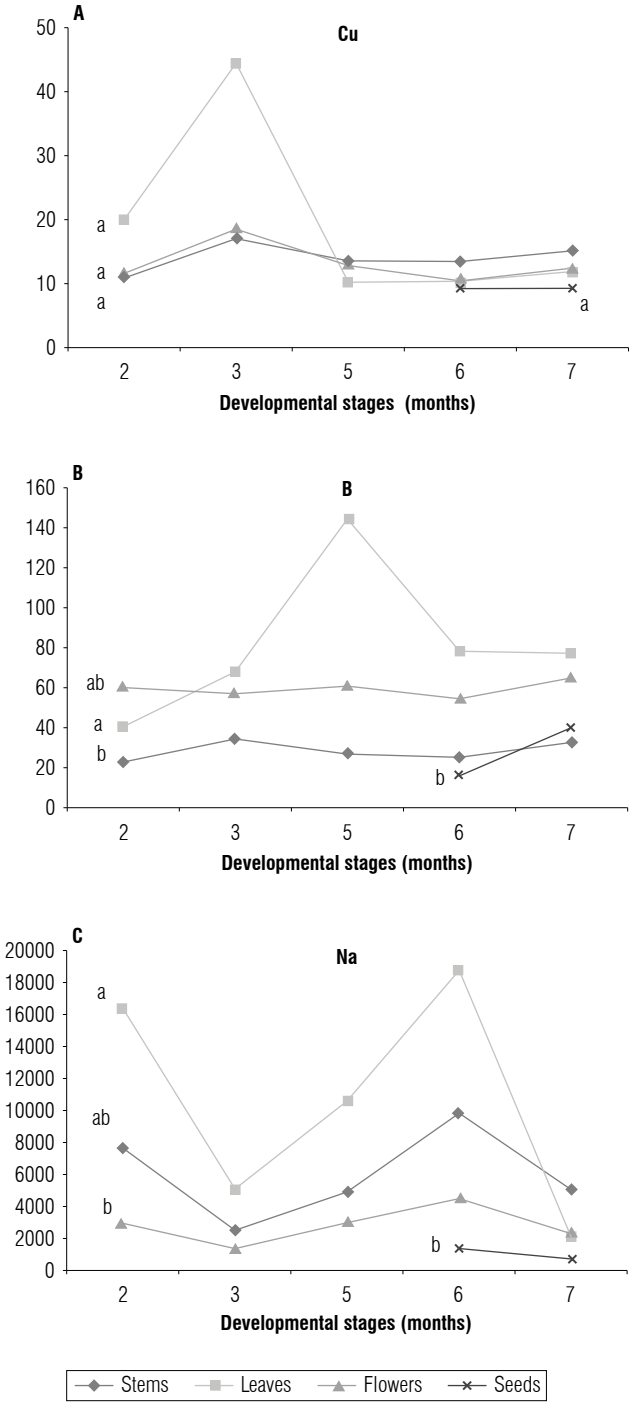

FIGURE 2. A) Copper (Cu), B) boron (B), and C) sodium (Na) contents (mg kg^{-1}) in leaves, stems, flowers and seeds of the calendula plant at different development stages (months). Means with the same letter do not differ significantly according to the Duncan test ($P<0.05$).

The higher concentrations in leaves and stems reveal the high activity of Na in osmotic control processes and turgor generation. It can replace K in many species with its similar functions and concentrations in chloroplasts, exercising phosphorylation functions in photosynthesis (Salisbury & Ross, 1994; Marschner, 2003). The high levels of Na in the soil possibly facilitated its absorption by the plant; so, the accumulation in the tissues without observing damage allows us to elucidate that the calendula prefers this nutrient.

On the other hand, the absorption and mobility of nutrients depends on environmental factors, the soil, and the crop. The interaction in the soil-plant phase is mainly determined by the ionic and molecular forms that, in turn, are influenced by the atomic weight, valence, and type of movement in the soil (diffusion, root interception, and mass flow) (Jungk, 1996). In this research, the nutritional supply exceeded the critical levels for Colombian soils (Castro, 2004), facilitating rapid absorption by the crop, which translated into vigorous growth and development.

Nutrient content at different stages of calendula development

The analysis of variance (Tab. 4) found statistically significant differences for all the nutrients monitored in the different developmental stages (months) of calendula.

TABLE 4. Analysis of variance of the concentration of nutrients in different vegetative stages (months) of calendula.

Nutrient	R^2	C.V. %	Pr > F
Na	0.995560	4.047	0.0001 **
B	0.989133	2.685	0.0001 **
Cu	0.985783	5.351	0.0001 **
Fe	0.589444	77.251	0.0460 *
Mn	0.996410	3.27	0.0001 **
Zn	0.983665	3.717	0.0001 **

*significant, **highly significant, ns - not significant. C.V. - Coefficient of variation.

For Mn, Fe and Zn, the ANOVA found significant differences ($P<0.05$) between the stages of plant development (Fig. 3). The dynamics of Mn are in the shape of a parabola, from minor accumulations in the second month (41.41 mg kg^{-1}) reaching the highest value in the fifth (135.95 mg kg^{-1}) and then declining in the seventh (42.84 mg kg^{-1}). Manganese acts as a cofactor activating about 35 enzymatic reactions, with some of them related to photosynthetic activity and others involved in plant defense (Azcón-Bieto & Talón, 2013).

The behavior of Fe was characterized by a low concentration in the first months of cultivation, and 294.64 and 253.56 mg kg^{-1} in the second and third, respectively. Then, it accumulated the largest amount in the fifth month (1891.52 mg kg^{-1}) with a decrease in the following samplings, and a value of 1021.36 mg kg^{-1} in the seventh month.

The highest Zn concentrations in the plant accumulated in the second month with a value of 62.88 mg kg^{-1}, while in the coming months the demand gradually decreased with 43.01 mg kg^{-1} in the fifth month, 41.26 mg kg^{-1} in the sixth, and 132.56 mg kg^{-1} in the seventh month. One of the primary functions of Zn is the synthesis of tryptophan, the amino acid precursor of indoleacetic acid (IAA), which is a growth hormone (Navarro & Navarro, 2003).

For Cu, B and Na, the ANOVA also found significant differences (P<0.05) between the stages of development (Fig. 4). Like Zn, the plant steadily accumulated Cu, reaching its highest peak in the third month (26.66 mg kg^{-1}) and declining to a level similar to the initial level for the rest of the cycle, with 12.19, 11.43 and 13.16 mg kg^{-1} in the fifth, sixth and seventh months, respectively. This element is related to enzymes that participate in processes such as

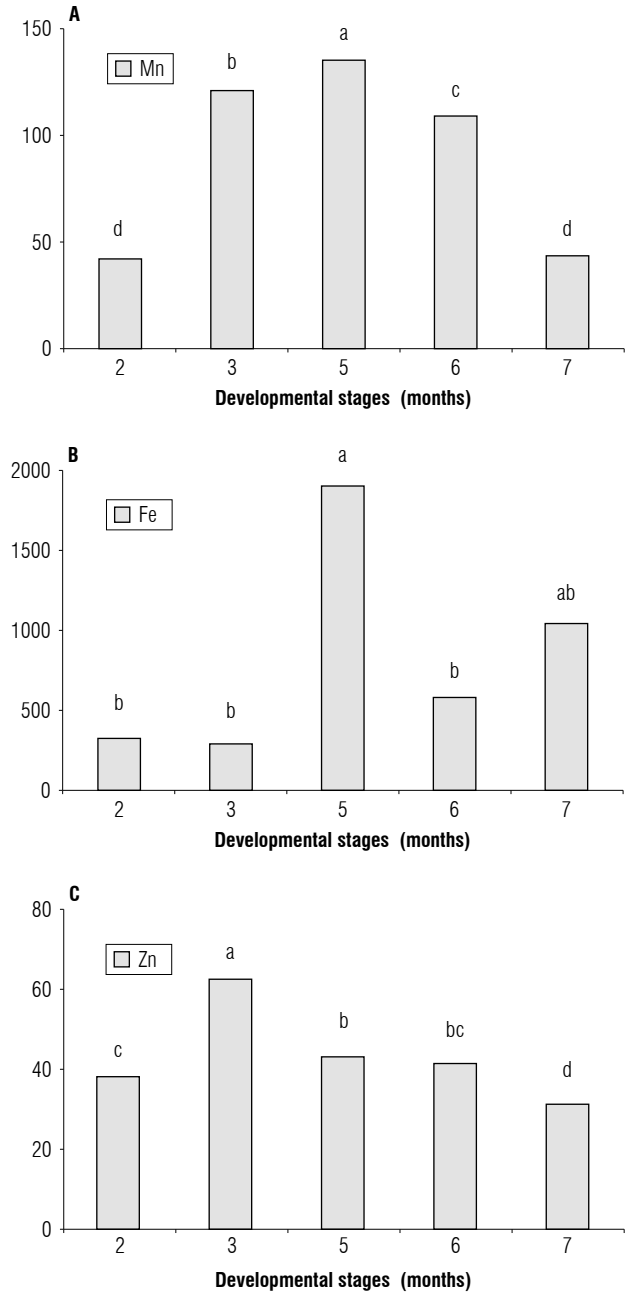

FIGURE 3. A) Mn, B) Fe, and C) Zn contents (mg kg^{-1}) at different developmental stages (months) of the calendula plant (leaves + stems + flowers + seeds). Means with the same letter do not differ significantly according to the Duncan test (P<0.05).

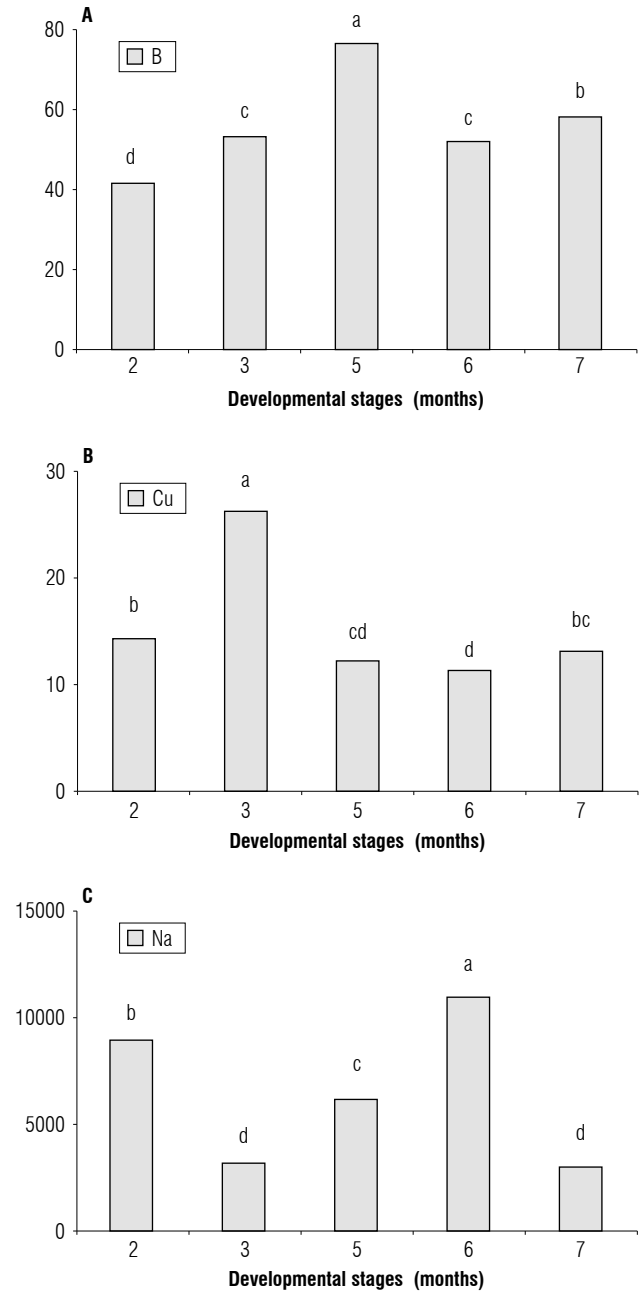

FIGURE 4. A) B, B) Cu, and C) Na contents (mg kg^{-1}) at different stages (months) of the calendula plant (leaves + stems + flowers + seeds). Means with the same letter do not differ significantly according to the Duncan test (P<0.05).

photosynthesis, mitochondrial respiration, cell wall metabolism, etc. (Hänsch & Mendel, 2009; Mendel, 2013).

The accumulated concentrations of B in the plant were statistically different between the evaluated stages. An increase was observed from the first sampling in the second month with 41.14 mg kg^{-1} until reaching the highest value in the fifth month (77.32 mg kg^{-1}). Then, a decrease was observed in the seventh month (58.25 mg kg^{-1}). Boron is required in the productive stage of fruit crops and basic grains for its participation in multiple functions, such as flowering, pollen germination, fruiting, synthesis of carbohydrates and hormones, and regulation of water absorption (Rao, 2009; Mejía, 2010).

In the case of Na, significant differences were recorded between the stages of development. The highest concentrations were recorded in the second (8951.16 mg kg^{-1}) and sixth months (11007.46 mg kg^{-1}), while the lowest occurred in the third (2947.17 mg kg^{-1}) and the seventh (3110.11 mg kg^{-1}) months. Sodium is required by many halophyte and certain C4 plants as a micronutrient that is mainly involved in osmosis (movement of water), ion balance, and chlorophyll synthesis. Additionally, it can substitute the functions of potassium under certain conditions (Brownell, 1980; Malavolta *et al*., 1997).

During the phenological stages of calendula (Fig. 5), the accumulated concentration of Na (32,135.16 mg kg^{-1}) at the end of the cycle was significantly higher than the rest of nutrients, followed by Fe (4,024.44 mg kg^{-1}), Mn (450.0 mg kg^{-1}), B (282.51 mg kg^{-1}), Zn (218.04 mg kg^{-1}) and Cu (77.61 mg kg^{-1}). The most demanded nutrients during some stages

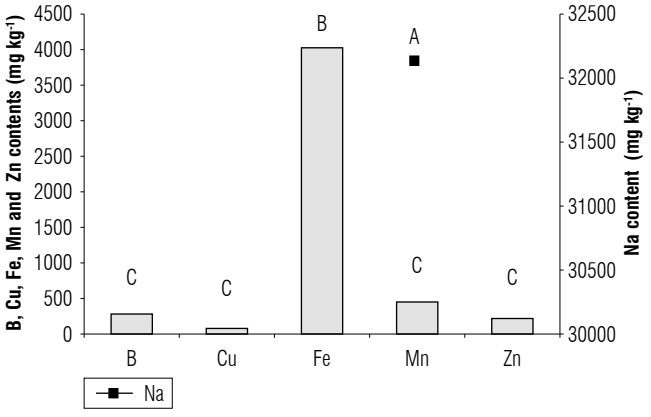

FIGURE 5. Concentration of essential (B, Cu, Fe, Mn and Zn) and beneficial (Na) nutrients in the calendula plant (leaves + stems + flowers + seeds) at different developmental stages (month 2 + 3 + 5 + 6 + 7). Means with the same letter do not differ significantly according to the Duncan test ($P<0.05$).

of calendula development were Fe, Mg and B (Teixeira *et al*., 2000). The concentrations of essential and beneficial elements varied constantly in the organs and phenological stages of calendula. Siddiqi and Glass (1981) have shown that the absorption efficiency and utilization efficiency of nutrients vary by multiple factors: the concentrations and availability of nutrients in the soil, fertilization, species to be cultivated, age and organ of the plant, photosynthetic capacity of the organ (root, fruit, leaf, and branches), etc. These findings allow the identification of the participation of each nutrient under field conditions, though Na is of great preference in the productive cycle of the crop.

Conclusions

The accumulation of nutrients differs between the plant organs, with the most elevated concentrations in the leaves (Na, Mn, B, Zn, Mn) and flowers (Fe). Foliar monitoring is an adequate tool for evaluating the nutrition of the crop.

The content of micronutrients and beneficial elements registered in the entire calendula cycle followed the order Na>Fe>Mn>B>Zn>Cu. Calendula demanded the largest amount of Na in the second and sixth months, B and Fe in the fifth, Cu and Zn in the third, and Mg in the third and fifth months, allowing the identification of the most demanding stages for improving fertilization programs.

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Author's contributions

BASR processed the information and prepared the presentation of the manuscript to be published. YSGV proposed the ideas and was in charge of the formulation and execution of the research. JCM obtained the funding and was a research advisor. CRB was also a research advisor and contributed to the publication of the manuscript.

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Naturally colored cotton irrigated with saline water at different growth stages

Algodón de color natural regado con agua salina en diferentes etapas de crecimiento

Andygley Fernandes Mota[1], Marcelo Tavares Gurgel[1], Kaline Dantas Travassos[2], Neyton de Oliveira Miranda[1*], Lucas Ramos da Costa[3], Miguel Ferreira Neto[1], and Nildo da Silva Dias[1]

ABSTRACT

This study aimed to evaluate the growth, production, and nutrition of naturally colored cotton (cultivar BRS Verde) irrigated with saline water in different growth stages. The trial was conducted with a randomized block design in which the treatments consisted of three irrigation water salinity levels applied throughout the crop cycle or alternately in three growth stages. The lowest-salinity water was drawn from the Arenito Açu aquifer in the state of Rio Grande do Norte, Brazil; the highest-salinity water was prepared to obtain salinity similar to the water drawn from the Calcário Jandaíra aquifer, and an intermediate salinity was obtained from the mixture of equal volumes of the lowest and highest-salinity waters. The application of the lowest-salinity water is recommended in all growth stages due to the increases of about 19% in cotton growth and 40% in yield compared to the application of intermediate or highest-salinity water. The application of the lowest-salinity water in the vegetative stage and the intermediate-salinity water in the following stages is an alternative to using good quality water throughout the cycle, despite the decreases of about 7% in growth and 16% in yield. The nutrition of cotton plants irrigated with saline water throughout the cycle or in some growth stages was marked by an increase of up to 86% in the cotton leaf sodium content, a decrease in the leaf potassium content of up to 21% and increases between 24% and 188% in leaf micronutrient content when the highest-salinity water replaced that with the lowest salinity.

Key words: *Gossypium hirsutum* L., salinity, water quality, lint yield.

RESUMEN

Este estudio tuvo como objetivo evaluar el crecimiento, producción y nutrición de algodón de color natural (cultivar BRS Verde) regado con agua salina en diferentes etapas de crecimiento. El ensayo se realizó en un diseño de bloques al azar en el que los tratamientos consistieron en tres niveles de salinidad del agua de riego aplicados a lo largo del ciclo del cultivo o alternativamente en tres etapas de crecimiento. El agua de menor salinidad se extrajo del acuífero Arenito Açu en el estado de Rio Grande do Norte, Brasil; el agua de mayor salinidad se preparó para obtener una salinidad similar a la del agua extraída del acuífero Calcário Jandaíra, y la salinidad intermedia se obtuvo de la mezcla de volúmenes iguales de las aguas de menor y mayor salinidad. Se recomienda la aplicación de agua de menor salinidad en todas las etapas de crecimiento debido a los incrementos de aproximadamente 19% en el crecimiento del algodón y 40% en el rendimiento comparado con la aplicación de agua de intermedia o mayor salinidad. La aplicación de agua de menor salinidad en la etapa vegetativa y de agua de salinidad intermedia en las siguientes etapas es una alternativa al uso de agua de buena calidad durante todo el ciclo, a pesar de las disminuciones de alrededor de 7% en el crecimiento y 16% en el rendimiento. La nutrición de las plantas de algodón regadas con agua salina durante todo el ciclo o en algunas etapas de crecimiento estuvo marcada por un aumento de hasta un 86% en el contenido de sodio de la hoja de algodón, una disminución del contenido de potasio de la hoja de hasta un 21% y aumentos entre un 24 y 188% en el contenido de micronutrientes de las hojas cuando el agua de mayor salinidad reemplazó a la de menor salinidad.

Palabras clave: *Gossypium hirsutum* L., salinidad, calidad del agua, rendimiento de pluma.

Introduction

Herbaceous cotton (*Gossypium hirsutum* L.) is one of the main commercial crops in Brazil. Lint cotton production in the 2016-2017 and 2017-2018 crop seasons amounted to 1.53 and 2.01 million metric t, respectively. The lint cotton yield in the Brazilian northeastern region ranged between 1600 and 1800 kg ha^{-1} in 2017-2018, similar to the nationwide yield. Global lint cotton production in the 2017-2018 growing season was around 27 million t, while India, China, and the United States were the largest producers (CONAB, 2018).

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The most widely planted cotton cultivar in the northeastern region of Brazil is CNPA 7H. The cultivar BRS Verde, launched in 2002, differs only by a gene that promotes the green color of the fiber, inherited from the North American cultivar Arkansas Green (Carvalho *et al*., 2011). BRS Verde was developed by the National Center for Cotton Research of EMBRAPA and has a low height and a cycle of 130 to 140 d.

Growing naturally colored cotton is important for generating employment and income for small farmers in the region. The use of naturally colored fibers dispenses with the need for dyes in the final phase of the thread or fabric manufacturing. This reduces the environmental impact of the dyeing process and allows the production of organic fabrics as well as the elimination of allergy risks to sufferers. Therefore, an interest in using clothing made from naturally colored cotton is growing worldwide. The sale price of naturally colored cotton fiber can be twice that of white fiber (Carvalho *et al*., 2011; 2014).

The semiarid region of northeastern Brazil has a strong potential for irrigated agriculture, but many of its water sources are of inferior quality due to high levels of salts. These waters can be used only in situations of high demand and after previous studies, since they can cause soil salinity problems in the medium and long term, compromising agricultural productivity and the environment (Holanda *et al*., 2010). Therefore, it is necessary to manage soil and water rationally in terms of water quality and water savings.

The water used for irrigation in the western region of the state of Rio Grande do Norte comes from shallow wells located in the geological formation called "Calcário Jandaíra", where water quality and cost of extraction are low. In the region, water is also extracted from deep wells, located in the "Arenito Açu" formation that does not contain excess salts but has high extraction costs (Medeiros *et al*., 2007). Excess salts in irrigation water cause plant stress and can impair physiological and biochemical functions, causing disturbances in water relations, changes in the absorption and utilization of essential nutrients (Braz *et al*., 2019), stunted plant growth, and yield reduction.

Salinity poses a great risk to cotton productivity, especially in arid or semiarid regions. Although cotton is considered tolerant, salinity can impair its growth due to osmotic effects, nutritional imbalance, and toxicity of Na and Cl^- for the plant's metabolism. Other effects are the alteration in the extensibility of the cell wall and salt accumulation in the apoplast. In general, moderate salinity does not impair the photosynthesis or transpiration of cotton plants but increasing salinity can decrease the nitrogen leaf content. Benefits to photosynthesis due to a small increase in salinity are attributed to the osmotic adjustment exerted by Na and Cl^-. Variations in the balance between Na and K levels in cotton leaves are evident effects of salt stress, but Na retention in roots is a probable mechanism of cotton salt tolerance (Ahmad *et al*., 2002).

In general, cotton biomass production is affected by increased salinity, but this response differs between different genotypes. The sensitivity of the crop is higher during germination and early development, especially in the 6-leaf stage of the seedling, but tolerance increases in the following growth stages. Among the reported effects of increased salinity are a reduction in vegetative growth, although roots may grow more at moderate salinity; a reduction in seed cotton yield, especially if irrigated with saline water in the early stages; a reduction in the number of bolls, although the weight of the seeds is less affected by salinity; a decrease in cotton fiber quality, and a decrease in the oil content that may tend to rise with small increases in irrigation water salinity and then to decrease with greater salinity increases (Ahmad *et al*., 2002).

In this context, this study was carried out to evaluate the growth, production, and nutrition of naturally colored cotton (cultivar BRS Verde) irrigated with water with different salt levels in different growth stages.

Materials and methods

The research was carried out from October 2011 to February 2012 at the experimental farm of the Federal Rural University of the Semi-arid Region, located in Mossoró, RN, Brazil (5°03'37" S, 37°23'50" W), at an altitude of 18 m a.s.l. The climate of the region is semiarid, megathermal, and with water deficits during most of the year. The average annual rainfall is 674 mm, of which about 550 mm occurs between February and May. The mean annual relative humidity is 68.9%, while the mean annual temperature is 27.7°C (Vanomark *et al*., 2018). The soil of the experimental area is a typic hapludult (Soil Survey Staff, 2014).

The experiment was carried out according to a completely randomized block design with five replicates. The description of treatments is shown in Table 1. Each plot consisted of four rows of 7 m in length, each containing 47 plants. The useful area for evaluations included the two central rows of the plot, discarding a plant at each end.

TABLE 1. Description of treatments combining three levels of water salinity applied in three cotton growth stages.

	Cotton growth stages		
	Vegetative (0-30 DAS)	Reproductive (31-90 DAS)	Maturation (91 DAS - harvest)
Treatments	Water salinity (dS m^{-1})		
T1	0.55	0.55	0.55
T2	2.16	2.16	2.16
T3	3.53	3.53	3.53
T4	0.55	2.16	2.16
T5	0.55	2.16	3.53
T6	0.55	3.53	3.53

DAS - days after sowing.

The lowest-salinity water (S1 - 0.55 dS m^{-1}) was drawn from a well from the Arenito Açu aquifer, at an average depth of 1000 m. The highest-salinity water (S3 - 3.53 dS m^{-1}) was prepared from water S1 to which 3.975 kg of NaCl and 3.966 L of $CaCl_2.2H_2O$ were added per 1000 L of water to represent the salinity of water from the Calcário Jandaíra aquifer according to Medeiros (1992). The intermediate-salinity water (S2 - 2.16 dS m^{-1}) was obtained by mixing equal volumes of waters S1 and S3.

The naturally colored cotton cultivar BRS Verde was sown with a spacing of 0.15 m between plants and 0.9 m between rows, resulting in a population of 74,000 plants ha^{-1}. Of the total amount of fertilizers, 20% of the urea and 50% of the KCl were applied at planting. The remaining KCl was applied via fertigation at 15 d after emergence (DAE), while the rest of the urea was applied at 15 and 40 DAE, also by fertigation.

The crop was irrigated twice a day with a drip irrigation system whose tubes, with self-compensating emitters, had an average flow of 1.8 L h^{-1} and a 95% distribution coefficient. The water depth was determined by the Penman-Monteith method using reference evapotranspiration and crop coefficients of 0.70 for the germination stage, 0.85 for the vegetative stage, 1.00 for the reproductive stage, and 0.95 for the maturation stage (Bezerra *et al*., 2010). The climatological normal for the years 1981 to 2010 (INMET, 2018) showed daily potential evapotranspiration of around 7.2 mm for October and November, 7.6 mm for December and January, and 7.1 mm for February.

At 100 DAE, the following crop growth characteristics were determined in each plot: plant height (PH), stem diameter (SD), and number of leaves (NL). The number of bolls and lint yield were determined in one row in the useful area of each plot. The first sampling was carried out when 60% of the bolls in the experimental area were open.

The evaluation of the nutritional status of the plants included the determination of macronutrients (Ca, Mg, P, K, N, and Na) and micronutrients (Mn, Zn, Fe, and Cu). For this, the fifth leaf below the apex was collected from 20 plants per plot at 65 DAE, as recommended by Malavolta *et al*. (1997).

The data were subjected to an analysis of variance to verify differences among treatments using the t-test (P<0.01 or 0.05). The means of the variables that showed a significant effect of the treatments were compared using the Tukey test (P<0.01 or 0.05). We used the SISVAR software for all analyses (Ferreira, 2014).

Results and discussion

The application of water with different salinities in different growth stages had a significant effect (P<0.01) on plant height and stem diameter of BRS Verde cotton (Fig. 1), with coefficients of variation (CV) of 8.9% and 9.4%, respectively. The effects observed on the number of leaves (CV = 27.0%), lint yield (CV = 37.5%), and number of bolls (CV = 41.6%) were not statistically significant according to the t-test.

There was a decrease in plant height (Fig. 1A) and stem diameter (Fig. 1B) when the saline waters (T2 - 2.16 dS m^{-1} and T3 - 3.53 dS m^{-1}) were applied throughout the crop cycle instead of the lowest-salinity water (T1 - 0.55 dS m^{-1}). However, a significant difference was only observed among the treatments that applied the lowest-salinity water (T1) at all growth stages and the treatments that applied the highest-salinity water (T3) or the intermediate-salinity water (T2) at all stages. Treatments T2 and T3, which did not differ in plant height and stem diameter, showed the lowest mean values.

Growth impairment of BRS Verde cotton due to water salinity was reported by Sousa Júnior *et al*. (2005), who observed a decrease of 3.23% in stem diameter and 5.68% in plant height per unit increment of the electrical conductivity of water (ECw) from 2.0 to 9.5 dS m^{-1}. The decrease in plant height of cotton cultivar BR 1 subjected to increased irrigation water salinity levels was attributed by Graciano *et al*. (2011) to the reduction in the osmotic potential of the soil solution. This reduction can be due to the high concentration of sodium (as observed in Fig. 3B), hindering the absorption of water and nutrients by the plants. The effect of sodium on the height of cotton plants can also be attributed to a cationic imbalance, such as the reduction in the K/(Ca + Mg) ratio (Queiroz & Büll, 2001).

A reduction in stem diameter was also observed in Jatropha plants subjected to an increase in ECw from 0.6 to 3.0 dS m^{-1} (Nery *et al*., 2009). Castor bean plants (Cavalcanti *et al*., 2005) showed a reduction in this variable of 1.45% for each unit increase in ECw at 80 DAE. However, Costa *et al*. (2013) found no difference in the diameter of the castor bean stem when they applied water with ECw of up to 3.66 dS m^{-1}, according to the growth stage. The effects of the different salinity levels are due to management conditions, soil and climate characteristics, plant species, genotypes of the same species, and plant growth stages (Suassuna *et al*., 2017).

Cotton plants irrigated with the lowest-salinity water throughout the cycle (T1) showed greater height and stem diameter than when irrigated with waters whose salinity varied according to the growth stage. On the other hand, irrigation with different salinity waters in different growth stages (T4, T5, and T6) showed better results than the use of saline waters (T2 and T3) throughout the cycle. This may be due to the fact that cotton tolerance to salinity increases as growth stages progress (Khorsandi & Anagholi, 2009).

The number of leaves (Fig. 1C) showed a response to salinity different from plant height and stem diameter. Although not statistically different, a greater number of leaves was observed when irrigation water salinity was different according to the growth stage (T4, T5, and T6) than when water salinity was the same in all stages (T1, T2, and T3). The highest mean (61 leaves) was obtained when the lowest-salinity water was applied in the vegetative stage, and the intermediate-salinity water was applied in the other stages (T4). This treatment had 16% more leaves than T1, which used the lowest-salinity water in all stages. This indicates that if the plants grow well while receiving the lowest-salinity water in the vegetative stage, it would be advantageous to apply moderate-salinity water in the other stages (T4), or even to apply moderate salinity water in the reproductive stage and the highest-salinity water in the maturation stage (T5). According to Khorsandi and Anagholi (2009), to compensate for the delay in development after saline stress, cotton plants restart the vegetative growth and emit new leaves, which reactivate the photosynthesis and start a new cycle to produce flowers and bolls.

The cotton production parameters of lint yield (Fig. 2A) and number of bolls (Fig. 2B) did not show a significant effect of treatments, even after data transformation of the number of bolls, which did not adjust to the normal distribution, according to the Shapiro-Wilk test. These results were explained by Ribeiro-Oliveira *et al*. (2018) in a study on data transformation. The behavior of data on the number of bolls was similar to that of lint yield, stem diameter, and plant height when water of the same salinity was applied throughout the cotton cycle (T1, T2, and T3). When irrigated with the lowest-salinity water in all growth stages (T1), lint yield and number of bolls were 40% higher than in T3, in which the highest-salinity water was used at all stages.

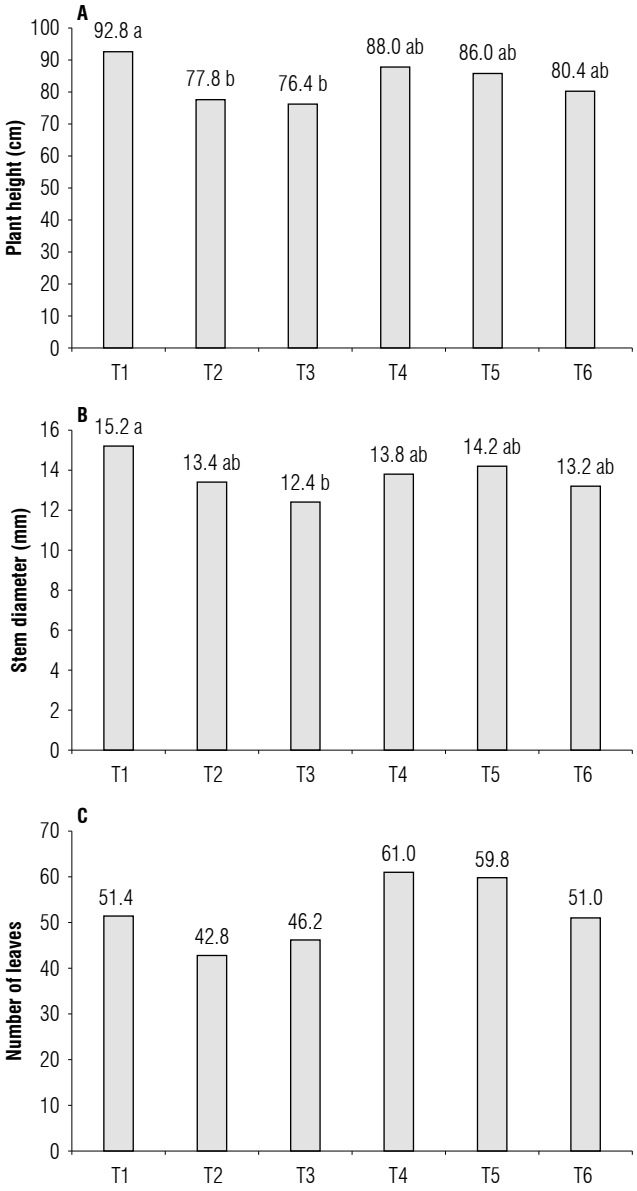

FIGURE 1. A) Comparison of means of plant height, B) stem diameter, and C) number of leaves at 100 d after emergence of naturally colored cotton subjected to three irrigation water salinity levels in different growth stages. T1 (S1 (0.55 dS m^{-1}) water in the three stages); T2 (S2 (2.16 dS m^{-1}) water in the three stages); T3 (S3 (3.53 dS m^{-1}) water in the three stages); T4 (S1 in the vegetative stage (V), and S2 in the reproductive (R) and maturation (M) stages); T5 (S1 in V, S2 in R, and S3 in M), and T6 (S1 in V, and S3 in R and M).

According to Ahmad *et al*. (2002), long-term irrigation with saline water caused a decrease in the number of bolls per plant and seed cotton yield. The reduction in the number of bolls was due to the reduction of fruiting sites and a greater fall of bolls. In this regard, Jácome *et al*. (2003) tested several cotton genotypes irrigated with increasing levels of water salinity and observed a significant reduction in the number of bolls per plant of BRS Verde and of the lint yield of CNPA 7H. Similarly, Sousa Júnior *et al*. (2005) observed a linear decrease in the number of bolls of BRS Verde cotton when the ECw of irrigation water increased from 2.0 to 9.5 dS m^{-1}.

However, the second-largest yield was observed when the lowest-salinity water was applied in the vegetative stage, the intermediate-salinity water was applied in the reproductive stage, and the highest-salinity water was applied in the maturation stage (T5). The treatment T5 had a 13% lower lint yield and 11% lower number of bolls than T1. According to Khorsandi and Anagholi (2009), the application of saline water in the vegetative stage is more harmful to cotton plants, since they do not recover when suffering moderate to severe damages, while plants are less affected in the reproduction and maturation stages.

Although there were no statistical differences, the lowest values of the production variables were observed in the T6 treatment, in which the highest-salinity water was applied in the reproductive and maturation stages after the application of the lowest-salinity water in the vegetative stage. Given the existence of two water sources with different salinities in the region, and that the farmers in the area face problems of availability of good quality water and soil salinization, the results obtained in T5 indicate that the application of good quality water in the vegetative stage and the mixture of the water from the two sources in the reproductive and maturation stages should be better studied as an alternative to improve cotton growth and production. This is in agreement with Khorsandi and Anagholi (2009) and Soares *et al*. (2018). These authors recommend the application of good quality water until full crop establishment, followed by the application of intermediate-salinity water in the reproductive stage. The highest-salinity water is not so harmful in the maturation stage because plants have already formed their reproductive organs, guaranteeing the production of bolls and because the plants are more tolerant of salinity during the growth of bolls than during the vegetative and reproductive stages.

The treatments had a significant effect on the leaf contents of sodium (P<0.01 - Fig. 3B), potassium (P<0.01 - Fig. 3C), phosphorus (P<0.05 - Fig. 3D), magnesium (P<0.05 - Fig. 3E), and calcium (P<0.05 - Fig. 3F). These variables showed CV values of 15.8%, 10.9%, 20.3%, 11.6%, and 15.3%, respectively. No significant effect was observed on leaf nitrogen content (CV = 16.2%) (Fig. 3A), which is the most limiting nutrient to plant growth. No treatment achieved a leaf nitrogen content of 32 g kg^{-1}, which is the appropriate level according to Dechen *et al*. (2007). This occurred in the treatments receiving saline water since the effect of salinity reduces nitrogen absorption, mainly in the form of NO_3^-, and restricts its load in the xylem (Miller *et al*., 2007).

The sodium content of leaves (Fig. 3B) increased when replacing good quality irrigation water (T1) with saline water in all growth stages (T2 and T3). The accumulation of sodium in plant shoots in response to the increase in salt concentration in the soil solution is an important

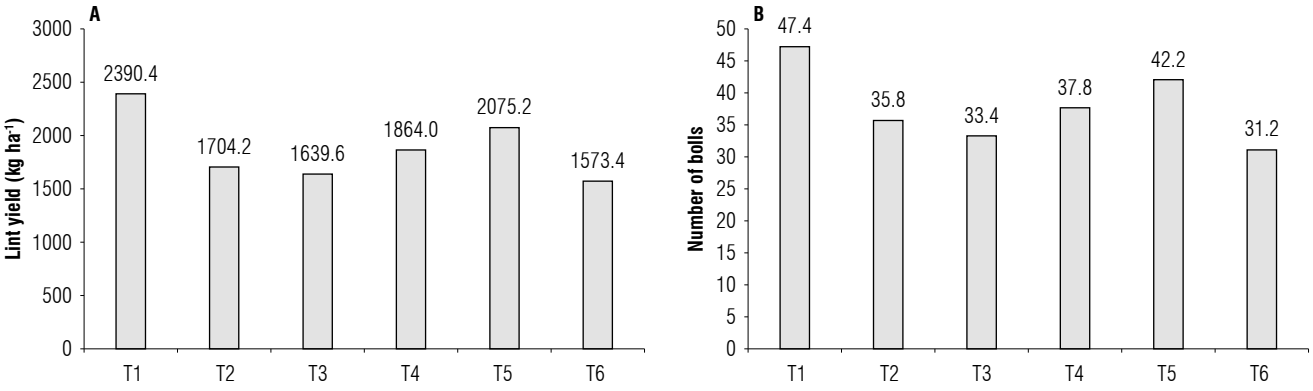

FIGURE 2. Comparison of means for A) lint yield, and B) number of bolls of colored cotton subjected to three irrigation water salinity levels in different growth stages. T1 (S1 (0.55 dS m^{-1}) water during the three stages); T2 (S2 (2.16 dS m^{-1}) water during the three stages); T3 (S3 (3.53 dS m^{-1}) water during the three stages); T4 (S1 in the vegetative stage (V), and S2 in the reproductive (R) and maturation (M) stages); T5 (S1 in V, S2 in R, and S3 in M), and T6 (S1 in V, and S3 in R and M).

mechanism of plant tolerance to salinity (Queiroz & Büll, 2001). The highest leaf sodium content was observed when the highest-salinity water was applied in all stages (T3), similar to T6, which received the lowest-salinity water in the vegetative stage and the highest-salinity water in the reproductive and maturation stages. When comparing this treatment with treatments that received the lowest-salinity water only in the vegetative stage (T4, T5, and T6), there was no difference in the sodium leaf content when the highest-salinity water (T5) or the intermediate-salinity water (T4) was applied during the reproductive period. Additionally, the leaf contents of sodium of these treatments were similar to those of treatments T1 and T2.

The potassium leaf content (Fig. 3C) declined when the lowest-salinity irrigation water (0.55 dS m^{-1}) was replaced by saline water (2.16 and 3.53 dS m^{-1}) in all growth stages. However, when applying waters of different salinities in different growth stages, the T4 treatment (which used the lowest-salinity water in the vegetative stage and

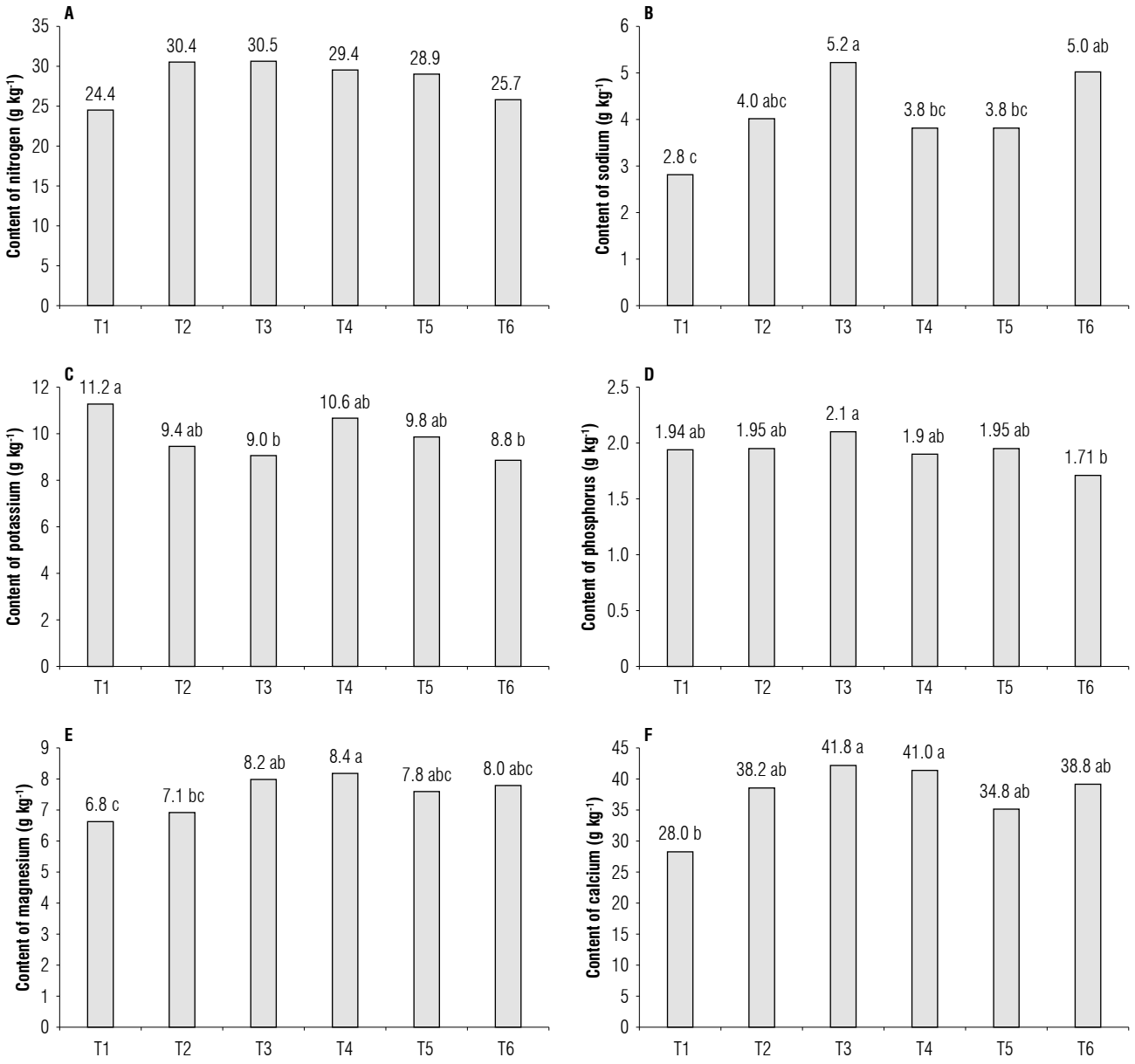

FIGURE 3. Comparison of means for the leaf contents of A) nitrogen, B) sodium, C) potassium, D) phosphorus, E) magnesium, and F) calcium of colored cotton subjected to three irrigation water salinity levels in different growth stages. T1 (S1 (0.55 dS m^{-1}) water in the three stages); T2 (S2 (2.16 dS m^{-1}) water in the three stages); T3 (S3 (3.53 dS m^{-1}) water in the three stages); T4 (S1 in the vegetative stage (V), and S2 in the reproductive (R) and maturation (M) stages); T5 (S1 in V, S2 in R, and S3 in M), and T6 (S1 in V, and S3 in R and M).

intermediate-salinity water in the other stages), showed slightly lower results than T1 and higher results than saline water treatments (T2, T3, T5, and T6). Despite this, T1 and T4 differed significantly only from the treatments that applied the highest-salinity water (T3 and T6).

According to Queiroz and Büll (2001), the increase in soil sodium content reduces the absorption of potassium by cotton plants, as occurred in the treatments that used saline water in this study. Inhibition of potassium uptake due to high sodium concentration in the soil solution is one of the main disturbances caused by salinity. This inhibition may explain why the potassium leaf content was lower than 14 to 16 g kg^{-1} in all treatments, which is considered the appropriate content by Dechen *et al*. (2007).

Although the crop was not fertilized with phosphorus, its content in cotton leaves (Fig. 3D) was greater than the range of 2.0 to 2.5 g kg^{-1} considered adequate by Malavolta (2004). However, T6, which received the highest-salinity water in the reproductive and maturation stages, showed significantly lower leaf phosphorus contents than T3. When the irrigation water salinity was the same in all crop stages, the leaf phosphorus content increased from T1 (0.55 dS m^{-1}) to T2 (2.16 dS m^{-1}) and to T3 (3.53 dS m^{-1}) which was the treatment with the highest phosphorus content. However, a reduction in phosphorus content of plant tissue was expected due to the ionic force effect, which reduces phosphate activity in the soil solution. Additionally, the high adsorption of phosphate, whose solubility decreases when NaCl increases in the soil, may contribute to the lower content of this macronutrient (Sharpley *et al*., 1992). According to Zoz *et al*. (2009), an increase in phosphorus availability to plants may occur if the salt accumulation causes an increase in pH and a reduction in phosphorus adsorption in the soil.

In the treatments that received the lowest-salinity water only in the vegetative stage, the leaf phosphorus content was similar to that in the treatments that received the intermediate-salinity water in the reproductive and maturation stages (T4), the intermediate-salinity water in the reproductive stage, and the highest-salinity water in the maturation stage (T5). These treatments showed a higher leaf phosphorus content than T6 that received the highest-salinity water in the reproductive and maturation stages. Considering that treatments T6 and T3 received the highest-salinity water from the beginning of the reproductive stage, the higher leaf P content of T3 may be due to increased soil pH or to plant adaption to stress before the reproductive stage. According to Grattan and Grieve (1998), the effect of salinity on plant phosphorus content depends on the genotype, phenological stage, phosphorus content in the substrate, type of salts, and salinity level.

The contents of magnesium in the leaf tissue of all treatments (Fig. 3E) were higher than the range of 4.0 to 5.0 g kg^{-1} considered adequate by Malavolta (2004). Our results showed a significant increase as the lowest-salinity irrigation water (T1 - 0.55 dS m^{-1}) was replaced by saline waters (T2 - 2.16 dS m^{-1} and T3 - 3.53 dS m^{-1}). This increase might be due to the magnesium contained in saline waters.

The leaf contents of magnesium observed in treatments T4, T5, and T6 that received saline waters in some stages of their growth were similar to that observed in T3 that received the highest-salinity water in all stages. However, Queiroz and Büll (2001), who studied higher levels of water salinity, observed a reduction in the absorption of calcium and magnesium in five cotton cultivars due to the increased salinity of the irrigation water. According to these authors, the increase in the concentration of sodium in the soil solution promotes a reduction in the absorption of calcium and magnesium due to the effect of competitive inhibition between these cations during absorption.

Calcium contents in the cotton plant tissue (Fig. 3F) were also above the range considered adequate by Malavolta (2004), which is between 30 and 40 g kg^{-1}. Only T1 that received the lowest-salinity water in all growth stages had a slightly lower calcium content than that considered adequate. This observation illustrates the trend of an increase in leaf calcium contents when saline water (2.16 or 3.53 dS m^{-1}) was applied in all stages or in the reproductive and maturation stages. One aspect that must be taken into account is that in the preparation of saline water used in this study, the high levels of calcium in the water of the Calcário Jandaíra aquifer were reproduced.

The treatments irrigated with waters of different salinities in different growth stages of BRS Verde cotton had a significant effect on the leaf contents of zinc ($P<0.01$), copper, and manganese ($P<0.05$), but not on the iron content (Fig. 4). These variables showed CV values of 30.5, 7.2, 20.0, and 24.6%, respectively.

The zinc content in cotton leaves was generally above the range considered adequate, which is from 10 to 15 mg kg^{-1} (Malavolta *et al*., 1997; Galrão, 2002), while the iron and copper contents were within the appropriate ranges, which are from 50 to 250 mg kg^{-1} for iron (Malavolta *et al*., 1997; Galrão, 2002) and from 5 to 15 mg kg^{-1} for copper

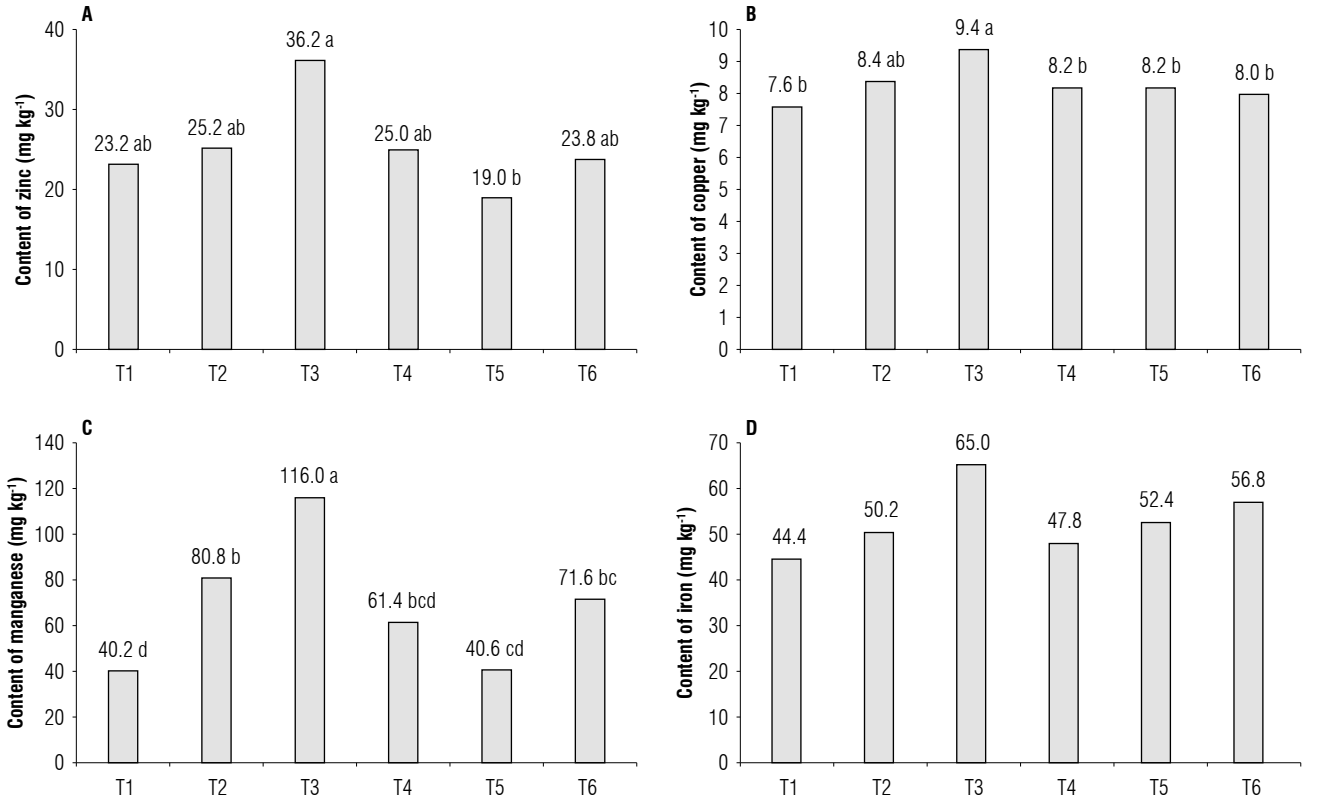

FIGURE 4. Comparison of means for the foliar contents of the micronutrients A) zinc, B) copper, C) manganese, and D) iron of colored cotton subjected to three irrigation water salinity levels in different growth stages: T1 (S1 (0.55 dS m^{-1}) water in the three stages); T2 (S2 (2.16 dS m^{-1}) water in the three stages); T3 (S3 (3.53 dS m^{-1}) water in the three stages); T4 (S1 in the vegetative stage (V), and S2 in the reproductive (R) and maturation (M) stages); T5 (S1 in V, S2 in R, and S3 in M), and T6 (S1 in V, and S3 in R and M).

(Carvalho, 2007). The manganese content is not considered to be toxic to cotton, since Foy *et al*. (1995) observed manganese toxicity only in cotton plants with leaf contents greater than 1500 mg kg^{-1}.

A clear tendency of increase in leaf contents of zinc (Fig. 4A), copper (Fig. 4B), manganese (Fig. 4C), and iron (Fig. 4D) with increased irrigation water salinity was observed when comparing the different salinity applied in all cotton growth stages (T1, T2, and T3). The increases observed between treatments T1 (water of 0.55 dS m^{-1} in all stages) and T3 (water of 3.53 dS m^{-1} in all stages) were 24%, 46%, 56%, and 190% for copper, iron, zinc, and manganese, respectively.

According to Hu and Schmidhalter (2001), the ability to metabolize micronutrients efficiently depends on the plant's genotype, but salinity alters micronutrient availability due to an increase in the solubility of these elements. Additionally, changes in soil pH occur that also influence micronutrient solubility. This information is corroborated by Grattan and Grieve (1992), who reported that micronutrient concentration in plant shoots is also influenced by soil mineralogy, type of plant and tissue, level of salinity, micronutrient concentration in the medium, and environmental conditions. These authors reported increases due to salinity in the shoot contents of zinc, manganese, and iron in diverse crops.

When the cotton was irrigated with the lowest-salinity water in the vegetative stage and with saline waters in the other growth stages (T4, T5, and T6), the micronutrient contents differed little among the treatments. In general, the contents in these treatments were lower than in the T3 treatment, in which the highest-salinity water (3.53 dS m^{-1}) was used in all growth stages.

Conclusions

The recommendation to apply the lowest-salinity water in all growth stages is based on the increase in cotton growth and yield of around 19% and 40%, respectively, compared to the application of the intermediate or highest-salinity water. The application of the lowest-salinity water in the vegetative stage and intermediate-salinity water in the following stages is an alternative to using good quality water

throughout the cycle, despite the decreases of about 7% in growth and 16% in yield. The nutrition of cotton plants irrigated with saline water throughout the cycle or in some growth stages was marked by an increase of up to 86% in the leaf sodium content, a decrease in the leaf potassium content of up to 21% and increases between 24% and 188% in leaf micronutrient content when the highest-salinity water replaced that with the lowest salinity.

Author's contributions

AFM and MTG formulated the research goals and aims and managed the project. AFM, MTG, and KDT developed and designed the methodology. AFM, KDT, and LRC conducted the experiment. AFM, MTG, and MFN carried out the statistical analysis. AFM, MTG, NOM, and NSD wrote the original draft. MFN, NOM, and NSD reviewed the draft and translated it into English.

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Rhizospheric rhizobia with potential as biofertilizers from Cuban rice cultivars

Rizobios rizosféricos con potencial como biofertilizantes a partir de cultivares cubanos de arroz

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ABSTRACT

Rice biofertilization with *Rhizobium* increases the growth and yield of the crop. However, evidence for this has not been observed in Cuban rice cultivars. This research aimed to typify two *Rhizobium* isolates, considering the use of different carbon sources, their tolerance to stress conditions, and the ability to promote the growth and development of rice. Two *Rhizobium* sp. isolates (Rpr11 and 5P1) were used and their facility to grow on different carbon sources, pH, and salinity levels was determined. The effect of the inoculation of these bacteria on the growth and yield of rice was evaluated under controlled, greenhouse, and field conditions. Both isolates grew on mannitol, glycerol, maltose, and fructose at the highest concentrations of NaCl (1.0, 1.5 and 2.0%). The isolate 5P1 grew at all evaluated pH levels, especially at pH 5.0 and pH 8.0. The inoculation of both isolates increased the plant biomass and the potassium content. The plants inoculated with 5P1 had the highest contents of nitrogen, total chlorophyll, carbohydrates and proteins, and grain yield. This study is the first in Cuba that shows the beneficial effect of *Rhizobium* inoculation on the physiology and yield of rice.

Key words: *Rhizobium*, grass, salinity, acidity, growth, yield.

RESUMEN

La biofertilización de arroz con *Rhizobium* incrementa el crecimiento y rendimiento del cultivo. Sin embargo, en cultivares cubanos de arroz no se han observado tales evidencias. El objetivo de esta investigación fue tipificar dos aislamientos de *Rhizobium* considerando el uso de diferentes fuentes de carbono, su tolerancia a condiciones de estrés y la capacidad de promover el crecimiento y el desarrollo del arroz. Se emplearon dos aislamientos de *Rhizobium* sp. (Rpr11 y 5P1) y se determinó su capacidad para crecer en diferentes fuentes carbonadas, pH y niveles de salinidad. Se evaluó el efecto de la inoculación de estas bacterias sobre el crecimiento y rendimiento del arroz en condiciones controladas, de invernadero y de campo. Ambos aislamientos crecieron en manitol, glicerol, maltosa y fructosa y en las mayores concentraciones de NaCl (1.0, 1.5, y 2.0%). El aislamiento 5P1 creció en todos los niveles de pH, especialmente en pH 5.0 y pH 8.0. La inoculación de ambos aislamientos incrementó la biomasa y el contenido de potasio en las plantas. Las plantas inoculadas con 5P1 mostraron un mayor contenido de nitrógeno, clorofilas totales, carbohidratos y proteínas, y rendimiento del grano. Este estudio es el primero en Cuba que demuestra el efecto benéfico de la inoculación de *Rhizobium* en la fisiología y el rendimiento del arroz.

Palabras clave: *Rhizobium,* gramínea, salinidad, acidez, crecimiento, rendimiento.

Introduction

Rhizobia are bacteria that establish a symbiotic association with leguminous plants. As a result of complex molecular communication between the bacteria and the plant, nodules are formed on the roots or stem where rhizobia perform biological nitrogen fixation (Taiz *et al*., 2015). However, some studies reveal the beneficial effects of rhizobia on non-leguminous plants such as corn (*Zea mays* L.), lettuce (*Lactuca sativa*), tomatoes (*Solanum lycopersicum*) and wheat (*Triticum* spp.) (García-Fraile *et al*., 2012; Flores-Félix *et al.,* 2013). Furthermore, rhizobia have been also found associated with rice (*Oryza sativa* L.) as rhizospheric and endophytic bacteria. The rhizobia-rice interaction differs in many ways from that established with leguminous plants. These differences are fundamentally related to gene induction, cell-cell signaling, the infection process, and bacteria distribution inside the vegetable tissue (Chen *et al.,* 2015; Wu *et al*., 2018).

Bradyrhizobium, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Rhizobium* are rhizobia genera that have been mostly associated with rice. These microorganisms increase the growth of grass mainly by phytostimulation (production of indole acetic acid and gibberellins) and enhance physiological mechanisms such as photosynthesis (Yanni

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et al., 2001; Chi *et al*., 2005; Chen & Zhu, 2013). Previous studies report that rhizobia inoculation increases rice yield, even with lower doses of nitrogen than those recommended (Osorio Filho *et al.,* 2016, Lemes dos Santos *et al.,* 2019). However, studies about the potential of rhizobia strains to obtain inoculants that increase rice yields in Cuban cultivars and that decrease mineral fertilization are scarce.

In Cuba, rice is a prioritized crop since annual consumption is around 72 kg per capita, one of the highest in Latin America (Galán, 2017). However, imports are the main source for supplying the cereal in this country (FAO, 2019). Cuban rice cultivars INCA LP-5 and INCA LP-7 are widely distributed in the country (Galán, 2017) due to their positive characteristics that ensure high grain yields and resistance to diseases.

Only four reports about the rhizobia/non-leguminous crop interactions have been reported for Cuba. Two of them showed the beneficial effect of rhizobia inoculation on the growth and yield of corn. However, the rhizobia used in these trials were isolated from legume nodules and not from the crop's rhizosphere itself (Pérez-Pérez *et al.,* 2019). The other two studies explored the rhizobia-rice interaction but did not show the effect of rhizobia inoculation on the physiology and yield of rice (Hernández Forte & Nápoles García, 2017, 2019). Therefore, Cuban rice cultivar biofertilization with *Rhizobium* could be an alternative for reducing contamination and improving rice yield production and soil fertility in the country.

About 30% of Cuban soils and 15% of the country's agricultural area are affected by acidity and salinity, respectively. Around 104,000 ha dedicated to rice cultivation in Cuba are affected by salinity (Mesa, 2003). To increase productivity, mineral fertilizers are irrationally used (Goulding, 2016; Toledo, 2016). The use of tolerant microorganisms for stressful soil conditions could be an alternative for decreasing the application of mineral fertilizers and increasing rice yields. This research aimed to typify two *Rhizobium* isolates, considering the use of different carbon sources as a nutrient, their tolerance to stress conditions, and the ability to promote the growth and development of rice.

Materials and methods

Biological material

We used two isolates, Rpr11 and 5P1, belonging to the genus *Rhizobium* (accession numbers: MT387213 and MT759831, respectively) from the bacteria collection of the Laboratory of Microbiology of the Department of Plant Physiology and Biochemistry at the National Institute of Agricultural Sciences (INCA), Cuba. Isolate Rpr11 was obtained from rice cultivar INCA LP-5 rhizoplane (Hernández Forte & Nápoles García, 2017) and isolate 5P1 from rhizospheric soil of rice cultivar INCA LP-7 plants. Both cultivars were cultivated under flood conditions, in a petroferric nodule ferruginous gleysol soil from Pinar del Río, Cuba (Hernández Jiménez *et al*., 2015). The isolates were inoculated on 5 ml of yeast-mannitol (YM) medium (Vincent, 1970) and kept under shaking conditions at 150 rpm for 16 h at 30°C. The optical density (OD) (λ = 600 nm) of the inoculum was adjusted to 0.05.

Certified rice seed cultivars INCA LP-5 and INCA LP-7 were used in inoculation tests under controlled and greenhouse conditions. Seeds were disinfected and pregerminated with 70% ethanol for 5 min and 6% (v/v) sodium hypochlorite for 30 min. Then, they were washed ten times with sterile distilled water, put onto plates with water agar medium (0.8%, m/v), and incubated at 30°C for 3 d in the dark (Hernández Forte & Nápoles García, 2019).

Rhizobium sp. growth on different carbon sources

Several multiplication tactics were carried out with the *Rhizobium* isolates. Microbial growth was determined in tubes with 4.5 ml of YM medium and in four variants where mannitol was replaced by maltose, lactose, glycerol and fructose (10 g L^{-1}). Every medium was inoculated with 500 µl of inocula and incubated in a thermostated shaker (HEIDOLPH-UNIMAX-2010, Schwabach, Germany) at 150 rpm and 30°C for 24 h. The OD (λ = 600 nm) was measured every 2 h for 24 h. Five replicates of each isolate were used in each culture medium with different carbon sources.

Rhizobium sp. growth at different pH and salinity levels

Tubes containing 4.5 ml of YM medium with three pH levels (4.0, 5.0, and 8.0) and three concentrations of sodium chloride (1.0%, 1.5%, and 2.0%) were used. The tubes were inoculated with 500 µl of inocula and incubated at 150 rpm at 30°C. Yeast-mannitol medium with pH 6.8 and 0.01% sodium chloride (Vincent, 1970) was used as a positive control. The inocula OD (λ = 600 nm) with different pH levels was determined every 2 h for 16 h, whereas the readings of inoculum with different concentrations of sodium chloride were performed every 2 h for 24 h. The pH and salinity of the culture medium were not controlled during the evaluation period. Three replicates were used for each isolate and for each pH and salinity condition.

Effect of *Rhizobium* sp. inoculation on rice growth

In vitro growth conditions

Disinfected and pre-germinated rice seeds were placed in pots with 0.21 kg of non-sterilized petroferric nodule ferruginous gleysol soil. Three seeds were placed in each pot. The chemical characterization of the soil showed a slightly acidic pH, medium organic matter content, high levels of phosphorus, adequate contents of calcium, magnesium, and sodium (Hernández *et al*., 2015), and a low potassium level (Paneque Pérez *et al*., 2010) (Tab. 1).

Rice seedlings of cultivars INCA LP-5 and INCA LP-7 were inoculated with 300 µl of *Rhizobium* sp. isolates Rpr11 and 5P1 at $5x10^9$ colony-forming units (CFU) ml^{-1}. Rice seedlings inoculated with sterile YM medium were considered as a negative control. Ten pots were used for each treatment in a completely randomized design. The pots were placed in trays containing diluted Hoagland nutrient solution (1:2). The plants were grown in a 12 h light/12 h dark photoperiod at 26°C/22°C (day/night) and 70% relative humidity. At 7 d after inoculation (DAI), two plants were removed leaving only one plant per pot.

At 50 DAI the following variables were evaluated: plant height (cm), root length (cm), shoot dry weight (g), and root dry weight (g). The content of N, P and K in shoots and roots (%) was also determined from three samples of 0.2 g of the dry weight of each plant part per treatment. The samples were digested with sulfuric acid (H_2SO_4) and the color was subsequently developed with Nessler's reagent to determine the N content and with molybdenum blue for P and K (Paneque Pérez *et al*., 2010).

Greenhouse conditions

Pots containing 1.2 kg of non-sterilized petroferric nodule ferruginous gleysol soil were used. The inoculation of the seedlings was carried out similarly to the test under controlled conditions. Two control treatments were used in the experiment. The negative control consisted of seedlings inoculated with sterile YM medium. As a positive control, the *Herbaspirillum seropedicae* Z67 strain was inoculated at the same volume and concentration as *Rhizobium*. Six pots were used for each treatment in a completely randomized design.

The plants were irrigated every other day with running water. Two plants were removed 7 DAI, leaving one plant per pot. At 70 DAI, the following variables were evaluated: plant height (cm), root length (cm), shoot dry weight (g), and root dry weight (g). The relative index of total chlorophyll content (SPAD) was measured in the flag leaf and other randomly chosen leaves, using a chlorophyll reader (SPAD-502, Konica Minolta, China). The total soluble carbohydrates (mg g^{-1}) were determined by the anthrone technique (Leyva *et al*., 2008) and proteins (µg g^{-1} fresh weight) were quantified by the microLowry method (Sun, 1994) in leaves and roots. In all cases, six replicates were evaluated per treatment.

Field trial

A field experiment was carried out at the Basic Technological-Scientific Unit "Los Palacios" in Pinar del Rio, Cuba (22°44' N, 83°45' W). The experimental area has petroferric nodule ferruginous gleysol soil (Hernández *et al*., 2015) with the following chemical properties: pH (in water) of 6.46, 2.86% organic matter, 46.80 mg kg^{-1} of P_2O_5 and 0.18 $cmol_c$ kg^{-1} of K^+.

Rice plants were obtained from certified INCA LP-7 rice seeds. The seeds were sown in plastic trays (60 cm length x 30 cm width x 3 cm depth) with a 5400 cm^3 mixture of petroferric nodule ferruginous gleysol soil and organic matter (1:1). Triple superphosphate (27 g m^2), urea (7 g m^2) and potassium chloride (4 g m^2) were applied to the mixture. After 5, 10 and 15 d, urea and potassium chloride were applied again at the same concentration. One thousand six hundred plants were cultivated in each tray and irrigation was carried out with permanent watering.

Fifty plants were collected at 28 d, and the roots were embedded into the inoculum base of the selected *Rhizobium* strain (5 x 10^9 CFU ml^{-1}) for 10 min. Non-inoculated plants were used as a negative control of the experiment. The plants were taken to the plots (total area of 9 m^2) and sowing was carried out with one plant per node, leaving 25 cm between plants.

Before transplantation and 10 d after transplantation, 20% of the recommended nitrogen fertilization (Ministerio

TABLE 1. Chemical characteristics of petroferric nodule ferruginous gleysol soil used in inoculation of rice plants.

pH (KCl)	OM (%)	P_2O_5 (mg 100 g^{-1} soil)	Ca^{2+}	Mg^{2+}	Na^+	K^+
			($cmol_c$ kg^{-1})			
6.57 ± 0.27	3.05 ±0.70	75.1 ± 6.5	11.62 ± 0.97	4.75 ± 0.1	Traces	0.80 ± 0.02

OM - organic matter.

de la Agricultura, 2014) was applied to the plants. Plants with 100% mineral fertilizer (Ministerio de la Agricultura, 2014) were used as positive controls and non-inoculated and non-fertilized plants were used as an absolute control. A randomized block design with three replicates for each treatment was used.

Fifteen plants were randomly selected and the tiller number was determined at 35, 42, 50, 57, 63, 71, 78, 85, 92, 99 and 105 d after transplanting. At 105 d, plants were harvested and the number of filled grains per panicle, the weight of 1000 grains (g), and yield (t ha^{-1}) (14% grain moisture) were determined.

Statistical analysis

Absorbance values obtained in the growth tests and multiplication dynamics at different carbon sources, pH levels, and salinity, as well as the data from the inoculation tests under controlled and greenhouse conditions were subjected to the normality test (Bartlett test) and homogeneity of variance (Kolmogorov-Smirnov test). A simple classification analysis of variance was applied with the Tukey HSD (inoculation assay under controlled and greenhouse conditions) or Duncan (assay under field conditions) mean comparison tests for $P<0.05$. The Statgraphic Plus program version 5.0 was used for statistical processing of the data and Microsoft Excel 2010 for its representation.

Results

Rhizobium uses different carbon sources as nutrients

The results showed that isolate Rpr11 growth in glycerol was lower than in the rest of the carbon sources at 8 h. However, the isolate showed increased growth in this alcohol after 20 h. The isolate Rpr11 showed lower growth in maltose and fructose than in mannitol from 12 h to 20 h after which no differences were found between mannitol and fructose (Fig. 1A).

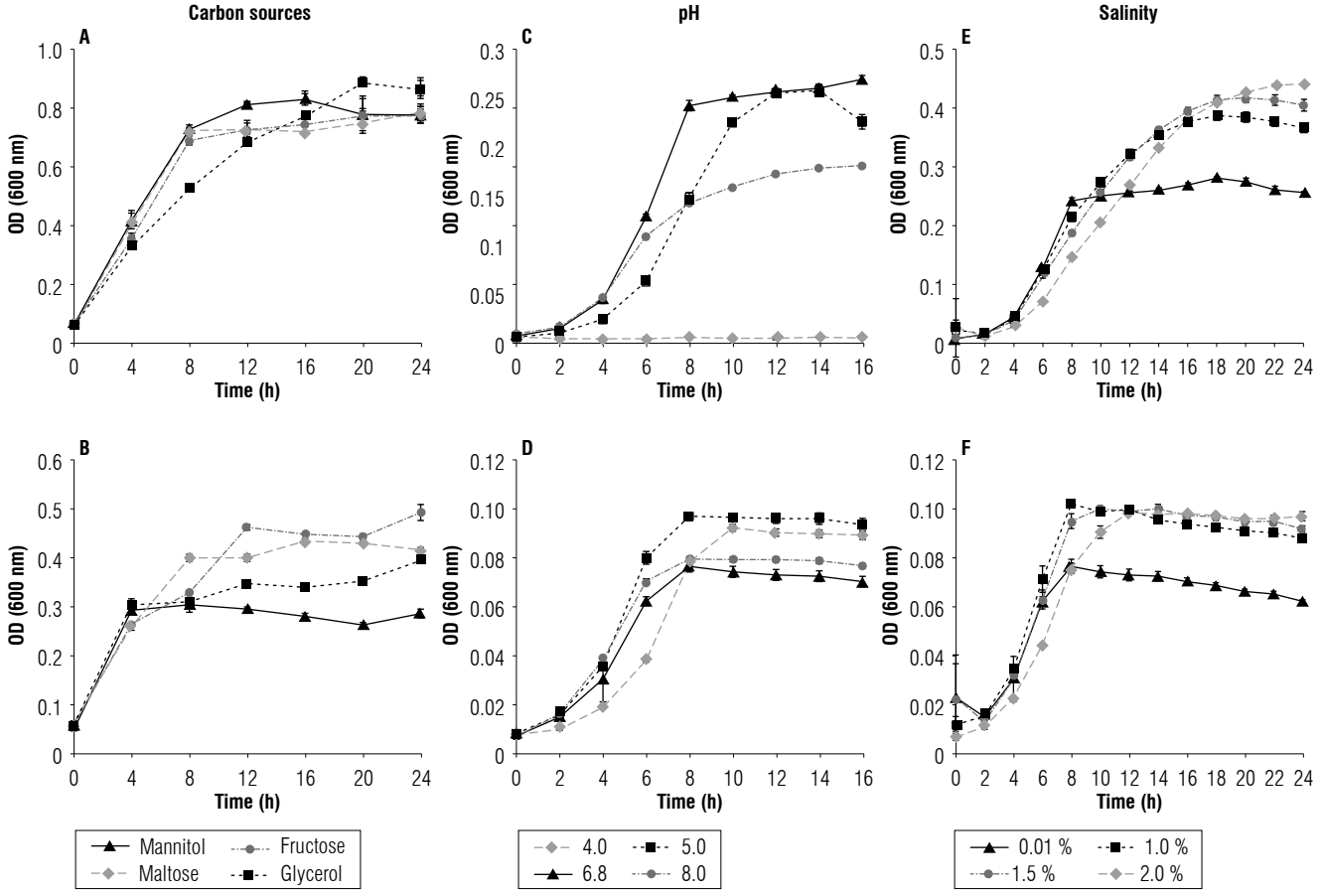

FIGURE 1. Multiplication dynamics of isolates Rpr11 (A, C, E) and 5P1 (B, D, F) in medium with different carbon sources, pH and NaCl concentrations. The data points and bars represent the means and standard errors of the mean from three replicates at each sampling time (Tukey HSD $P<0.05$, n = 3). OD - optical density.

Mannitol and glycerol caused the highest growth of isolate 5P1 at 4 h. However, the bacteria increased the growth in fructose and maltose after 8 h. This isolate showed higher growth in fructose, maltose, and glycerol than in mannitol after 12 h. No differences were observed in the growth of 5P1 in fructose and maltose from 16 h to 20 h. This isolate showed greater growth in fructose than in the rest of the carbon sources at 24 h (Fig. 1B). It can be summarized that both isolates can use mannitol, glycerol, maltose, and fructose as carbon sources.

Rhizobium isolates tolerate acidity, basicity, and salinity conditions in the medium

The isolate Rpr11 showed the highest growth at pH 6.8 after 6 h. However, no differences were observed between pH 6.8 and pH 5.0 between 12 h and 14 h. The bacteria did not grow at pH 4.0 (Fig. 1C). The isolate 5P1 grew at the lowest pH levels, although this isolate could multiply at all the tested pHs. The most acidic condition allowed a better growth of this bacterium (Fig. 1D).

Regarding the dynamics at different salinity levels, isolate Rpr11 showed higher growth in 1.0% and 1.5% of NaCl than did the control treatment from 12 h. Similar behavior was observed after 14 h, when the isolate had the greatest growth at 1.0%, 1.5% and 2.0%. The lowest bacterial growth occurred at 2.0% NaCl, from 6 h to 10 h. However, this isolate had the greatest growth in the last two hours of the assay (Fig. 1E).

The isolate 5P1 showed a similar performance with increased growth in the most saline media after 10 h. No differences were displayed in the media with 1.0%, 1.5% and 2.0% of NaCl from 12 to 24 h (Fig. 1F).

Rhizobium promotes the growth of rice plants under *in vitro*, greenhouse and field conditions

Under controlled conditions, an inoculation assay was carried out to determine the effect of inoculation with isolates Rpr11 and 5P1 on rice plant growth. The results showed that plants inoculated with isolates Rpr11 and 5P1 increased the root and shoot dry weight, respectively (Tab. 2).

The inoculation of *Rhizobium* sp. isolates increased the nutrient content in the shoot of rice plants (Fig. 2). Plants inoculated with isolate Rpr11 showed an increase in the potassium content (Fig. 2A), while those treated with 5P1 showed a higher nitrogen and potassium content than non-inoculated rice plants (Fig. 2B).

On the other hand, the results of the inoculation assay with the isolate 5P1 on the cultivar INCA LP-7 under greenhouse conditions showed that the inoculation increased plant height, root length, shoot dry weight, and relative index of total chlorophyll, total soluble carbohydrates, and total soluble protein contents (Fig. 3, Tab. 3).

The plant inoculation with 5P1 and *H. seropedicae* Z67 produced a similar beneficial effect on rice growth (Fig. 3, Tab. 3). However, the plants inoculated with 5P1 showed a higher total carbohydrate content in leaves than plants inoculated with the reference strain *H. seropedicae* Z67.

Inoculation with 5P1 under field conditions showed a higher tiller number than the absolute control plants. Fertilized plants showed the highest tiller number at all evaluation levels (Fig. 4).

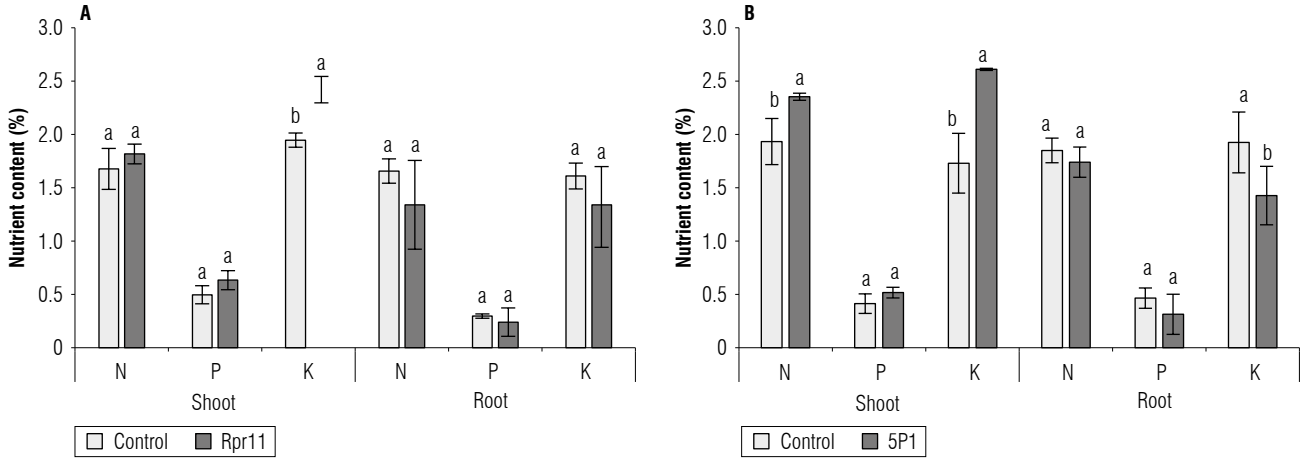

FIGURE 2. Effect of Rpr11 and 5P1 inoculation on the nutrient content of *O. sativa* L. A) cv. INCA LP-5 and B) INCA LP-7, respectively, at 50 d after inoculation (DAI) under controlled conditions. The bars represent the means ± the standard errors of the mean from nine shoot and root nutrient content sample replicates according to the Tukey HSD test ($P<0.05$, n = 9).

TABLE 2. Effect of Rpr11 and 5P1 inoculation on *O. sativa* L. cv. INCA LP-5 and INCA LP-7 growth at 50 d after inoculation (DAI) under controlled conditions.

Treatments	Height (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)
		INCA LP-5		
Control	53.2 ± 1.3 a	25.2 ± 1.2 a	0.61 ± 0.03 a	0.33 ± 0.02 b
Rpr11	52.5 ± 1.4 a	25.0 ± 1.1 a	0.69 ± 0.05 a	0.40 ± 0.03 a
		INCA LP-7		
Control	59.3 ± 1.9 a	22.5 ± 2.0 a	0.33 ± 0.04 b	0.19 ± 0.03 a
5P1	62.1 ± 1.4 a	24.7± 2.1 a	0.43 ± 0.02 a	0.23 ± 0.02 a

Control plants were inoculated with sterile yeast-mannitol medium. Means with the same letter in the same column are not statistically different according to the Tukey HSD test ($P<0.05$, n = 10).

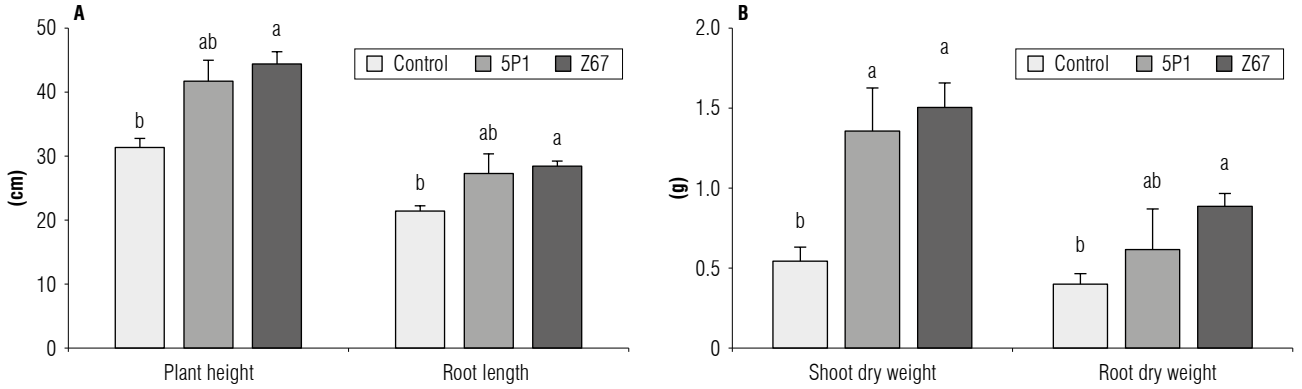

FIGURE 3. Effect of 5P1 and *H. seropedicae* Z67 (reference strain) inoculation on A) plant height and root length and B) shoot dry weight and root dry weight of *O. sativa* L. cv INCA LP-7, 70 d after inoculation (DAI) under greenhouse conditions. The bars represent the means ± the standard errors of the mean from six sample replicates (Tukey HSD $P<0.05$, n = 6).

TABLE 3. Effect of 5P1 and *H. seropedicae* Z67 inoculation on chlorophyll, carbohydrate, and protein contents of *O. sativa* L. cv INCA LP-7, at 70 d after inoculation (DAI) under greenhouse conditions.

Treatments	Chlorophyll (SPAD)	Total soluble carbohydrates (mg g^{-1})	Total soluble proteins (μg g^{-1})	
		Leaf	Leaf	Root
Control	24.1 ± 0.4 b	1.93 ± 0.04 b	23.20 ± 0.46 b	6.39 ± 0.22 b
H. seropedicae Z67	26.2 ± 0.6 a	1.90 ± 0.08 b	30.32 ± 0.46 a	8.71 ± 0.46 a
5P1	27.2 ± 1.4 a	2.36 ± 0.07 a	32.37 ± 0.98 a	7.83 ± 0.33 a

Control plants were inoculated with sterile yeast-mannitol medium. SPAD - relative index of total chlorophyll content. Means with the same letter in the same column are not statistically different according to the Tukey HSD test ($P<0.05$, n = 6).

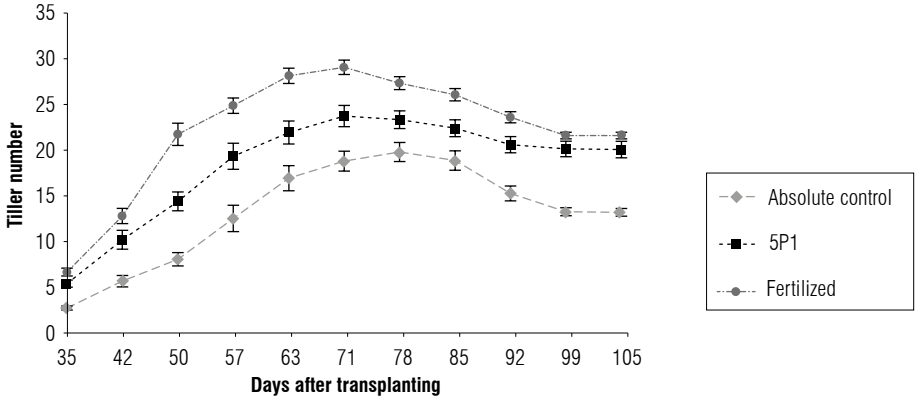

FIGURE 4. Tiller number of *O. sativa* L. cv. INCA LP-7 from 35 to 105 d after transplanting under field conditions. The absolute control treatment corresponded to non-inoculated plants with 50% mineral fertilization. The fertilized treatment corresponded to plants treated with mineral fertilization following the instructions of the Ministerio de la Agricultura (2014). The data points and bars are the means and standard errors of the mean from 15 replicates at each sampling time according to the Tukey HSD test ($P<0.05$, n = 15).

TABLE 4. Effect of 5P1 inoculation and mineral fertilization on 1000 seed weight and crop yield of *O. sativa* L. cv. INCA LP-7, at 105 d after transplanting under field conditions.

Treatments	Grains filled per panicle	1000 grain weight (g)	Grain yield (t ha^{-1})	Increase (absolute control) (%)
Absolute control	30.8 ± 0.9 c	29.9 ± 0.8 a	1.0 ± 0.1 c	-
Fertilized	111.7 ± 2.5 a	29.2 ± 0.4 a	7.7 ± 0.2 a	87.0
5P1	103.5 ± 1.8 b	28.2 ± 0.8 a	6.4 ± 0.1 b	84.4

Absolute control - non-inoculated and non-fertilized plants. Fertilized plants - 100% mineral fertilization, according to the instructions of the Ministerio de la Agricultura (2014). Means with the same letter in the same column are not statistically different according to the Duncan test ($P<0.05$, n = 15).

The maximum tiller number of fertilized plants inoculated with 5P1 was observed at 71 d after transplanting, while in the absolute control plants it occurred at 78 d. Rice tillering decreased in all treatments and remained constant from 99 to 105 d after transplanting.

No differences were observed in the weight of 1000 grains between treatments. However, plants inoculated with 5P1 increased the number of filled grains per panicle and the grain yield by more than 80% compared to the control. The fertilizer treatment allowed the greatest increases of filled grains per panicle and grain yield (Tab. 4).

Discussion

Rhizobium sp. isolates Rpr11 and 5P1 are able to use different carbon sources, tolerate stress conditions, and promote growth of rice plants. Nutritional studies of microorganisms are essential for designing culture media that meets their physiological needs, allowing a greater multiplication of the active ingredient in the inoculum. Viability, effectiveness, and efficiency are the most important characteristics of these bioproducts (Praveen Biradar & Santhosh, 2018).

The rhizobia inoculum requires a minimum bacterial concentration of 10^8 CFU ml^{-1} or g^{-1} (De Gregorio *et al*., 2017). The carbon source choice is essential to increase bacterial growth and keep its viability in the inoculum. This is especially relevant for heterotrophic bacteria such as rhizobia (Zafar *et al*., 2017).

The results showed the versatility of two *Rhizobium* sp. isolates for growing in four carbon sources. It has been reported that rhizobia strains isolated from wild legumes (*Genista microcephala* and *Argyrolobium uniflorum*) grow in different sugars as a carbon source (Dekak *et al*., 2018). The use of different sugars as carbon and energy sources constitutes advantages for the bacteria since they can survive saprophytically to compete in the rhizosphere colonization.

The multiplication dynamics suggest that *Rhizobium* sp. isolates differ in their ability to use carbon sources, regardless of the fact that both belong to the *Rhizobium* sp. group. This is especially evident when bacterial growth was compared in the glycerol, maltose and fructose. Several bacteria prefer some sugars over others as carbon sources, since they have the necessary enzymes to oxidize them in the culture medium. Recent studies show the diversity of *Rhizobium* strains for using different carbon sources (Degefu *et al*., 2018; Dekak *et al*., 2018).

The growth of isolates 5P1 and Rpr11 in fructose and maltose as carbon sources offers the possibility of using relatively cheap raw materials to replace mannitol, the main component of the YM medium. The use of refined sugar, which is rich in fructose, could be an alternative for the industrial production of inocula with both rhizobia isolates. Chemical or enzymatic processing of starch as the main reserve material in plants constitutes a maltose source for this purpose (Wang *et al*., 2015).

Acidity and salinity affect rhizobia viability and the infection and colonization processes (Plá & Cobos-Porras, 2015; Shahid *et al*., 2018). However, inoculation with tolerant rhizobia improves the establishment of some crops in forest areas and perennial legumes such as pigeon pea (*Cajanus cajan*) (Manet *et al*., 2016; Sethi *et al*., 2019).

Most rhizobia grow optimally at a pH of 6-7. The methods used to isolate these bacteria confirm it (Koskey *et al*., 2018). Therefore, it is expected that the growth of both studied *Rhizobium* isolates would be lower at acidic pH than at pH 6.8. However, the growth of isolates Rpr11 and 5P1 at pH 5.0 and the isolate 5P1 at pH 4.0 demonstrates their tolerance to acidity. Usually, *Rhizobium* is a bacterium that acidifies the culture medium as it grows, and the pH of the culture medium may decrease with its growth. Therefore, isolates such as 5P1 could have higher tolerance to acidity conditions. Recent studies show the ability of *Rhizobium* to live under acid conditions (Pádua Oliveira *et al*., 2017; Tullio *et al*., 2019).

The synthesis of acid shock proteins and lipopolysaccharides and the proton exclusion to extracellular space explains the ability of bacteria to survive and multiply under acidic conditions (Geddes *et al.,* 2014; Hawkins *et al.,* 2017). This constitutes an additional advantage that allows plant growth-promoting bacteria (PGPB) to compete during rhizosphere colonization. This advantage is especially relevant because of rhizosphere acidification due to organic acids and proton production from root exudates (Conte & Walker, 2011).

The *Rhizobium* genus produces acids in the culture medium (Koskey *et al*., 2018) that could explain the growth of isolates Rpr11 and 5P1 at pH 8. However, the results showed that these bacteria displayed greater growth at pH 5.0 than at pH 8.0. Previous studies indicate a similar behavior of rhizobia strains from *Desmodium triflorum* nodules (Bécquer *et al*., 2017).

Salinity tolerance was another trait identified in the studied *Rhizobium* isolates. Around 9.30 % of Pinar del Río soils, the origin of these isolates, are affected by salinity (1.0% of salts approximately) (Mesa, 2003). In this research, the tolerance of two *Rhizobium* isolates to different NaCl concentrations (1.0, 1.5 and 2.0%) was studied. These concentrations are lower than those found in previous studies with rhizobia (Cardoso *et al.,* 2017; Franzini *et al*., 2019; Nohwar *et al*., 2019) but higher than those found in the studied soils. NaCl is not the only salt that contributes to soil salinity (Shao *et al.,* 2019); however, it is used in many PGPB characterization studies to determine the salt bacteria tolerance in the culture medium (Numan *et al*., 2018; Jiang *et al*., 2020).

Salinity decreases the colonization of roots by rhizobia (Tewari & Sharma, 2020). However, rhizobia tolerant to salinity could survive, grow, and effectively associate with their plant hosts (Yanni *et al*., 2016). This seems to be the case of isolate 5P1 that had the highest values of optical density when it was cultured at the highest concentration of NaCl. Tolerance to salinity may be due to a plasmid-mediated resistance and salt resistance can be rapidly transferred from tolerant to sensitive bacteria (Kajić *et al*., 2016).

Around 8000 ha dedicated to rice production in Cuba are affected by salt excess, a factor that decreases crop yield (Lamz Piedra & González Cepero, 2013). This is an opportunity to establish biofertilization strategies with salinity-tolerant microorganisms such as 5P1 and Rpr11. Isolating *Rhizobium* strains adapted to stressful conditions and increasing their concentration in these soils from their inoculation could have a positive ecological effect on ecosystems.

Isolate Rpr11 increases the growth of rice plants of the cultivar INCA LP-5 (Hernández Forte & Nápoles García, 2019). So, in this research the effect of inoculation of this bacterium on the nutrient content of rice plants was determined. The positive contribution of isolate 5P1 inoculation on the plant rice cultivar INCA LP-7 growth was shown for the first time. The acidity tolerance of both *Rhizobium* sp. isolates could explain their establishment on the slightly acidic petroferric nodule ferruginous glaysol soil used, which is similar to the soil where both bacterial isolates originated (Hernández Forte & Nápoles García, 2017). The non-sterilization of the soil used would suggest diverse interactions between the inoculated bacteria and the resident microbiota, which is a very important aspect in plant-microorganism interaction (Čapek *et al.,* 2018).

Growth promotion in non-leguminous plants such as rice, sorghum (*Sorghum bicolor*), and corn (*Zea mays*) when inoculated with rhizobia is already known (Solaiman *et al*., 2011; Bécquer *et al*., 2012; Singh *et al*., 2013). The production of indole acetic acid, gibberellins, and vitamins of the B group are some mechanisms that rhizobacteria use to increase plant height, shoot dry weight, and root dry weight in rice plants (Gopalakrishnan *et al*., 2015). Some of these mechanisms could explain the positive effects of inoculation with isolates Rpr11 and 5P1 in rice plants cv. INCA LP-5 and INCA LP-7, respectively.

The increase of root dry weight in rice plants cv. INCA LP-5 inoculated with isolate Rpr11 under controlled conditions could have favored greater potassium absorption, mainly when its concentration was low in petroferric nodule ferruginous gleysol soil. The inoculation of rice plants with strains of rhizobia causes the modification of the roots, favoring the expanded root architecture (Yanni & Dazzo, 2015). This allows us to explore a larger reservoir of nutrients from the existing resources in the rhizosphere; and, thus, increase the absorption of nutrients and the dry weight of plants. Previous research reports that *Rhizobium* inoculation increases the nutrient content in plants since it promotes root growth and enhances the plant´s ability to absorb it (Osorio Filho *et al.,* 2016). This last case could be the mechanism used by isolate 5P1 which increased the potassium and nitrogen content in the rice shoot without promoting root growth.

Taiz *et al*. (2015) report that chlorophyll synthesis is closely related to nitrogen availability in soil and to the

plant's ability to absorb it. Therefore, the increase of the nitrogen content in the shoot of rice plants cv. INCA LP-7 inoculated with isolate 5P1 could explain the increase of the chlorophyll content. A higher content of these molecules enhances photosynthesis, allowing the synthesis of carbohydrates (Degiovanni *et al*., 2010), an effect shown with the inoculation of isolate 5P1 in rice plants cv. INCA LP-7 under greenhouse conditions. The enhancement of photosynthesis is one of the main mechanisms that explains the growth promotion of rice inoculated with *Rhizobium* (Chi *et al*., 2010).

Photosynthesis also influences the synthesis of carbohydrates to provide a reduction power for nitrogen assimilation (Degiovanni *et al*., 2010). The positive effect of isolate 5P1 inoculation on the chlorophyll content and nitrogen absorption could explain the increase in the content of total soluble protein in rice plants. The proteomic analyses show that the inoculation of rice plants with rhizobia induces the production of plant proteins that contribute to a better yield compared to non-inoculated plants (Chi *et al.,* 2010).

The nitrogen from the decomposition of soil organic matter enhanced with 5P1 could be one possible source of nitrogen that rice plants use to increase protein synthesis in leaves and roots. Previous studies demonstrate the positive effect of rhizobia inoculation as nitrogen-fixing bacteria on plant growth and yield. The inoculation of some *Rhizobium* strains allows an increase of dry matter in rice shoots with a decrease of 40% of the nitrogen dose (Osorio Filho *et al.,* 2016; Lemes dos Santos *et al.,* 2019). Therefore, the fixed nitrogen could also be another nitrogen source for rice plants inoculated with *Rhizobium* sp. isolate 5P1.

Regarding the effects of 5P1 inoculation on rice production areas in Cuba, the number of panicles and filled grains per panicle depends on the number of effective tillers, parameters that constitute some of the components that explain rice yield (Degiovanni *et al*., 2010). Therefore, the positive effect on the number of tillers could explain the increase in grain yield obtained in plants inoculated with isolate 5P1.

In rice, tillering requires high amounts of nitrogen (Degiovanni *et al*., 2010). This explains why fertilized treatment had the highest number of tillers and, therefore, the highest yield. Although the application of low doses of chemical fertilizer and the inoculation with the 5P1 strain did not surpass the fertilized treatment in any of the evaluated variables, they surpassed the control treatment. Similar results are described by Yanni and Dazzo (2010) who obtain a higher tillering in plants fertilized with N-fertilizers compared to the treatments inoculated with rhizobia. However, the combination of both treatments was even more effective. Previous research confirms that the application of PGPB such as *Bacillus*, *Pseudomonas* and *Rhizobium* to rice and wheat (*Triticum aestivum* L.) increases the tiller number and yields with low nitrogen fertilization under field conditions (Tan *et al.,* 2015; Gusain & Sharma, 2019; Saber & Qader Khursheed, 2020).

The effect of *Mesorhizobium* sp. inoculation on rice has been previously studied. One study determined that the inoculation with this bacterium does not produce statistical differences between the control and inoculation treatments in the number of grains per panicle and grain yield when 60 kg ha^{-1} were used (Hahn *et al.,* 2016). Other authors report that *Mesorhizobium* sp. associated with the recommended nitrogen dose provides the same rice grain yield as the recommended crop dose (Lemes dos Santos *et al.,* 2019). Rice plants inoculated with isolate 5P1 allowed a higher number of grains per panicle than those obtained with *Mesorhizobium* sp. The action of PGPBs on rice plants is variable since several factors may contribute to the different responses of rice to PGPBs inoculation, such as the bacterial strain, edaphoclimatic conditions, and the specificity of the plant genotype (Buzo *et al*., 2019).

Yanni and Dazzo (2015) reported lower means of increased yield in five rice cultivars inoculated with *Rhizobium leguminosarum* bv. *trifolii* than those obtained with isolated 5P1 in rice cv. INCA LP-7. These authors also emphasize the importance of looking for adequate concentrations of N-fertilizers and inoculum to ensure a balanced supply of nitrogen, since an excess of this element favors the overproduction of extra non-reproductive tillers that do not contribute to rice yield.

Therefore, the results of this study show the potential of the isolate 5P1 as promising bacteria for making a biofertilizer to inoculate rice, especially under acidity and salinity conditions.

Conclusions

This research revealed the potential of *Rhizobium* sp. associated with a Cuban rice cultivar to use multiple carbon sources as nutrients to tolerate acidity, basicity and salinity conditions and promote rice growth. This study is the first in Cuba to show the beneficial effect of *Rhizobium* inoculation on the physiology, growth and yield of a Cuban rice cultivar.

Author's contributions

IHF formulated the overarching research goals and aims, developed the methodology, created the models, validated the overall replication/reproducibility of results/experiments and other research outputs, conducted the research process, and created and/or presented the published work, specifically the initial draft (including substantive translation). IHF and ORH curated the data for initial and later reuse. IHF and LAML performed the experiments or data/evidence collection. RPP implemented the computer programs and the statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data. MCNG and RPP performed the critical review, commentary, or revision of the manuscript. MCNG led and managed the research activity planning and execution and acquired the financial support for the project leading to this publication.

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Effect of a friction-reducing additive on the drip irrigation uniformity with sugarcane vinasse

Efecto de un aditivo reductor de fricción sobre la uniformidad del riego por goteo con vinaza de caña de azúcar

Lucas Grogenski Meloca[1] and André Luiz Justi[1*]

ABSTRACT

Fertigation using vinasse, a high nutrient residue, is a viable form of complementary soil nutrition. However, it represents a dangerous risk of contamination if not properly disposed of. The objective of this study was to evaluate the irrigation and fertigation uniformity using vinasse in a drip irrigation system with and without the addition of polyacrylamide (friction-reducing polymer) applied at a concentration of 0.01 kg m^{-3} (10 mg L^{-1}). The tests consisted of collecting flow from 16 drippers in the system. Four were selected from each of the four lateral lines (first emitter, those located at 1/3 and 2/3 of the length, and the last one). Uniformity was obtained by the coefficient of distribution uniformity (CDU), Christiansen's uniformity coefficient (CUC), the total coefficient of variation (CVt), and the statistical uniformity coefficient (SUC). The CUC values after the addition of the polymer were 2.33% and 2.1% higher for water and vinasse, respectively. For the CDU, the addition of the polymer resulted in values of 6.07% and 5.3% higher for water and vinasse, respectively, and the SUC resulted in values of 3.99% and 3.83% for water and vinasse, respectively. We concluded that vinasse showed a lower average uniformity compared to water. However, when the friction-reducing agent was added, an increase was observed in the average uniformity in the drip irrigation system.

Key words: fertigation, polyacrylamide, application uniformity, irrigation evaluation.

RESUMEN

La fertirrigación con vinaza, un residuo rico en nutrientes, es una forma viable de nutrición complementaria del suelo. Sin embargo, representa un riesgo peligroso de contaminación si no se elimina correctamente. El objetivo de este estudio fue evaluar la uniformidad del riego y fertirrigación mediante el uso de vinaza en un sistema de riego por goteo con y sin la adición de poliacrilamida (polímero reductor de fricción) aplicada a una concentración de 0.01 kg m^{-3} (10 mg L^{-1}). Los ensayos consistieron en recolectar el flujo de 16 goteros en el sistema. Se seleccionaron cuatro de cada una de las cuatro líneas laterales (primer emisor, los ubicados a 1/3 y 2/3 de la longitud, y el último). La uniformidad se obtuvo mediante el coeficiente de uniformidad de distribución (CUD), el coeficiente de uniformidad de Christiansen (CUC), el coeficiente de variación total (CVt) y el coeficiente de uniformidad estadística (CUE). Los valores de CUC después de la adición del polímero fueron un 2.33% y un 2.1% más altos para el agua y la vinaza, respectivamente. Para el CUD, la adición del polímero resultó en valores de 6.07% y 5.3% más altos para agua y vinaza, respectivamente, y el CUE resultó en valores de 3.99% y 3.83% para agua y vinaza, respectivamente. Se concluyó que la vinaza presentó una uniformidad promedio menor en comparación con el agua. Sin embargo, cuando se agregó el agente reductor de fricción, hubo un aumento en la uniformidad promedio en el sistema de riego por goteo.

Palabras clave: fertirrigación, poliacrilamida, uniformidad de aplicación, evaluación del riego.

Introduction

There was a great production incentive in the sugar and alcohol industry in Brazil with the creation of PROÁLCOOL (National Ethanol Program), increasing pollution from refineries (Christofoletti *et al*., 2013). In Brazil, ethanol is used as fuel in the form of hydrated ethanol (mixture of alcohol and water) and is also added to gasoline as anhydrous ethanol (Milanez *et al*., 2008). With the rise in the utilization of biofuel vehicles, the cultivation of sugarcane has also grown in recent years. Brazil is the largest producer of sugarcane in the world with a forecast of 665.1 million t to be harvested for the 2020-2021 season. However, given the current scenario of the Covid-19 pandemic, there was a reduction in production compared to the previous harvest (7.9%), although production of 32.9 billion L of ethanol is still expected (CONAB, 2020).

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The introduction of ethanol to the market as a biofuel and a sustainable alternative to replace non-renewable fossil fuels (EPE, 2017) has drawn attention to research in the agricultural area. However, the generation of effluents such as vinasse, is an inevitable consequence (Macedo, 2007). Freire and Cortez (2000) state that vinasse is the main residue of the distillation process in the sugar and ethanol industry as a result of the fermentation process. For each liter of ethanol produced, 10 L of vinasse are generated, thus creating a massive amount of this residue. Studies of vinasse show a great nutritional potential due to its composition (Barros *et al*., 2010), with benefits such as increased K^{+} and Mg^{+2} content in soils (Silva *et al*., 2019). Additionally, this subproduct may be applied in crops through fertigation (Silva *et al*., 2007). Using vinasse with the correct management benefits soil fertility and crop development (Chitolina & Harder, 2020).

The Sugarcane Technology Center (CTC), located in Piracicaba, SP, Brazil, carried out studies on the characterization of vinasse. The first study was performed in 1995, with 64 samples in 28 plants in the State of São Paulo, and the second was carried out in 2007. Table 1 shows the variation in the characterization of the composition of sugar cane vinasse.

TABLE 1. Sugarcane vinasse characterization.

Description	Values
CaO (mg L^{-1})	71 - 2614.7
BOD (mg L^{-1})	5,879 - 75,330
COD (mg L^{-1})	9,200 - 97,400
Fe (mg L^{-1})	2 - 200
P (mg L^{-1})	<10 - 188
Glycerol (% v/v)	0.26 - 2.50
MgO (mg L^{-1})	97 - 1,112.9
Mn (mg L^{-1})	1 - 12
N (mg L^{-1})	81.2 - 1,214.6
Ammoniacal N (mg L^{-1})	0.4 - 220.0
pH	3.5 - 4.9
K (mg L^{-1})	814 - 7,611.5
Sulfate (mg L^{-1})	92.3 - 3,363.5
Sulfite (mg L^{-1})	5 - 153
Zn (mg L^{-1})	<0.5 - 4.6
T (°C)	65 - 110.5
Cu (mg L^{-1})	<0.2 - 3.2
Al (mg L^{-1})	<5.0 - 120.0

BOD - biochemical oxygen demand; COD - chemical oxygen demand. Adapted from Elia Neto and Nakahondo (1995) and Elia Neto and Zotelli (2008).

The composition, organic matter concentration and chemical composition of vinasse may vary according to the mode of product preparation, the fermentation method, the type of material used for fermentation, among other parameters (Robertiello, 1982). Freire and Cortez (2000) support this statement due to the great variability in the chemical composition of vinasse, as it contains large amounts of organic matter and potassium, calcium, and sulfate, low levels of nitrogen, phosphorus, and magnesium, and low concentrations of micronutrients.

Although the use of vinasse as a fertilizer may provide several benefits, attention should be paid to the problems that its application may cause. Several authors cite vinasse as a pollutant (Christofoletti *et al*., 2013), and its composition is considered a factor of importance that may cause changes in the aquatic flora and fauna of rivers and lakes. Additionally, large quantities of this residue may affect soil properties (physical, biological, and chemical). Applying vinasse in an uncontrolled manner may cause profound changes in soil properties, from salinization and changes in the nutritional balance to ion leaching into groundwater (Ribeiro *et al.,* 2010). The basic rate of water infiltration in the soil may show a reduction of up to 40% in soils with the uncontrolled application of vinasse (Dalri *et al*., 2010). Thus, the Environmental Company of the State of São Paulo (CETESB), in its standard P4.231 (CETESB, 2006), indicates the recommended values for the application of vinasse in the soil to prevent modifications resulting from the excessive use of the product.

Unfortunately, fertigation used in plants is not always treated in an appropriate and technical way, considering the quantity, quality and time required for each irrigation. Sprinkler irrigation using a self-propelled system with hydraulic cannon is a common method used with vinasse; nevertheless, its application uniformity is low (Bebé *et al*., 2009). Drip systems are a more efficient alternative since they irrigate only a part of the soil surface, directly in the root region and with low amounts of water. Thus, these systems have a low flow with high frequency, keeping the soil always close to field capacity (Bernardo *et al*., 2006).

Fertigation using vinasse requires an adequate dimensioning of the irrigation system that transports fluids to the crops. Hydraulic parameters must be considered, such as pressure drops in pipes and channels due to the way this subproduct is applied (Justi *et al*., 2012). These hydraulic factors affect not only the efficiency of the system but also the fixed and variable costs, like piping and electricity. The economic aspect of vinasse application may not be

advantageous when inadequately measured, showing the importance of studies related to head loss.

Scientists have tried to find possible ways to reduce the friction factor inside the ducts. In 1948, the British chemist B.A. Toms demonstrated a diluted polymer solution that changed the flow pressure without changing the flow (Virk *et al*., 1967; Bizotto *et al*., 2011). Researchers started using these polymers in the 80's, creating new possibilities for study. According to Bizotto and Sabadini (2008), the application of polymers prevents the formation of swirls and reduces the loss of kinetic energy in the flow, with both resulting in reduced friction. The use in drip irrigation may or may not affect the uniformity of application in the drip system with irrigation and fertigation with vinasse. This study aimed to evaluate the influence of polyacrylamide as a friction-reducing additive on drip irrigation and fertigation using water and sugarcane vinasse.

Materials and methods

The experimental setup of the drip irrigation system consisted of a recycling system of water and vinasse with a canvas adapted for collecting liquids. The system was placed in a wooden structure with dimensions of 5.00 m length x 1.08 m width x 1.55 m height at the Advanced Campus Jandaia do Sul, Federal University of Parana - UFPR (Brazil).

The dripper tube (model Manari, Petroisa®, Avare, SP, Brazil) was non-compensating, with nominal flow of 1.5 L/h, a 0.1 m gap between drippers and 98.1 kPa of service pressure. The irrigation system consisted of four dripper tubes of 4.60 m long for a total of 46 emitters per line.

The system layout was arranged so that the pipes could be coupled to a pump set (model QB60, GAMMA®, Quatro Barras, PR, Brazil), with a maximum flow of 36 L/min (6×10^{-4} m³/s) and output suppression of 313.6 kPa, connected to a 200 L reservoir. The suction tube diameter was 2.54 cm (1 inch) in PVC, with 2.54 cm (1 inch) filter coupled to a 2.54 cm ball valve (1 inch) located at the outlet reservoir that is responsible for controlling the flow and pressure of the system. The system was monitored using a Bourdon pressure gauge maintained at 98.1 kPa.

The tests used two fluids, water and sugar cane vinasse with and without the friction-reducing polymer and the addition of polyacrylamide (FLONEX 9051 SI, SNF, Brazil) at a concentration of 0.01 kg m^{-3} (10 mg L^{-1}). This material is

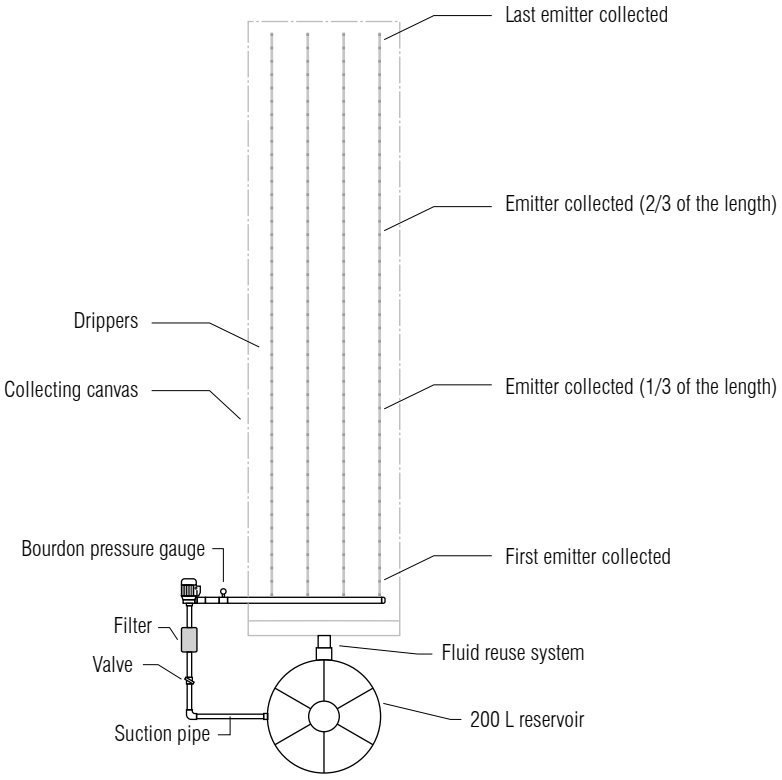

FIGURE 1. System layout assembled for the tests.

presented in the form of a light powder with color ranging from white to slightly pink, an apparent specific mass of 0.80 g cm^{-3}, viscosity of 500 cP at a concentration of 5 g L^{-1}, 200 cP at 2.5 g L^{-1}, and 80 cP at 1.0 g L^{-1}, and 90% purity. The experiment layout is shown in Figure 1.

Drip flow rates were collected using the methodology proposed by Keller and Karmeli (1975), in which the flow rates of the 16 drippers within the irrigation system are determined by selecting four drippers from four lateral lines (first emitter of the lateral line, those located at 1/3 and 2/3 of the length, in addition to the last lateral dripper).

Flow collecting was performed manually through the volume of each selected dripper after 4 min. A total of 200 irrigation cycles was carried out divided into water, vinasse, and both fluids with the addition of polyacrylamide. Each irrigation cycle had 16 flow samples for a total of 3200 samples. The statistical coefficients used for the evaluation of uniformity (Keller & Karmeli, 1975) were determined according to Equations 1-4.

$$CDU = \frac{qn}{qm} \times 100 \quad (1)$$

where CDU is the coefficient of distribution uniformity (%), qn is the average flow 25% lower from emitters (L/h), and qm is the average flow rates of emitters (L/h) resulting in a value directly proportional to the uniformity of the system (Keller & Karmeli, 1974). The classification proposed by ASAE (1996) was used, in which CDU is "excellent" when higher than 90%, "good" when between 75-90%, "regular" when between 62-75%, "poor" when between 50-62%, and "unacceptable" when the value is below 50%.

$$CUC = \left[1 - \frac{\sum_{(i=1)}^{n} \left| X_i - \overline{X} \right|}{n \times \overline{X}}\right] \times 100 \quad (2)$$

where CUC represents the Christiansen's uniformity coefficient (%), X_i is the volume obtained in order collector i (L), $\overline{X}$ is the average volumes obtained from the collectors (L), and n is the number of collectors. For the CUC, values above 90% are considered "excellent", between 80-90% are considered "good", between 70-80% are considered "regular", between 70-60% are considered "poor", and below 60% are considered "unacceptable" (Bernardo *et al.*, 2006).

$$CVt = \frac{SD}{qm} \quad (3)$$

where CVt is the total coefficient of variation (dimensionless), SD is the standard deviation of flows (L/h), and qm is the average flow (L/h). This coefficient of variation is used to calculate the statistical uniformity coefficient (SUC) by Equation 4. Table 2 shows the classification for this coefficient.

$$SUC = 100 \times (1 - CVt) \quad (4)$$

TABLE 2. Classification for the statistical uniformity coefficient (SUC).

Classification	SUC (%)
Excellent	>90
Good	80-90
Regular	70-80
Poor	60-70
Unacceptable	<60

Adapted from Favetta and Botrel (2001).

Results and discussion

The descriptive statistics considered the mean, mean standard error, standard deviation, minimum, first quartile, median, third quartile and maximum values for the CUC, CDU and SUC calculated for the evaluated variables liquid (water and vinasse) and polyacrylamide (with or without friction-reducing agent) (Tabs. 3-5). When the polyacrylamide was added, the CUC increased by 2.33% for water and 2.1% for vinasse. In relation to the CDU, for water the increase was 6.07% and for vinasse it was 5.3%. As for the SUC, there was an increase of 3.99% for the analysis with water and 3.83% for vinasse.

TABLE 3. Descriptive statistics of the Christiansen's uniformity coefficient (CUC) for liquid and polymer.

	CUC (%)			
	Water	Water*	Vinasse	Vinasse*
Average	89.28	91.41	87.62	89.46
Standard deviation	1.70	0.51	3.10	1.71
Variance	2.90	0.26	9.58	2.92
Minimum	85.15	90.23	76.49	84.66
1st Quartile	88.03	91.11	85.52	88.86
Median	89.83	91.47	87.73	89.80
3rd Quartile	90.70	91.79	90.14	90.71
Maximum	91.66	92.44	94.18	91.99
Amplitude	6.51	2.21	17.69	7.33

* Liquid with added friction-reducing agent (polyacrylamide).

The results for the CUC were classified as "excellent" for the flow of water with polyacrylamide, and "good" for the other variables. Considering the ideal values in the literature, only water with polyacrylamide obtained the expected results. For the CDU and SUC values, the results were more

sensitive. For SUC, all results were within what was classified as "good" and "very good"; water with polyacrylamide came close to "excellent" (over 90%) (ASAE, 1996; Favetta & Botrel, 2001).

TABLE 4. Descriptive statistics of the coefficient of distribution uniformity (CDU) for liquid and polymer.

	CDU (%)			
	Water	**Water***	**Vinasse**	**Vinasse***
Average	81.22	86.15	77.67	81.79
Standard deviation	4.16	1.15	6.52	3.87
Variance	17.29	1.33	42.44	15.00
Minimum	70.72	82.89	54.87	69.63
1st Quartile	78.45	85.34	72.85	81.03
Median	81.95	86.29	77.48	83.28
3rd Quartile	84.77	86.95	83.28	84.43
Maximum	86.51	88.10	90.07	86.16
Amplitude	15.79	5.21	35.2	16.53

* Liquid with added friction-reducing agent (polyacrylamide).

TABLE 5. Descriptive statistics of the statistical uniformity coefficient (SUC) for liquid and polymer.

	SUC (%)			
	Water	**Water***	**Vinasse**	**Vinasse***
Average	85.85	89.28	83.16	86.35
Standard deviation	3.21	0.59	4.98	2.99
Variance	10.30	0.35	24.82	8.94
Minimum	76.71	87.61	69.27	78.06
1st Quartile	84.01	88.95	79.70	86.10
Median	86.82	89.41	83.67	87.46
3rd Quartile	88.68	89.76	87.91	88.22
Maximum	89.52	90.22	92.70	89.39
Amplitude	12.81	2.61	23.43	11.33

* Liquid with added friction-reducing agent (polyacrylamide).

Since the drip system tended to clog, external and internal agents affected the general uniformity, causing changes in tests 13 and 26. Vinasse has a high content of organic matter and particles in suspension that caused the clogging of the emitters and filter (Fig. 2), especially in tests using vinasse without polyacrylamide. The system suffered blockages, verified by signs of change in the pump pressure, suction and visually perceptible obstruction of the emitters. The cleaning procedure consisted of removing all the vinasse from the system to wash it with water, making it recirculate within the tubes. Additionally, the emitters were unblocked and the filter was cleaned. When the CDU showed low values, some factors directly affected the results, such as quality control in the manufacturing processes, handling failure, physical changes in components, and aging and clogging of emitters (Merriam & Keller, 1978), which was observed in this experiment, as the drippers clogged (Fig. 2).

Cunha *et al.* (2006) observed the same clogging problem with wastewater from the pulping of filtered coffee fruits that was found with fertigation using vinasse. The CUC started with a value of 95.96% and, after 144 h, a reduction of 76% was observed. In the case of CDU, the reduction was 100%, going from an initially "excellent" result to "unacceptable" at the end of the period.

FIGURE 2. Screen filter clogged with particles from vinasse.

The clogging of emitters has several possible causes, such as the quality of water or drained fluid (Nakayama & Bucks, 1991). This was confirmed in this experiment by the rapid clogging by particles of vinasse duo to its high load of organic matter (Fig. 2). Zhou *et al.* (2017) stated that the clogging of emitters by the presence of organic material and microorganisms is one of the barriers to the development of drip irrigation, especially when using wastewater. Even with this issue, the results confirmed what was stated in theory. It is possible to notice that the addition of polyacrylamide caused the uniformity to increase, in both water and vinasse. The addition of the polymer to vinasse caused uniformity to reach higher values than those observed in water without the addition of this polymer. In controlled experiments, Oliveira and Villas Bôas (2008) and Silva and Silva (2005) obtained higher uniformities for the application of dripping water, maintaining 97.70% for CUC and 76% for micro sprinkling.

Figures 3-5 show the comparison between the addition or not of polyacrylamide to both liquids (water and vinasse) for CUC, CDU, and SUC, respectively. Figures 3A, 4A and 5A show the uniformities for pure water and water

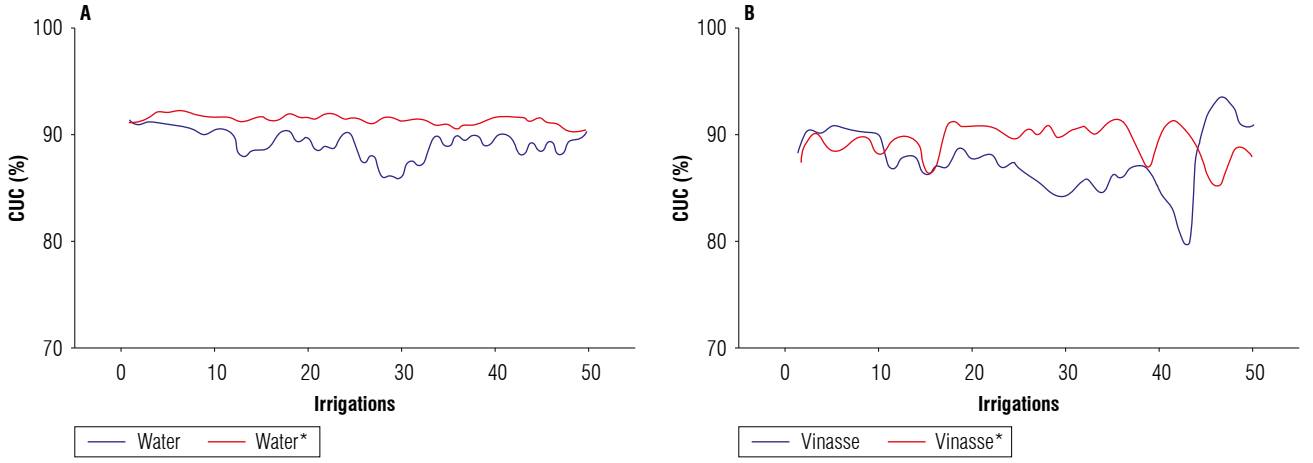

FIGURE 3. Comparison of the Christiansen's uniformity coefficient (CUC) with addition and without addition of polyacrylamide in A) water and B) vinasse. *****Liquid with added friction-reducing agent (polyacrylamide).

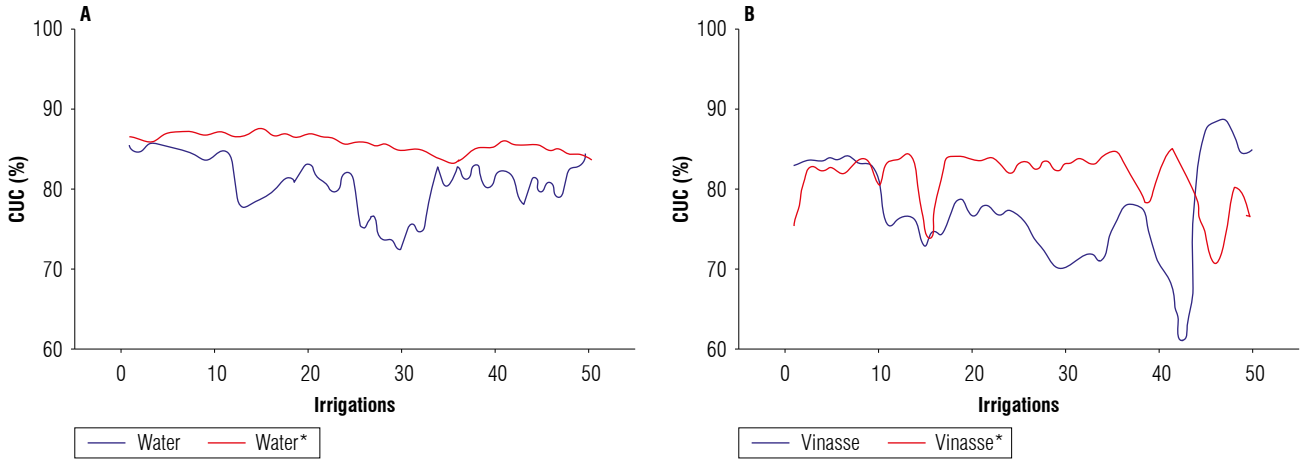

FIGURE 4. Comparison of the coefficient of distribution uniformity (CDU) with addition and without addition of polyacrylamide in A) water and B) vinasse. *Liquid with added friction-reducing agent (polyacrylamide).

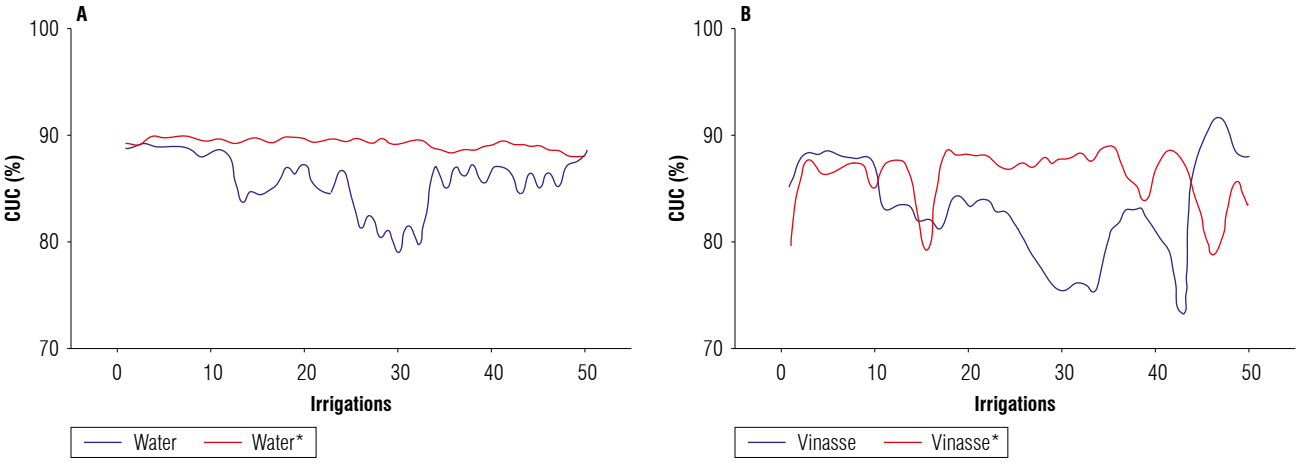

FIGURE 5. Comparison of the statistical uniformity coefficient (SUC) with addition and without addition of polyacrylamide in A) water and B) vinasse. *Liquid with added friction-reducing agent (polyacrylamide).

with polyacrylamide, while Figures 3B, 4B and 5B show the uniformity of vinasse with and without the addition of polyacrylamide.

In all cases listed above, the addition of the polymer caused an increase in uniformity, optimizing the system. The positive results of the polymer are similar to those obtained by Justi *et al.* (2017) when comparing the effect of polyacrylamide in tests with a variation of flow and diameters 2.54 cm, 1.905 cm, and 1.27 cm (1, ¾, and ½ inches) using water and vinasse in polyethylene pipes. In that study, the authors obtained an increase in flow values only with the addition of the polymer. Al-Yaari *et al.* (2009), when studying the reduction of friction in the flow of oil and water, found friction reductions of up to 65%, positively confirming that the use of friction-reducing polymer in pipes may also affect irrigation uniformity. Even for CDU that is an extremely sensitive coefficient (Merriam & Keller, 1978), an increase of up to 5% in the uniformity average was verified, emphasizing the role of the friction-reducing agent within the system.

The uniformity of vinasse is, in general, less than ideal; however, the conditions become more advantageous with the addition of polyacrylamide since uniformity is increased, reducing operating costs. This justifies the use of vinasse from a technical perspective.

Conclusions

The evaluation of irrigation systems is of paramount importance due to the necessity for saving resources and preserving the environment through the sustainable use of liquids of lesser quality than water, such as vinasse that may be used as a biofertilizer. Based on the results obtained in the present study, the average of uniformity coefficients analyzed (CUC, CDU and SUC) of water were 1.89%, 4.57% and 3.23%, higher than those found in fertigation with vinasse without the polymer, as expected due to the characteristics of the fluids. However, the uniformity coefficients were higher both in water and in vinasse when adding polyacrylamide.

The results for vinasse with the addition of the polymer exceeded by 0.2%, 0.7%, and 0.58% the values of polymer-free water for CUC, CDU and SUC, showing the efficiency and positive influence of the addition of the polymer in the evaluation of fertigation with vinasse.

For further studies, we suggest evaluating the flocculating effect of polyacrylamide on sugar cane vinasse in different dilutions and how the polymer may have an impact on physicochemical characterization and irrigation.

Conflict of interest statement

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

Author's contributions

ALJ designed the experiments and reviewed the translation. LGM carried out the field experiments and wrote the article. Both authors contributed to the data analysis and reviewed the manuscript.

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Optimization of lactic acid production by *Lactobacillus plantarum* strain Hui1 in a medium containing sugar cane molasses

Optimización de la producción de ácido láctico por *Lactobacillus plantarum* cepa Hui1 en un medio que contiene melaza de caña

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ABSTRACT

The aim of this study was to optimize lactic acid production by a native strain (Hui1) of *Lactobacillus plantarum* isolated from a Peruvian Amazon fruit (*Genipa americana*) in a medium supplemented with an agroindustrial by-product such as sugar cane molasses. Optimization was performed though one-factor-at-a-time studies followed by the Placket-Burman and central composite designs. The data were analyzed by using the Statistica® 10 software. Several carbon, nitrogen and ion sources were tested, and the optimum concentration of lactic acid achieved was 84.2 g L^{-1} in a medium containing as follows (in g L^{-1}): meat extract, 18.69; tryptone, 7.88; sugar cane molasses, 140; calcium carbonate, 15; dipotassium phosphate, 1; manganese phosphate, 0.03; sodium acetate, 5, and magnesium sulphate, 0.2. In addition, a high degree of conversion from sugar cane molasses to lactic acid was obtained ($Y_{p/s}$ 0.898 g g^{-1}). These results indicate the potential of *Lactobacillus plantarum* strain Hui1 to produce lactic acid in a medium supplemented with sugar cane molasses, an underutilized industrial by-product.

Key words: fermentation, response surface methodology, by-products, industry.

RESUMEN

El objetivo de este estudio fue optimizar la producción de ácido láctico por una cepa nativa (Hui1) de *Lactobacillus plantarum*, aislada de un fruto de la Amazonía peruana (*Genipa americana*), en un medio suplementado con un subproducto agroindustrial como la melaza de caña de azúcar. La optimización se realizó mediante el estudio de un factor a la vez seguido de los diseños de Placket-Burman y compuesto central. Los datos se analizaron utilizando el software Statistica® 10. Se evaluaron varias fuentes de carbono, nitrógeno y iones, alcanzándose una producción de ácido láctico de 84.2 g L^{-1} en un medio que contenía (en g L^{-1}): extracto de carne, 18.69; triptona, 7.88; melaza de caña de azúcar, 140; carbonato de calcio, 15; fosfato dipotásico, 1; fosfato de manganeso, 0.03; acetato de sodio, 5; y sulfato de magnesio, 0.2. Además, se obtuvo un alto grado de conversión de melaza de caña de azúcar a ácido láctico ($Y_{p/s}$ 0.898 g g^{-1}). Estos resultados indican el potencial de la cepa de *Lactobacillus plantarum* Hui1 para producir ácido láctico en un medio suplementado con melaza de caña de azúcar, un subproducto industrial subutilizado.

Palabras clave: fermentación, metodología de superficie de respuesta, subproductos, industria.

Introduction

Lactic acid (2-hydroxypropanoic acid) has multiple applications in the pharmaceutical, food, and chemistry industries as a food additive, pH regulator, and poly lactic acid precursor, among others (De Oliveira, Coelho, *et al*., 2018). In the last few years, the use of lactic acid has notably increased, and around 30% of the production is used for the development of biopolymers (Sengupta *et al*., 2020).

Lactic acid can be produced by chemical synthesis and microbial fermentation. The chemical pathway gives as a result an equal concentration of the racemic mixture (L and D stereoisomers) (De Oliveira, Coelho, *et al*., 2018). Fermentation, depending on the microorganism, results in the production of either one of the stereoisomers or in a variable composition of the mixture. Moreover, 90% of the lactic acid production worldwide is carried out by fermentation since it is more cost effective (De Lima *et al*., 2009; Coelho *et al*., 2011).

The genus *Lactobacillus* comprises a group of bacteria widely distributed in nature. This genus is characterized by its high tolerance to acidic pH values and its capacity to

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produce important concentrations of lactic acid, especially *Lactobacillus plantarum* (Hwang *et al.*, 2012; Behera *et al.*, 2018). However, *Lactobacillus plantarum* is an heterofermentative microorganism and requires a complex medium with several nutrients, including fermentable carbohydrates, vitamins, amino acids and minerals (Coelho *et al.*, 2011). During lactic acid production by this bacterium, the selection of carbon and nitrogen sources in the medium formulation is fundamental (Brinques *et al.*, 2010).

In recent years, lactic acid production using a number of industrial by-products from banana, corn, potato, wheat, etc., has been reported (Mufidah *et al.*, 2017). In this context, sugar cane molasses, a by-product from the sugar industry formed by fermentable sugars (sucrose, glucose and fructose, 50% w/v), nitrogen (0.5-0.9% w/v) and minerals, could be used as a substrate for the production of this compound (Coelho *et al.*, 2011). Sugar cane molasses is typically used as a low-cost animal food. However, it is also used to produce ethanol and has great potential in the production of chemicals (Dotaniya *et al.*, 2016). The production of this raw material in countries such as China is around 3 million t per year (Sun *et al.*, 2019), which makes it available for use in different industrial bioprocesses in a circular economy context.

The aim of this study was to optimize the lactic acid production by *Lactobacillus plantarum* strain Hui1 isolated from a Peruvian Amazon fruit (*Genipa americana*), in a medium supplemented with sugar cane molasses. Different components were tested, and optimization was achieved by the response surface methodology (RSM).

Materials and methods

Inoculum and culture preparation

Lactobacillus plantarum strain Hui1 was isolated from huito (*Genipa americana*), a fruit from the Peruvian Amazon. This strain was isolated and identified by our research group (Laboratorio de Biologia Molecular, Facultad de Farmacia y Bioquimica, Universidad Nacional Mayor de San Marcos) through partial sequencing of 16S rDNA (GenBank accession number: KY977384). The stock cultures were maintained in De Man, Rogosa and Sharpe (MRS) medium supplemented with 20% (v/v) glycerol at -20°C. The MRS medium composition was as follows (in g L^{-1}): glucose, 20; peptone, 10; yeast extract, 5; meat extract, 10; sodium acetate, 5; ammonium citrate, 2; dipotassium phosphate, 5; magnesium sulphate heptahydrate, 0.1, and manganese sulphate tetrahydrate, 0.05.

The pre-inoculum was grown in the MRS medium at 30°C for 24 h. Then, the inoculum was prepared by transferring 2 ml of the pre-inoculum into an Erlenmeyer flask containing 20 ml of MRS medium. The culture was incubated for 10 h at 37°C and then the cell concentration was determined by the correlation between the optical density (OD) and the cell density ($1x10^8$ colony-forming units (CFU) ml^{-1}) (McFarland curve). The cells were concentrated by centrifugation at 2000 *g* for 10 min, then washed twice with 50 mM phosphate buffer at pH 7.0, and finally re-suspended in 1 ml of the same buffer. The OD measurements were carried out at 620 nm. The inoculum cell concentration used in this work was 10^9 CFU ml^{-1}. All the fermentations were carried out in 250 ml flasks containing 50 ml of culture medium as the final volume.

Sugar cane molasses pre-treatment

Sugar cane molasses were obtained from the company Ajinomoto Peru S.A. Sugar cane molasses was heated at 70°C for 10 min in a water bath and filtered through Whatman filter paper of 11 µm. Subsequently, the concentration of total sugars was analyzed by the modified Anthrone method described by Rodríguez (1987). A concentration of 100 g of total sugars per liter of culture medium (100 g TS L^{-1}) was equivalent to 140 g L^{-1} of sugar cane molasses.

One-factor-at-a-time

The effect of the following 16 factors on the lactic acid production by *Lactobacillus plantarum* strain Hui1 was evaluated independently as follows: fructose, galactose, glucose, lactose, maltose, cellobiose, sucrose, Tween 80, sodium acetate, ammonium citrate, starch, and sugar cane molasses as carbon sources, as well as meat extract, yeast extract, casein tryptone, and bacteriological tryptone as nitrogen sources. This was done to decide the significant factors in lactic acid production. For these assays, 2.6 g L^{-1} of MRS medium was supplemented either with 12 g L^{-1} of each carbon source or with 4 g L^{-1} of each nitrogen source (Chauhan *et al.*, 2007). Additionally, the medium containing each nitrogen source was also supplemented with glucose (12 g L^{-1}). In the case of the media with carbon sources such as ammonium citrate and sodium acetate, they were also supplemented with glucose (12 g L^{-1}). After fermentation, the lactic acid concentration was determined by acid-base titration.

Effect of calcium carbonate

To determine the effect of calcium carbonate on the lactic acid production, 2.6 g L^{-1} of MRS medium was supplemented with 140 g L^{-1} of sugar cane molasses and 15 g L^{-1}

of calcium carbonate (Dumbrepatil *et al*., 2008; Pejin *et al*., 2015). The pH of the medium was adjusted to 6.5 with 1 M NaOH and the culture was incubated at 37°C for 5 d at 50 *g*. Samples were collected at 22, 46, 72, 94 and 118 h of fermentation. The concentration of lactic acid was determined using the modified ferric chloride method (Pacios *et al*., 2009).

Plackett-Burman design

The four significant factors from the one-factor-at-a-time study, as well as the effect of ions, including Mg^{2+}, Mn^{2+} and K^{1+} (magnesium sulphate, manganese sulphate and dipotassium phosphate) were analyzed through a Plackett-Burman design that was divided into two levels with two central points and a replicate (Tab. 1). Cultures were carried out in 25 ml flasks containing 20 ml of medium supplemented with 140 g L^{-1} of sugar cane molasses and 15 g L^{-1} of calcium carbonate (Chauhan *et al*., 2007; Coelho *et al*., 2011). The pH of the medium was adjusted to 6.5 and the cultures were incubated at 37°C for 5 d and 50 *g*. Data obtained were analyzed by using the Statistica® 10 software.

TABLE 1. Plackett-Burman design for lactic acid production by *Lactobacillus plantarum* strain Hui1.

Independent variables	Low level	Central level	High level
	g L^{-1}		
	-1	0	+1
Meat extract	1.0	5.5	10.0
Casein tryptone	1.0	5.5	10.0
Ammonium citrate	0.0	1.0	2.0
Sodium acetate	0.0	2.5	5.0
Magnesium sulphate	0.0	0.1	0.2
Manganese sulphate	0.0	0.025	0.05
Dipotassium phosphate	0.0	1.0	2.0

Central composite design

The two significant factors from the Placket-Burman design, the meat extract and the casein tryptone, were optimized by applying the central composite design (CCD) methodology at five levels, including five replicates for the central point (Tab. 2). A total of 13 experiments were carried out in 25 ml flasks containing 20 ml of medium with the following composition (in g L^{-1}): sugar cane molasses, 140; calcium carbonate, 15; dipotassium phosphate, 1; manganese sulphate, 0.03; sodium acetate, 5; and magnesium sulphate, 0.1. The medium was adjusted to a pH of 6.5, and the cultures were incubated at 37°C, for 5 d and 50 *g*. The model is represented by the quadratic Equation 1:

$$Y = \beta_0 + \Sigma\beta iXi + \Sigma\beta iiXi^2 + \Sigma\beta ijXiXj \quad (1)$$

where Y is the predicted lactic acid response in g L^{-1}, β_0 is the intercept, βii is the interaction coefficient, and the independent variables correspond to Xi and X_iX_j. According to Table 2, X_i corresponds to X_1 (meat extract in g L^{-1}) and X_j corresponds to X_2 (tryptone in g L^{-1}).

TABLE 2. Range and levels of the variables used in the central composite design.

Variable (g L^{-1})	Code	Range and levels				
		- 1.414*	-1	0	1	1.414*
Meat extract	X_1	12.34	14	18	22	23.66
Casein tryptone	X_2	5.38	6	7.5	9	9.62

*The values ±1.414 correspond to α obtained from √2.

The analysis of variance (ANOVA) was carried out and the values of the F-test, correlation coefficient (R), coefficient of determination (R^2), t-student and the lack-of-fit were determined. The results were obtained with a 95% confidence level (P<0.05). Then, three-dimensional response surface curves were plotted to assess the interaction among components. Finally, the optimum values of the medium components determined by RSM were tested experimentally, to validate the optimization methodology. Statistical analysis was performed using the Statistica® 10 software.

Analytical methods

Quantitative determination of lactic acid

After each fermentation of the media resulted from the one-factor-at-a-time, the Plackett-Burman, and central composite designs, the culture media were centrifuged at 4000 *g* for 5 min, recovering the supernatant liquid and discarding the pellet. The supernatants were analyzed by either of the two methods used for the quantitative determination of lactic acid. The first was the AOAC 947.05 method (AOAC, 2019). This was carried out as follows: one drop of phenolphthalein was added to 10 ml of the supernatant and then the mixture was titrated with 0.1 N NaOH. The lactic acid was calculated and expressed as g of lactic acid L^{-1} of culture. The second method was a colorimetric assay using ferric chloride (Pacios *et al*., 2009). In this method, 1.5% (w/v) calcium carbonate was added to the supernatant, and the mixture was heated to 50°C for 5 min. Then, this suspension was centrifuged at 2800 *g* for 10 min, and 1 ml of 61.6 mM ferric chloride was added to 1 ml of the recovered supernatant. The ferric lactate concentration was determined by spectrophotometry at 440 nm.

Biomass calculations

The biomass concentration was determined by turbidimetry diluting 0.5 ml of culture in 50 mM phosphate buffer at pH 7.0. The absorbance was measured at 620 nm and the cell concentration was determined by comparison with the OD and the cell density ($1x10^8$ CFU ml^{-1}) (McFarland curve).

Total sugars calculations

The total sugar concentration was determined using the modified Anthrone method (Rodríguez, 1987). The samples obtained from the fermentation were previously centrifuged at 4000 *g* for 5 min, and 500 ml of the supernatant were collected and placed in 2 ml microtubes on ice. Then, 1 ml of the Anthrone reagent was added. The mixture was subsequently homogenized and placed on ice; then, the tubes were transferred to a boiling water bath for 5 min. Afterwards, the tubes were placed on ice for 1 min. The measurement was carried out at a wavelength of 640 nm.

Determination of $Y_{p/s}$

The $Y_{p/s}$ is the yield of substrate in product, considering that P_0 (initial lactic acid in g L^{-1}), S_0 (initial total sugars in g L^{-1}), P_f (final lactic acid in g L^{-1}) and S_f (final total sugars in g L^{-1}) are the initial and final concentrations of product and substrate of the fermentation. It is the ratio of the lactic acid produced by consuming the total sugars present in the cane molasses in the fermentation medium (Eq. 2).

$$Y_{p/s} = \frac{P_f - P_o}{S_o - S_f} = \frac{Lactic\ acid\ produced\ (g)}{Total\ sugars\ consumed\ (g)} \quad (2)$$

Results and discussion

One-factor-at-a-time

This study was carried out to identify the carbon and nitrogen sources with a significant effect on the lactic acid production by the *Lactobacillus plantarum* strain Hui1. Figure 1 shows that all carbon sources were fermented except for starch. From these results, it can be observed that sugarcane molasses enhanced the lactic acid production 4.8-fold compared to the control (MRS medium). Molasses are low-cost agro-industrial by-products whose composition includes 40-60% sugars (approximately 11% reducing sugars, 34% sucrose), proteins, vitamins, and minerals (Sindhu *et al.*, 2016). The high sugar content, combined with their low market price, make them eligible to be used instead of synthetic sugars (glucose, fructose, and others). However, the composition of molasses will depend on different factors such as the variety of sugarcane, climate, and the process employed for sugar extraction (Sindhu *et al.*, 2016). The fermentation by *Lactobacillus plantarum* with molasses from different places (Komesu *et al.*, 2017), and sugarcane bagasse (Lino *et al.*, 2018), has a high potential in the production of lactic acid.

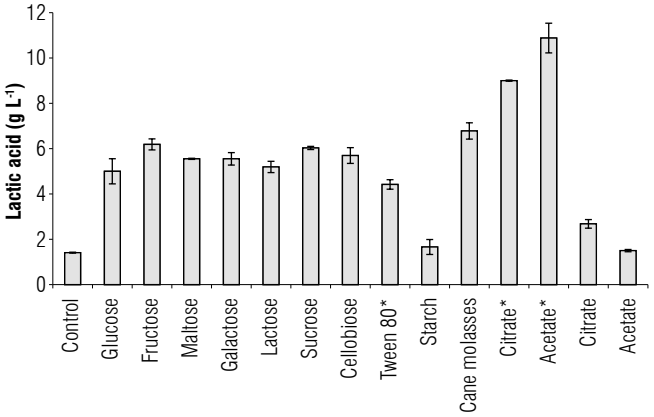

FIGURE 1. Effect of carbon sources on the lactic acid production by *Lactobacillus Plantarum* strain Hui1. *Supplemented with 12 g L^{-1} of glucose. Error bars represent the standard deviation from the mean (n = 3).

Figure 2 shows the effect of nitrogen sources on the production of lactic acid by the *Lactobacillus plantarum* strain Hui1. The greatest production of lactic acid was obtained with meat extract and tryptone; for this reason, they were selected for the Plackett-Burman design (Fig. 2). The nitrogen source plays a fundamental role on the growth of *Lactobacillus* strains. Several *Lactobacillus* strains are unable to synthesize some amino acids and, therefore, require a medium supplemented with them (Papizadeh *et al.*, 2020). Nitrogen from animal or bacterial origin represents an important source of these amino acids, low molecular weight peptides, and growth factors that protein hydrolysates provide. Safari *et al.* (2012) showed that *Lactobacillus plantarum* PTCC1058 had good growth in a medium containing peptones from the hydrolyzed wastes compared with the MRS control medium. They also indicated that *Lactobacillus plantarum* requires amino acids such as arginine, isoleucine, tyrosine, valine, and pantothenic acid for optimal growth. According to Solval *et al.* (2019), growth and the amount of lactic acid produced by *Lactobacillus plantarum* NRRL B-4496 in MRS medium without a nitrogen source (0.22 h^{-1}; 3.13 h and 1.73%) were lower than in the MRS control medium (0.48 h^{-1}; 1.44 h and 0.60%); thus, the absence of a nitrogen source affects the maximum growth rate, doubling time and lactic acid production.

The meat extract shows a higher concentration of vitamins, salts and approximately 57.83% of proteins in its

composition. On the other hand, tryptone contains a variety of essential amino acids with 13% of total nitrogen content in its composition. High nitrogen content allows a better growth of bacteria and, as a result, a greater production of lactic acid (Abedi & Hashemi, 2020).

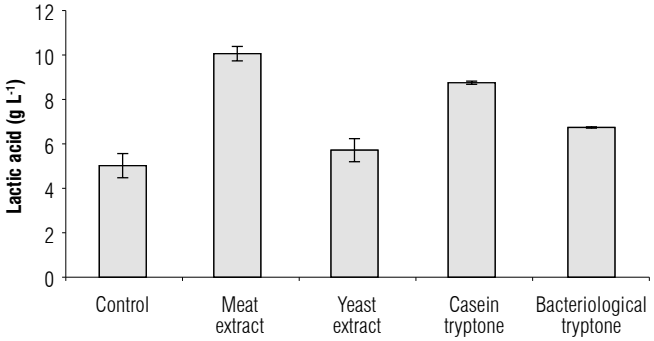

FIGURE 2. Effect of nitrogen sources on the lactic acid production by *Lactobacillus plantarum* strain Hui1. Error bars represent the standard deviation from the mean (n = 3).

Effect of calcium carbonate on lactic acid production

Figure 3 shows the effect of calcium carbonate on lactic acid production in the MRS medium supplemented with 140 g L^{-1} of sugar cane molasses. The maximum concentration of lactic acid was achieved at 72 h. Lactic acid concentration was remarkably higher in the presence of calcium carbonate than in the absence of this compound (3.5-fold). This could be explained due to the calcium carbonate regulating the pH of the culture during the fermentation process as it is considered a neutralizing agent (Yang *et al.*, 2015). According to Pejin *et al.* (2015), the *Lactobacillus* cells have a high viability due to the effect of calcium carbonate at the end of fermentation, and allow an increase in the lactic acid yield. Thus, Cubas-Cano *et al.* (2019) reported a lactic acid yield of 94% in a controlled pH fermentation, and a yield of 41% when pH was not controlled in the process.

Lactobacillus plantarum strains can function at low pH conditions (Iorizzo *et al.*, 2016); however, the acidification of the medium from a high accumulation of lactic acid causes an inhibition by-product that is not suitable for lactic acid overproduction (Singhvi *et al.*, 2018; Mousavi & Mousavi, 2019); also, the *Lactobacillus* growth is hampered by maintaining the transmembrane pH gradient (Othman *et al.*, 2017).

For these reasons, the use of calcium carbonate is necessary and, compared to other neutralizing agents (ammonia, sodium hydroxide, calcium hydroxide and potassium hydroxide), is a great alternative since it does not generate calcium sulphate as a by-product or cause cell toxicity by ammonia (Yang *et al.*, 2015).

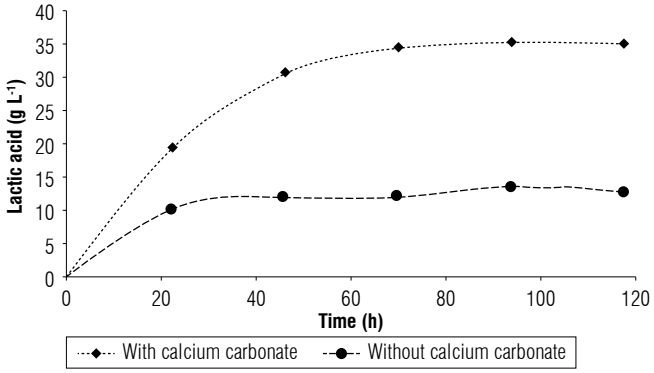

FIGURE 3. Effect of calcium carbonate on the lactic acid production from *Lactobacillus plantarum* Hui1.

The Plackett- Burman design

In this experiment, the concentrations of sugar cane molasses and calcium carbonate were kept constant at 140 and 15 g L^{-1}, respectively. Seven factors selected from the one-factor-a-time assays were tested, including meat extract and casein tryptone as nitrogen sources, ammonium citrate and sodium acetate as carbon sources, and Mg^{2+} (magnesium sulphate), Mn^{2+} (manganese sulphate) and K^{1+} (dipotassium phosphate) as ions. According to the ANOVA and the Pareto chart from the Plackett-Burman design (Tab. 3 and Fig. 4, respectively), the meat extract, casein tryptone, dipotassium phosphate, and manganese sulphate significantly influenced the lactic acid production (P<0.05). Both the meat extract and casein tryptone were important components in the culture medium due to their high concentration of peptides, amino acids, and vitamins. Therefore, as sugar cane molasses has a low organic nitrogen content, the concentrations of meat extract and casein tryptone used can be increased to improve lactic acid yields and the growth of *Lactobacillus plantarum* in a medium containing sugar cane molasses (Lino *et al.*, 2018; Papizadeh *et al.*, 2020).

TABLE 3. Analysis of variance (ANOVA) of the Plackett-Burman design for the lactic acid production by the *Lactobacillus plantarum* strain Hui1.

Factor	Sum of squares	Degrees of freedom	Square mean	F-value	*P*-value
Meat extract	816.41	1	816.41	134.15	0.0000[s]
Casein tryptone	179.92	1	179.92	29.56	0.0003[s]
Ammonium citrate	0.10	1	0.097	0.02	0.9019
Sodium acetate	24.46	1	24.46	4.02	0.0728
Magnesium sulphate	4.24	1	4.24	0.69	0.4233
Manganese sulphate	171.72	1	171.72	28.22	0.0003[s]
Dipotassium phosphate	142.95	1	142.95	23.49	0.0007[s]
Error	60.86	10	6.09		
Total sum of squares	1400.66	17			

Significance level: P<0.05; R^2: 0.956; R^2 (adjusted): 0.926, and s: significant at P<0.05.

Manganese sulphate showed a positive effect on the lactic acid production due to Mn^{2+} being a cofactor of several *Lactobacillus* enzymes such as the RNA polymerase, lactate dehydrogenase (LDH), NADH oxidase and superoxide dismutase that allows improving the efficiency and productivity of the lactic fermentation (Cheng *et al*., 2014). Cheng *et al*. (2014) demonstrate the effect of the Mn^{+2} on the stimulation of LDH by directing the conversion of pyruvic acid to lactic acid, and they show that the production of lactic acid decreases in the absence of Mn^{2+}. However, although *Lactobacillus plantarum* CCFM436 tolerates high concentrations of Mn^{2+}, its growth is affected (Tong *et al*., 2017). For that reason, it is necessary to adjust the Mn^{2+} concentration to prevent a negative effect on the lactic acid production. Although dipotassium phosphate has a negative effect on the lactic acid production, it is kept in the culture since KPO_4^{2-} is an inorganic salt important for the metabolism of *Lactobacillus plantarum* as it interacts with metal ions (Mn^{2+} and Mg^{2+}), thus improving the tolerance of bacteria to oxidative stress (Correa Deza *et al*., 2017; Alcántara *et al*., 2018). Therefore, the manganese sulphate and dipotassium phosphate concentrations were kept constant in the medium (0.03 and 1 g L^{-1}, respectively).

Regarding Mg^{2+}, the concentration of magnesium sulphate was kept constant at 0.2 g L^{-1} in the medium. Lew *et al*. (2012) indicate that the interaction between Mn^{2+} and Mg^{2+} is related to the obtention of good production of organic acids in *Lactobacillus*. In another study, Lew *et al*. (2013) indicate that both factors (Mn^{2+} and Mg^{2+}) exert a synergistic effect to achieve a high production of lactic acid; therefore, despite the fact that in our case magnesium was not significant, it was kept at a constant value in the following experiments.

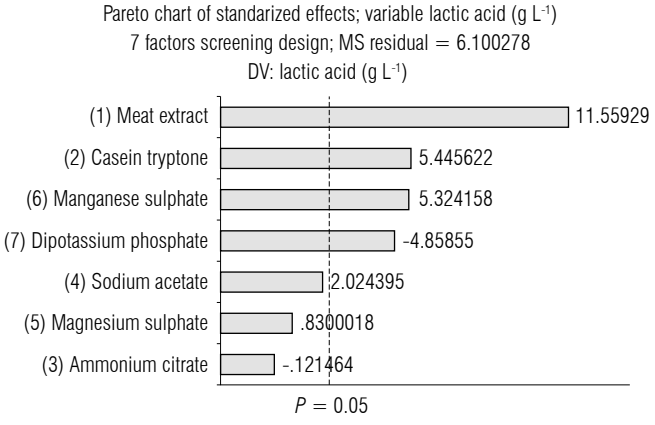

FIGURE 4. Pareto chart of the Placket-Burman design for the lactic acid production by *Lactobacillus plantarum* Hui1. MS residual - residual mean square and DV - dependent variable.

Central composite design

From the Placket-Burman design, the meat extract and casein tryptone were selected as the key factors for further optimization in lactic acid production by *Lactobacillus plantarum* Hui1. The ANOVA of the CCD (Tab. 4) shows that the lack of fit value was 2.94 and the *P*-value was not significant (0.07). According to this, the model fitted the data well, indicating that it can describe the influence of the factors on the response to predict lactic acid production.

TABLE 4. Analysis of variance (ANOVA) of the central composite design (CCD) for lactic acid production by the *Lactobacillus plantarum* strain Hui1.

Factor	Sum of squares	Degrees of freedom	Square mean	F-value	*P*-value
(1) Meat extract (L)	14.32	1	14.32	27.04	0.0001[s]
(2) Meat extract (Q)	106.36	1	106.36	200.81	0.0000[s]
(3) Casein tryptone (L)	6.74	1	6.74	12.73	0.0091[s]
(4) Casein tryptone (Q)	23.45	1	23.45	44.23	0.0002[s]
1L by 2L	0.02	1	0.02	0.03	0.8633
Residual	3.71	7	0.53		
Lack of fit	2.94	3	0.98	5.11	0.0745
Error	0.77	4	0.19		
Total sum of squares	143.59	12			

Significance level: $P<0.05$; $R^2 = 0.97$; R^2 (adjusted) = 0.95; L - linear; Q - quadratic; 1L by 2L: interaction between the two linear variables, and s: significant at $P<0.05$.

Equation 3 defines the fitted model, with the regression coefficients shown in Table 5. This quadratic model contains two linear terms, two quadratic terms and one factorial interaction.

$$Y = -48.0079 + 9.0512X_1 - 0.2444X_1^2 + 12.6581X_2 - 0.8161X_2^2 + 0.0108\,X_1\,X_2 \quad (3)$$

where Y is the predicted response (lactic acid concentration), and X_1 (meat extract) and X_2 (casein tryptone) are natural variables.

The meat extract and the casein tryptone had a significant effect ($P<0.05$) on the lactic acid production as well as positive coefficients (Tab. 5). Thus, an increase in the concentration of these compounds will result in an increase in lactic acid production. The interaction between the squared variables X_1^2 and X_2^2 was also significant ($P<0.05$), but their coefficients were negative, suggesting that at high concentrations of these factors (in relation to the quadratic form (Q) of the factors), obtaining lactic acid could be impaired. The X_1X_2 interaction was not significant ($P>0.05$). Additionally, the R^2 indicated that 97.42% of the variance could be explained by the quadratic model.

TABLE 5. Regression coefficients of the central composite design (CCD) for lactic acid production by the *Lactobacillus plantarum* strain Hui1.

Factor	Regression coefficients	Standard error	t (7)	*P*-value	-95% Confidence level	+95% Confidence level
Mean/Intercept	-48.0079	12.41799	-3.8660	0.0061^{s}	-77.3718	-18.6440
(1) Meat extract (L)	9.0512	0.77233	11.7194	0.0000^{s}	7.2249	10.8774
(2) Meat extract (Q)	-0.2444	0.01725	-14.1708	0.0000$_{s}$	-0.2852	-0.2036
(3) Casein tryptone (L)	12.6581	2.14595	5.8986	0.0006^{s}	7.5837	17.7324
(4) Casein tryptone (Q)	-0.8161	0.12264	-6.6545	0.0003^{s}	-1.1061	-0.5261
1L by 2L	0.0108	0.06065	0.1786	0.8633	-0.1326	0.1542

Significance level: $P<0.05$; L - linear; Q - quadratic; 1L by 2L: interaction between the two linear variables, and s: significant at $P<0.05$.

The graph of the response surface model (Fig. 5) showed that concentrations around 18 g L^{-1} of meat extract and 8 g L^{-1} of casein tryptone produced 86.23 g L^{-1} of lactic acid. Concentrations higher than 22 g L^{-1} of meat extract and 10 g L^{-1} of casein tryptone showed a reduction in the lactic acid production, demonstrating that nitrogen concentrations higher than 20 g L^{-1} were toxic for this *Lactobacillus plantarum* strain.

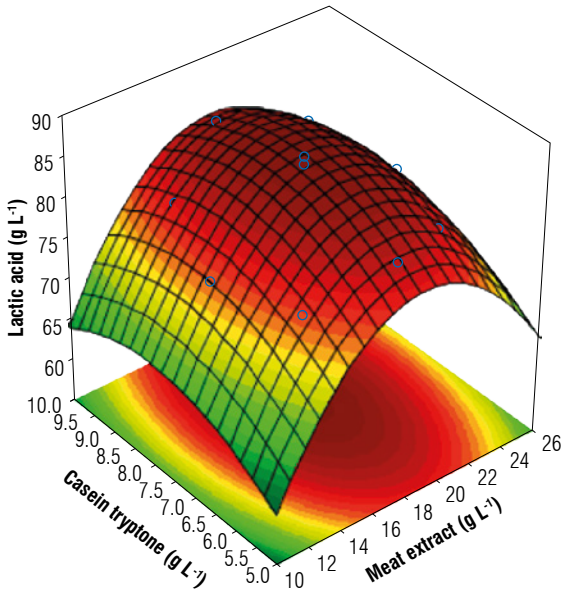

FIGURE 5. 3D response surface of the central composite design (CCD) showing the effects of the meat extract and casein tryptone on the lactic acid production by the *Lactobacillus plantarum* strain Hui1. MS pure error - mean square pure error and DV - dependent variable.

The maximum lactic acid production determined by CCD (86.44 g L^{-1}) was achieved using a medium containing 18.69 g L^{-1} of meat extract, 7.88 g L^{-1} of casein tryptone, 140 g L^{-1} of sugar cane molasses, 15 g L^{-1} of calcium carbonate, 1 g L^{-1} of dipotassium phosphate, 0.03 g L^{-1} of manganese sulphate, 5 g L^{-1} of sodium acetate and 0.2 g L^{-1} of magnesium sulphate. These results were tested and a lactic acid production of 84.2 g L^{-1} was obtained, demonstrating that the model was predictable.

Previous studies on the optimization of lactic acid production by different microorganisms are known (De Lima *et al.,* 2009, 41.42 g L^{-1}; Tripathi *et al.,* 2015, 39.2 g L^{-1}; Mufidah *et al.,* 2017, 28.01 g L^{-1}). In these studies, different industrial by-products were used, but the concentration of the lactic acid obtained was considerably lower than that in this study.

Other studies with *Lactobacillus plantarum* strains have shown that the nitrogen source improves the production of lactic acid, such is the case of Solval *et al.* (2019). They showed improved lactic acid production by *Lactobacillus plantarum* NRRL B-4496 in media containing high free amino acids and protein. Coghetto *et al.* (2016) obtained 21 g L^{-1} of lactic acid by *Lactobacillus plantarum* BL011 growing in soy bean acid residue (20 g L^{-1}), peptone (2 g L^{-1}), corn steep liquor (5 g L^{-1}), and yeast extract (15 g L^{-1}). These studies show the necessity for the fermentation medium to have a carbon source and a nitrogen source. Unban *et al.* (2019) found that gelatinized starchy waste and corn steep liquor were the two nutrients that significantly influence lactic acid production by *Lactobacillus plantarum* S21. Hence, corn steep liquor, a nitrogen source, could replace the meat extract and tryptone casein since these are considerably more expensive nitrogen sources in fermentation processes.

Some authors have worked using derived low-cost sugar media, such as Sikder *et al.* (2014), who reported a production of 89.5 g L^{-1} of lactic acid and $Y_{p/s}$ of 0.83 g g^{-1} by *Lactobacillus plantarum* NCIM 2912 starting with sugarcane juice (140 g L^{-1}) and yeast extract. This value is similar to that obtained in this study. Mousavi and Mousavi (2019) showed that lactic acid was the most significant product at the end of fermentation by *Lactobacillus plantarum* DSMZ 20174 using high fructose corn syrup. Zhang and Vadlani (2015) obtained a $Y_{p/s}$ of 0.87 g g^{-1} and 25 g L^{-1} of lactic acid by *Lactobacillus plantarum* ATCC 21028 from poplar hydrolysate (biomass-derived sugars).

In this case, molasses have been used as an efficient carbon source for the production of metabolites of industrial interest such as lactic acid (Papizadeh *et al*., 2020), with lower cost compared to sugarcane juice, since molasses is an industrial waste. Furthermore, as in the study of Coelho *et al.* (2011), supplementation with nitrogen sources was considered. These authors carried out the optimization of the medium composition in lactic acid production by *Lactobacillus plantarum* LMISM6 grown in molasses. A maximum lactic acid production of 94.8 g L^{-1} was obtained in a medium containing molasses, corn steep liquor, dipotassium phosphate and Tween 80 at concentrations of 193.50 g L^{-1}, 37.50 ml L^{-1}, 2.65 g L^{-1}, and 0.83 ml L^{-1}, respectively. Srivastava *et al.* (2014) showed a strong interaction between molasses and amino acids. High concentrations of nitrogen (>40 g L^{-1}) had a negative effect, while an increase in the concentration of cane molasses improved lactic acid production.

The $Y_{p/s}$ obtained at the end of the fermentation was 0.89 g g^{-1} and indicated that sugar cane molasses was a suitable substrate for lactic acid production by *Lactobacillus plantarum* strain Hui1. The production of lactic acid obtained in this study was similar to that achieved by De Oliveira, Rossell, *et al.* (2018) who obtained a $Y_{p/s}$ of 0.93 g g^{-1} by *Lactobacillus plantarum* CCT 3751 from hemicellulosic liquor from sugarcane bagasse, yeast extract (20 g L^{-1}), and sodium acetate (5 g L^{-1}).

Conclusions

The optimization of lactic acid production by *Lactobacillus plantarum* strain Hui1 was achieved by RSM obtaining a yield of 84.2 g L^{-1}. The results showed that *Lactobacillus plantarum* strain Hui1 has the potential to be used for the industrial production of lactic acid. In addition, this research demonstrated the feasibility of using an agro-industrial waste such as sugar cane molasses as a promising carbon source in the culture medium for lactic acid production. Finally, it is important to highlight that the replacement of synthetic sources with by-products in a culture medium contributes to reduce production costs. This work provides insights for continuing the search for low-cost sources for the medium culture formulation, mainly nitrogen sources.

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Author's contributions

SS conducted the research and investigation process, applied the statistical techniques to analyze the study data and developed the methodology. LAP formulated the overarching research goals and aims, conducted the research and investigation process, developed the methodology and verified the overall replication/reproducibility of results/experiments. JCFS conducted the research and investigation process, applied the statistical techniques to analyze the study data, and developed the methodology. CNFF formulated the overarching research goals and aims, conducted the research and investigation process, wrote the original draft, and carried out the critical review, commentary, or revision of the manuscript. HAG wrote the original draft and carried out the critical review, commentary, and revision of the manuscript. AIZP obtained the financial support for the project leading to this publication, managed and coordinated the research activity planning and execution, wrote the original draft, and carried out the critical review, commentary, and revision of the manuscript.

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SCIENTIFIC NOTE

The age of tomato plants affects the development of *Macrosiphum euphorbiae* (Thomas, 1878) (Hemiptera) colonies

La edad de las plantas de tomate afecta el desarrollo de las colonias de *Macrosiphum euphorbiae* (Thomas, 1878) (Hemiptera)

Vincenzo Trotta[1], Irene Toma[2], Pierluigi Forlano[1], Paolo Fanti[3], Juliana Durán Prieto[4*], and Donatella Battaglia[1]

ABSTRACT

We tested the hypothesis that the intensity and duration of *Macrosiphum euphorbiae* infestations in tomato depend on both the age (phenological stage) of the host plant and the initial number of aphids present in the colony. We compared the effects of three initial levels of infestation and two phenological stages of the plant (pre-flowering and flowering stages) on infestation curves. The position of the infestation peak over time was significantly affected by the plant phenological phase. Populations of *M. euphorbiae* reached the highest peak of abundance on plants infested at the pre-flowering stage compared to those subsequently infested. Within a phenological phase, the maximum abundance also varied according to the initial aphid density on the plant. The implications concerning the management of the pest in the field are briefly discussed.

Key words: aphids, curve of infestation, plant phenological stages, *Solanum lycopersicum*, trophic interactions.

RESUMEN

Se planteó la hipótesis que la intensidad y duración de las infestaciones de *Macrosiphum euphorbiae* en el tomate dependen simultáneamente de la edad (etapa fenológica) de la planta hospedera y del número inicial de áfidos de la colonia. Se compararon los efectos de tres niveles iniciales de infestación y dos fases fenológicas de la planta (fases de pre-floración y floración) sobre las curvas de infestación. La posición del pico de infestación a lo largo del tiempo estuvo afectada significativamente por la fase fenológica de la planta. Las poblaciones de *M. euphorbiae* alcanzaron su máximo pico de abundancia en las plantas infestadas en la fase de pre-floración comparadas con las plantas infestadas en fases posteriores. Dentro de la fase fenológica, la máxima abundancia varió también de acuerdo con la densidad inicial de áfidos en la planta. Las implicaciones concernientes al manejo de la plaga en campo se discuten brevemente.

Palabras clave: áfidos, curva de infestación, estados fenológicos de la planta, *Solanum lycopersicum,* interacciones tróficas.

Introduction

Aphids are r-strategist (Gadgil & Solbrig, 1972) insects characterized by their capacity for extremely rapid population growth together with a transient relationship with the host plant (Powell *et al.,* 2006). Aphid colonies typically decline after an initial period of rapid growth. This last phase is largely due to the production of winged morphs and is stimulated by crowding, the presence of natural enemies, and the decrease in plant quality (Müller *et al*., 2001; Irwin *et al*., 2007). The curve of infestation for the same aphid species can be dramatically affected by the host plant despite keeping all the other factors equal (Larocca *et al*., 2011).

Macrosiphum euphorbiae (Thomas, 1878) (Hemiptera: Aphididae) is an important aphid pest that causes the most significant direct damage among the aphid species that attack tomato (*Solanum lycopersicum* L. 1753) (Perring *et al*., 2018). In a previous study concerning the development of *M. euphorbiae* colonies on tomato plants, we observed that cultivars and water stress affect the peak but do not interfere with the length of the infestation (Rivelli *et al*., 2013). This result was confirmed by field observations (Colella *et al*., 2014).

Several models of aphid population dynamics, which exclude predator inflicted mortality as a regulating factor, identify the initial number of aphids as the main

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determinant of the width of the density curve and/or size and the timing of the peak of maximum density (Kindlmann *et al.*, 2007). In addition, some of these models take into account variations in the "aphid-carrying capacity" due to changes in host plant quality over time. Plant quality may largely depend on its phenology, which modifies the physiological priorities for resource allocation (Boege & Marquis, 2005).

In this study, we hypothesized that the initial number of aphids and/or the age of the plant are significant factors that can influence the duration and, consequently, the harmfulness of *M. euphorbiae* infestations in tomato.

Materials and methods

Tomato plants of the cultivar Rio Grande (pear-shaped processing tomato for paste and concentrated juice) were used for both *M. euphorbiae* rearing and the experiment. We bought tomato plants in polystyrene trays from a nursery that used seeds produced by OLTER (Piacenza, Italy). Seedlings were transplanted and grown individually in plastic pots (18 cm diameter, 19 cm height) with commercial soil (COMPO SANA® universal potting soil, International Kingenta Group, Italy).

Macrosiphum euphorbiae individuals were originally collected in a tomato crop in Scafati (Salerno, Italy) and reared on tomato plants at 20 ± 1°C, 65 ± 5% relative humidity, and a photoperiod of 18 h light:6 h dark.

The experiment was carried out at the University of Basilicata, Italy (40°36' N, 15°48' E), in the summertime, in a naturally lit and temperature-controlled greenhouse maintained at 20°C (with an oscillation between 15°C at night and 28°C at 12-14 pm). We compared three initial levels of infestation and two phenological phases of the plant. Nine plants were infested 7 d after transplant (pre-flowering stage; 18.0 ± 2.6 cm height; root biomass 0.10 ± 0.04 g; leaf biomass 0.43 ± 0.08 g) and 9 plants were infested 26 d after transplant (beginning of flowering; 39.5 ± 4.6 cm height; root biomass 1.5 ± 0.3 g; leaf biomass 6.4 ± 3.0 g). Three levels of initial infestation were obtained by placing 6, 10, and 15 *M. euphorbiae* adults (three replicates for each level of initial infestation: R1, R2, and R3), respectively, on plants. We selected this range of initial infestation to simulate a variable but incipient attack of the pest. We used apterous females for simplicity, even if it is more plausible that the infestation starts with winged females under natural conditions.

All plants were infested on the same day. The plants in the flower transition stage had been transplanted 19 d before the plants in pre-flowering. Plants were placed in the greenhouse in a completely randomized experimental design and regularly checked. The number of aphids in the whole plant was counted using a magnifying glass.

The infestation curves for each plant stage and each level of initial infestation were adjusted to second degree polynomials passing through the origin according to a "cumulative density model" (Kindlmann *et al.*, 2007). In the present study, we used the polynomial parameters to calculate the axes of the vertex of the second-degree polynomials: the theoretical values of maximum abundance of aphids (the Y axis) and of the day needed to reach the maximum abundance (the X axis). The theoretical values of the maximum number of aphids and of the days needed to reach it were then analyzed with factorial ANOVAs, with "stage" (7 and 26 d after transplant, i.e., pre-flowering and flowering stages) and the initial level of infestation as fixed effects. The same analyses were also performed on the observed values of the maximum number of aphids and of the days needed to reach it. All the analyses in this study were performed using the R 3.2.3 software (R Core Team, 2014).

Results and discussion

The response curves for the three replicates of the pre-flowering and flowering stages are shown separately for each level of initial infestation in Figure 1. In all cases, values of adjusted R^2 are significant and the adjustments to second degree polynomial provide plausible values for the vertex. Positions of the theoretical and observed vertices of all the curves (mean values of three replicates) with the respective standard errors are shown in Figure 2. The mean value of the peak and time when the peak occurred for each plant stage are shown in Tables 1 (theoretical values) and 2 (observed values).

Aphid population reached a much higher peak on plants infested after 7 d from transplant (pre-flowering) compared to those subsequently infested (flowering) (theoretical values: $F_{1,12} = 48.18$, $P<0.001$; observed values: $F_{1,12} = 83.28$, $P<0.001$). Significant differences in the maximum abundance were also detected for the level of infestation (theoretical values: $F_{2,12} = 4.45$, $P<0.05$; observed values: $F_{2,12} = 5.49$, $P<0.05$) but not for the interaction between the phenological stage and the level of infestation. These differences are mainly due to the peak reached on plants initially infested with 15 aphids on the 7th d (Figs. 1C and 2). The vertices of the other curves are all very close.

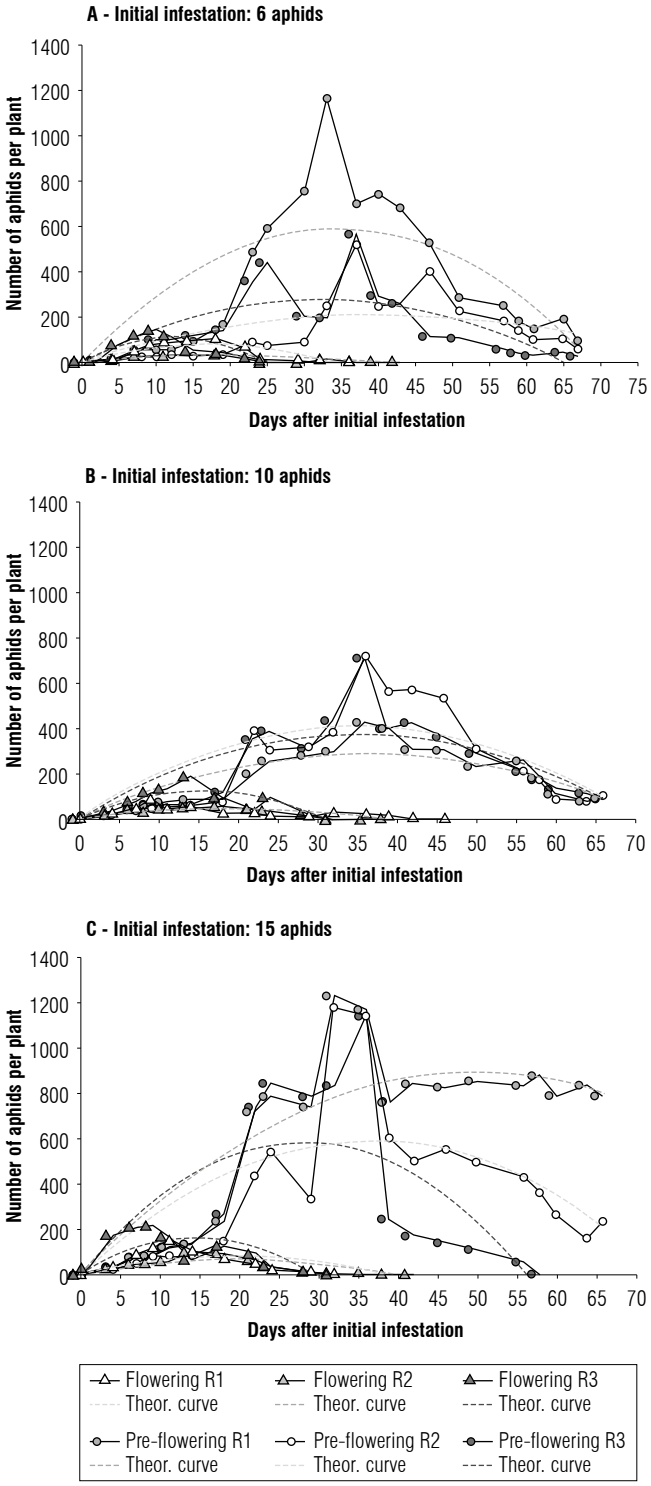

FIGURE 1. Curves of *Macrosiphum euphorbiae* infestation estimated for plants initially infested at the pre-flowering stage or at the beginning of flowering. A) Initial infestation: 6 aphids; B) initial infestation: 10 aphids; C) initial infestation: 15 aphids. Continuous lines connect the experimental points; dotted lines (theor.) represent the theoretical adjustments to second degree polynomial; R1, R2 and R3 are the three replicates for each level of initial infestation and stage.

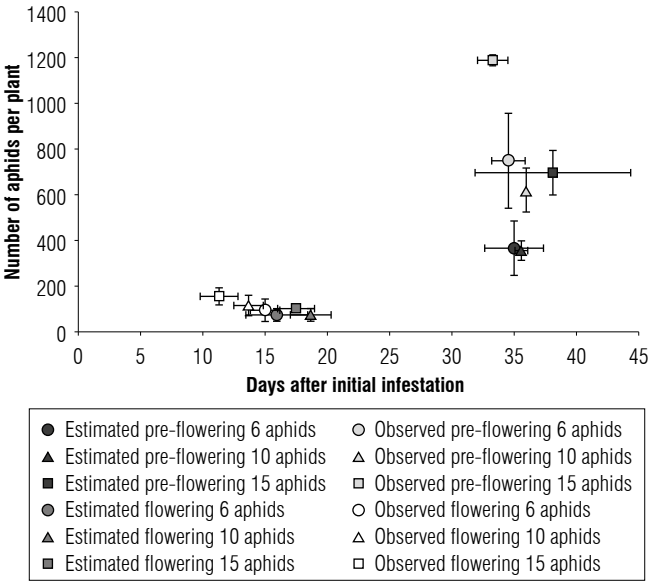

FIGURE 2. Maximum abundance of *Macrosiphum euphorbiae* colonies according to the initial infestation level (6, 10, or 15 aphids per plant) and the stage of the attacked plant (pre-flowering and flowering stages).

TABLE 1. Theoretical maximum abundance and time of maximum abundance according to the plant stage at the beginning of infestation and the initial number of aphids (mean ± standard error).

Plant stage at the beginning of infestation	Initial number of aphids	Time of maximum abundance (d)	Maximum abundance
Pre-flowering	6	35.05 ± 2.4	364.3 ± 120
Pre-flowering	10	35.63 ± 0.5	357.7 ± 36
Pre-flowering	15	38.16 ± 6.3	689.4 ± 102
Flowering	6	15.93 ± 2.4	71.7 ± 25
Flowering	10	18.65 ± 1.7	73.4 ± 26
Flowering	15	17.51 ± 1.4	99.0 ± 30

TABLE 2. Observed maximum abundance and time of maximum abundance according to the plant stage at the beginning of the infestation and the initial number of aphids (mean ± standard error).

Plant stage at the beginning of infestation	Initial number of aphids	Time of maximum abundance (d)	Maximum abundance
Pre-flowering	6	34.67 ± 1.3	745.3 ± 209
Pre-flowering	10	36.00 ± 0.0	617.7 ± 95
Pre-flowering	15	33.33 ± 1.3	1186.3 ± 26
Flowering	6	15.00 ± 3.0	94.3 ± 35
Flowering	10	13.67 ± 2.6	116.0 ± 39
Flowering	15	11.33 ± 1.5	154.0 ± 35

The position of the peaks of infestation over time (the day after infestation when the aphid population reached its maximum abundance) was significantly affected only by the plant stage at the start of infestation (theoretical values: $F_{1,12} = 57.35$, $P<0.001$; observed values: $F_{1,12} = 191$, $P<0.001$).

Our results show that the duration of *M. euphorbiae* infestation in tomato mainly depends on the phenological stage of the host plant (it is higher and persists longer on early infested plants), while the degree of infestation seems to be irrelevant considering the initial levels used in this experiment.

Plant defense significantly changes with plant development from the seedling to juvenile to mature and senescent stages (Barton & Boege, 2017). Ontogenetic changes in plant defenses observed in many species do not only concern the intensity of the defensive response but also the mechanisms involved. This can be partially explained by the fact that allocation costs and resource allocation priorities vary as plants develop (Boege & Marquis, 2005).

Two important changes that can impact the development of insect infestations occur during the flower transition: the negative regulation of herbivory-induced jasmonic acid (JA) signaling (Gaquerel & Stitz, 2017) and the increase of the C:N ratio in the phloem sap (Corbesier *et al*., 2002). The repression of the JA signaling-based induction explains why the ability of tomato plants to induce defenses against *Manduca sexta* (Linnaeus, 1763) is lost when the reproductive stage is reached (Wolfson & Murdock,1990). The down-regulation of the JA signaling pathway mainly favors chewing insects. On the other hand, the increase of the C:N ratio in the phloem sap is unfavorable for aphids since amino acid availability significantly affects their growth and reproduction (Ponder *et al*., 2000; Karley *et al*., 2002). In the case of aphids, the nutritional quality of the host plant can have an even higher impact than that of the induced defenses (Battaglia *et al*., 2013).

In addition to the nutritional aspects, we must consider that plants have evolved multiple defense strategies against aphids, including constitutive as well as inducible factors (Nalam *et al*., 2019) that may change during ontogenesis. For example, expanded leaves have greater trichome density and resistance in tomato plants in reproductive stages than in vegetative ones (Mymko & Avila-Sakar, 2019). Furthermore, herbaceous plants usually show a significant increase in secondary chemistry across the entire ontogenetic trajectory (Barton & Koricheva, 2010).

The ontogenetic changes in the nutritional quality of the plant and in the defense strategies may explain the greater development of aphid colonies we observed when we infested tomato plants in the pre-flowering stage compared to the flowering stage. Our results confirm the better performance of aphids on young plants previously reported for other aphid-plant systems, as in the case of *Diuraphis noxia* (Kurdjumov 1913) on barley (Ma & Bechinski, 2009), and *Myzus persicae* (Sulzer, 1776) and *M. euphorbiae* on potato (Karley *et al*., 2002).

The best performance of aphids on young plants has practical consequences on the development of aphid infestations in the field and, therefore, on the control strategies that can be implemented. In fact, the extent of aphid infestations not only depends on the number of winged forms that move from the winter host to the herbaceous crops but also, to a large extent, on the phenological stage in which crop plants are at the time of the aphid invasion. The anticipation of the sowing time, when possible, allows plants to reach a less suitable phenological stage before aphid colonization takes place. This could be a strategy for the control of aphids, as several field studies also suggest (Perring *et al*., 1988; John *et al*., 2017; Alam *et al*., 2020).

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Author's contributions

DB, PF, and VT were involved in planning and supervised the work; IT, PF, and JD performed the experiments; VT processed the experimental data and performed the analysis, and DB drafted the manuscript. All authors discussed the results and commented on the manuscript.

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SCIENTIFIC NOTE

Glyphosate and atrazine inhibit growth of *Azospirillum brasilense, Bacillus subtilis, Bacillus thuringiensis, Chromobacterium subtsugae* and *Saccharopolyspora spinosa*

El glifosato y la atrazina inhiben el crecimiento de *Azospirillum brasilense, Bacillus subtilis, Bacillus thuringiensis, Chromobacterium subtsugae* y *Saccharopolyspora spinosa*

David Ingsson Oliveira Andrade de Farias[1], Robson da Costa Leite[2], Evandro Alves Ribeiro[1], Albert Lennon Lima Martins[1], and Aloisio Freitas Chagas Júnior[1]

ABSTRACT

Glyphosate and atrazine are two herbicides used worldwide to ensure high yields in different types of crops. Despite the importance of herbicides, their application may have negative effects on non-target organisms, including bacteria used in biological control and biological nitrogen fixation. Therefore, this research aimed to analyze the *in vitro* effect of glyphosate and atrazine on the growth of bacteria *Azospirillum brasilense, Bacillus subtilis, Bacillus thuringiensis, Chromobacterium subtsugae,* and *Saccharopolyspora spinosa*. The design used was completely randomized, and the doses of the herbicides evaluated were 1.0, 2.0, 3.0 and 4.0 L ha^{-1}. The results showed that glyphosate and atrazine affected the development of the bacteria under study. Atrazine showed a lineal increasing effect between the doses used and inhibition of bacterial growth. Therefore, the dose of 4.0 L ha^{-1} of this herbicide was the one that showed the highest inhibition of bacteria, whereas glyphosate at a dose of 2.0 L ha^{-1} showed the highest inhibition of bacteria compared to doses of 1.0, 3.0 and 4.0 L ha^{-1}.

Key words: tolerance, biodegradation, pesticides, microorganisms.

RESUMEN

El glifosato y la atrazina son dos herbicidas utilizados en todo el mundo para garantizar una alta productividad en diferentes tipos de cultivos. A pesar de la importancia de los herbicidas, su aplicación puede causar efectos negativos en organismos no objetivo, incluyendo bacterias usadas en control biológico y fijación biológica de nitrógeno. Por lo tanto, esta investigación tuvo como objetivo analizar el efecto *in vitro* del glifosato y la atrazina sobre el crecimiento de las bacterias *Azospirillum brasilense*, *Bacillus subtilis, Bacillus thuringiensis, Chromobacterium subtsugae* y *Saccharopolyspora spinosa*. El diseño utilizado fue completamente al azar y las dosis de los herbicidas evaluadas fueron 1.0, 2.0, 3.0 y 4.0 L ha^{-1}. Los resultados mostraron que el glifosato y la atrazina afectaron el desarrollo de las bacterias estudiadas. La atrazina tiene un efecto lineal creciente entre las dosis utilizadas y la inhibición del crecimiento bacteriano. Por lo tanto, la dosis de 4.0 L ha^{-1} de este herbicida fue la que mostró la mayor inhibición de crecimiento de bacterias, mientras que el glifosato a una dosis de 2.0 L ha^{-1} mostró la más alta inhibición de crecimiento de bacterias en comparación con las dosis de 1.0, 3.0 y 4.0 L ha^{-1}.

Palabras clave: tolerancia, biodegradación, pesticidas, microorganismos.

Introduction

Human population growth has created a demand for crop areas that each year become more productive. However, pests, diseases, and weeds reduce productivity, making agricultural inputs essential for higher yields (Steffen *et al*., 2011). The application of herbicides is of great importance in world agriculture, as a technology widely used to guarantee high agricultural productivity (Hirakuri & Lazzarotto, 2014).

Glyphosate (N-(phosphonomethyl) glycine) is a post-emergent herbicide that exerts systemic control of a broad spectrum of weeds by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that is responsible for the synthesis of aromatic acids necessary for plant survival (Duke & Powles, 2008; Duke, 2018). Despite the low toxicity of this herbicide, its use can cause contamination of soils and water resources that affects non-target organisms (Haas *et al*., 2018).

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Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a pre-emergent or early post-emergent and selective herbicide with systemic action (Silva *et al*., 2017). It is widely used worldwide for weed control in the cultivation of corn, sorghum, and sugarcane (Fan & Song, 2014). Its chemical properties favor the contamination of surface and groundwater due to the high susceptibility of atrazine to leaching and runoff (Fan & Song, 2014).

Due to the fact that chemical control is the most currently used type of control in agriculture, its application has been a cause of concern for society. This is because with the increase in agricultural productivity there is also an awareness of the necessity to maintain environmental quality and human health (Simonato, 2018).

Biological control has acquired importance over the years, contributing to sustainability in the agroecosystem. Its advantages are the low damage to the environment and to human beings, and a greater specificity than chemical pesticides (Oliveira & Ávila, 2010; Wright, 2014).

Some microorganisms, such as those of the genera *Azospirillum, Bacillus, Saccharopolyspora*, and *Chromobacterium,* have been used in agriculture as growth promoters, biological nitrogen fixators, and biological control agents. These microorganisms have gained importance in the last years, contributing to the sustainability of agroecosystems and reducing damage to the environment and to humans (Palma *et al*., 2014; Wright, 2014; Caulier *et al*., 2019).

Despite the importance of herbicides for crop production, the application of these products (including those considered to be of low risk) has negative effects on non-target organisms, such as the microorganisms used in biological control. These effects can be direct, decreasing the abundance of plants, and indirect, impacting microorganisms. Therefore, studies on the harmful effects of pesticides on beneficial organisms are of great importance (Costa *et al*., 2014; Fonseca *et al*., 2015; Moscardini *et al*., 2015; Prosser *et al*., 2016).

The objective of this study was to analyze the relationship between glyphosate and atrazine applications and the growth of the bacteria *Azospirillum brasilense, Bacillus subtilis, Bacillus thuringiensis, Chromobacterium subtsugae and Saccharopolyspora spinosa*.

Material and methods

Study location

This research was conducted at the microbiology laboratory of the Federal University of Tocantins, University campus of Gurupi, Brazil (11º43' S, 49º04' N, at an altitude of 280 m a.s.l.). Five different types of bacteria were used: *Azospirillum brasilense*, *Bacillus subtilis, Bacillus thuringiensis, Chromobacterium subtsugae,* and *Saccharopolyspora spinosa*, from the mycological collection of the microbiology laboratory of the University. Each bacterium was evaluated separately to test the effects of the herbicides glyphosate (Roundup Original®) and atrazine (Atrazine nortox® 500 SC) on radial growth. These two herbicides are widely used in Brazilian agriculture.

Experimental design

We used a completely randomized design, in a 2x5 factorial arrangement with three replicates per Petri dish, in which factor A corresponded to the two types of herbicides and factor B to the doses of herbicides.

The herbicide doses were calculated according to the manufacturer's recommendation. The reference for glyphosate was a soybean crop and for atrazine a corn crop, using doses ranging from 1.0, 2.0, 3.0, and 4.0 L ha^{-1} and a control with only distilled water. The concentrations of the active ingredients for the respective doses were 180 g L^{-1}, 360 g L^{-1}, 540 g L^{-1}, and 720 g L^{-1} of glyphosate, and 250 g L^{-1}, 500 g L^{-1}, 750 g L^{-1}, and 1000 g L^{-1} of atrazine.

Procedures performed

The herbicide syrups were prepared with distilled and sterilized water with the respective concentrations of the herbicides. The bacteria were multiplied in potato dextrose agar (PDA) culture medium (250.0 g of potatoes, 20.0 g of dextrose, 20.0 g of agar, 250.0 mg of ampicillin to 1.0 L of distilled water) and incubated at 27°C for 7 d.

Subsequently, the bacteria were scratched onto Petri dishes (90 mm) containing the PDA culture medium. The herbicides were added using 10.0 mm diameter filter paper discs. The disks were soaked in the herbicide syrups corresponding to each dose, and then added to the culture medium containing the bacteria. Disks with distilled water were used for the control. After this process, the plates were kept in a biochemical oxygen demand (BOD) chamber (model BT 60 HR, BIOTHEC, Piracicaba, São Paulo, Brazil) under a temperature of 25°C.

The evaluations started 48 h after setting up the treatments, determining radial growth every 48 h, for a total of five evaluations. The measurements were performed with a digital caliper, determining the diameter (mm) of the bacterial growth inhibition halo in three orthogonal directions. If growth was not impeded, the value was equal to zero.

However, with an influence on growth, the total diameter was measured by discounting the value of the paper disk.

Statistical analysis

Data were subjected to an analysis of variance (ANOVA) and the means were compared by the Tukey test ($P \leq 0.05$). They were then subjected to a multivariate analysis using a principal component analysis (PCA) with the software R® version 3.5.3 (R Core Team, 2013). The graphs were plotted using the software SigmaPlot® version 10.0 (SYSTAT, 2014).

Results

According to the ANOVA, herbicides caused changes in bacterial growth (Tab. 1). For the herbicide variable only *C. subtsugae* showed differences between treatments. For the dose variable, there was a statistical difference for all bacteria. There was an interaction between herbicides and doses, except for *B. subtilis*.

According to Figure 1A and B, the bacteria *A. brasilense* and *B. subtilis* showed no statistical differences regarding the use of herbicides in the formation of the growth halo. For *A. brasilense,* the products acted linearly (Fig. 1A), so the highest dose caused a greater inhibition halo (10.40 mm).

Although the products are indifferent to the formation of halos, *B. subtilis* showed quadratic behavior (Fig. 1B), with the dose of 2 L ha^{-1} being the most harmful to the bacteria for both products (13.45 mm), 34.5% higher than the control.

Saccharopolyspora spinosa showed a difference for the dose only in the herbicide atrazine. It showed linear behavior (Fig. 1C) with a 12.90 mm halo for the dose of 4 L ha^{-1} that was 29% higher than the control. Glyphosate showed no statistical difference in terms of doses.

Bacillus thuringiensis and *C. subtsugae* showed differences in the use of glyphosate or atrazine. Atrazine showed linear

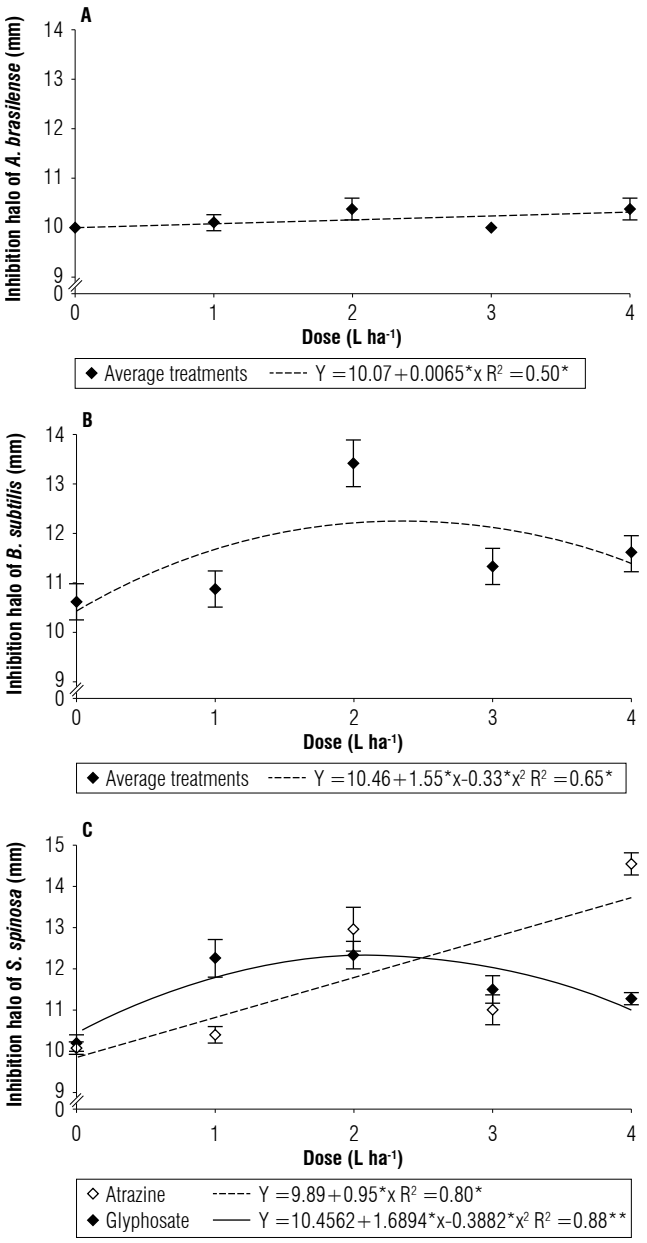

FIGURE 1. Bacterial growth inhibition halo of A) *Azospirillum brasilense*, B) *Bacillus subtilis*, and C) *Saccharopolyspora spinosa* under the effect of the herbicides glyphosate and atrazine (mean ± standard error).

TABLE 1. Analysis of variance of the diameter of the bacterial growth inhibition halo of the growth of bacteria under the influence of the application of glyphosate and atrazine.

Variable	Source of variation (MS)				Mean	CV (%)
	Herbicide (H)	Dose (D)	HxD	Residue		
	Degree of freedom					
	1	4	4	20		
Saccharopolyspora spinosa	0.87ns	7.16*	5.88*	1.64	11.65	11
Azospirillum brasilense	0.00ns	0.26*	0.07*	0.08	10.19	2.8
Bacillus subtilis	1.46ns	7.29**	1.10ns	1.14	11.60	9.19
Bacillus thuringiensis	0.01ns	15.42**	5.20**	2.09	12.73	11.35
Chromobacterium subtsugae	16.04**	19.74**	11.35**	0.63	12.45	6.41

MS - medium square; CV- coefficient of variation; **significant at 1% probability level ($P<0.01$); *significant at 5% probability level ($0.01 \leq P<0.05$); ns - not significant ($P>0.05$) according to the F test.

behavior, causing a greater inhibition halo with increasing doses. *Bacillus thuringiensis* showed a halo of 13.66 mm for the dose of 4 L ha^{-1}, representing an 18.8% increase compared to the control. Regarding the same bacteria, glyphosate showed quadratic behavior, with a higher reduction of bacterial growth than atrazine at doses of 1 and 2 L ha^{-1} (10.78 mm and 15.95 mm, respectively), and an increase of 27.39% compared to the control.

Chromobacterium subtsugae was similarly affected to *B. thuringiensis* (Fig. 2B). Glyphosate had a greater effect compared to atrazine only at the dose of 1 L ha^{-1}, with a halo of 12.33 mm which corresponds to an inhibition of 7.7% greater than atrazine and 19.7% compared to the control. The same dose of atrazine showed linear behavior and increased the inhibition halo with higher doses. The dose of 4 L ha^{-1} obtained an inhibition halo of 16.20 mm that was approximately 56% higher than the control.

According to the principal component analysis (PCA), the data represent a total of 100%. The greater the variance of the component, the greater its degree of importance. The first component (PC1) was responsible for 53.4% of the total variation of the analyzed characteristics regarding the source and doses of herbicides (Tab. 2). According to Hair Jr. *et al*. (2009), the percentage above 80% of the variance must be approached to determine the adequate number of components. This way, the first three components for the study were selected, which explained 96.6% of the total variance.

PC1 (53.4%) best represented the relationship between herbicide responses and doses and inhibition of bacterial growth, positively associated with *S. spinosa* (0.87), *A. brasilense* (0.77), *C. subtsugae* (0.68), *B. subtilis* (0.67), and *B. thuringiensis* (0.63). For the second component (PC2) (22.4%), the highest coefficients were *C. subtsugae* (0.64), *B. thuringiensis* (0.44), and *S. spinosa* (0.00), with *B. subtilis* (-0.44) and *A. brasilense* (-0.55) showing a negative correlation.

Regarding the third component (PC3), *B. thuringiensis* (0.61) and *B. subtilis* (0.57) showed the highest positive variance coefficients. In contrast, *S. spinosa* (-0.44), *A. brasilense* (-0.28) and *C. subtsugae* (-0.26) showed a negative correlation (Fig. 3).

Regarding the effects of treatments on the three main components (Tab. 3), for PC1 the treatments that showed the highest influence were atrazine 4 L ha^{-1} (2.14), atrazine 2 L ha^{-1} (1.99), and glyphosate 2 L ha^{-1} (1.65), and the lowest responses were observed in treatments atrazine 1 L ha^{-1} (-2.19), atrazine 0 L ha^{-1} (-2.06), and glyphosate 0 L ha^{-1} (-1.66). In PC2, the highest values were found in the treatment atrazine 3 L ha^{-1} (1.99) and the lowest in glyphosate 4 L ha^{-1} (-1.34) and atrazine 2 L ha^{-1} (-1.23). For PC3, the treatments with the lowest values were glyphosate 2 L ha^{-1} (1.64), atrazine 3 L ha^{-1} (1.12), and atrazine 4 L ha^{-1} (-1.66).

TABLE 2. Principal component analysis (PCA), eigenvalues (λi), and percentage of explained variance and cumulative variance (%) by components.

	Principal components				
	PC1	**PC2**	**PC3**	**PC4**	**PC5**
Eigenvalues	2.67	1.12	1.03	0.12	0.05
Explained variance (%)	53.4	22.4	20.8	2.5	0.9
Cumulative variance (%)	53.4	75.8	96.6	99.1	100

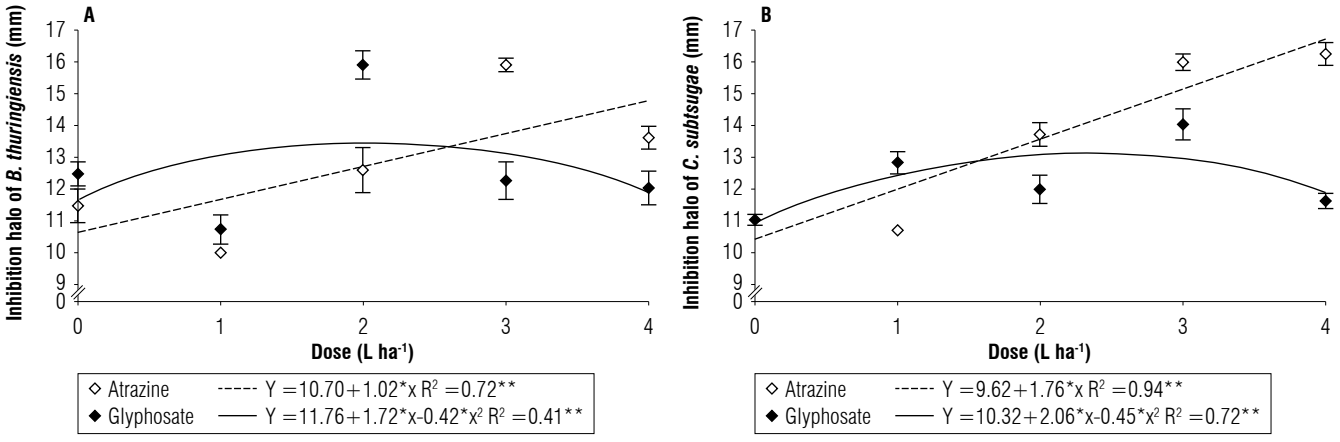

FIGURE 2. Bacterial growth inhibition halo of A) *Bacillus thuringiensis* and B) *Chromobacterium subtsugae* under the effect of the herbicides glyphosate and atrazine (mean ± standard error).

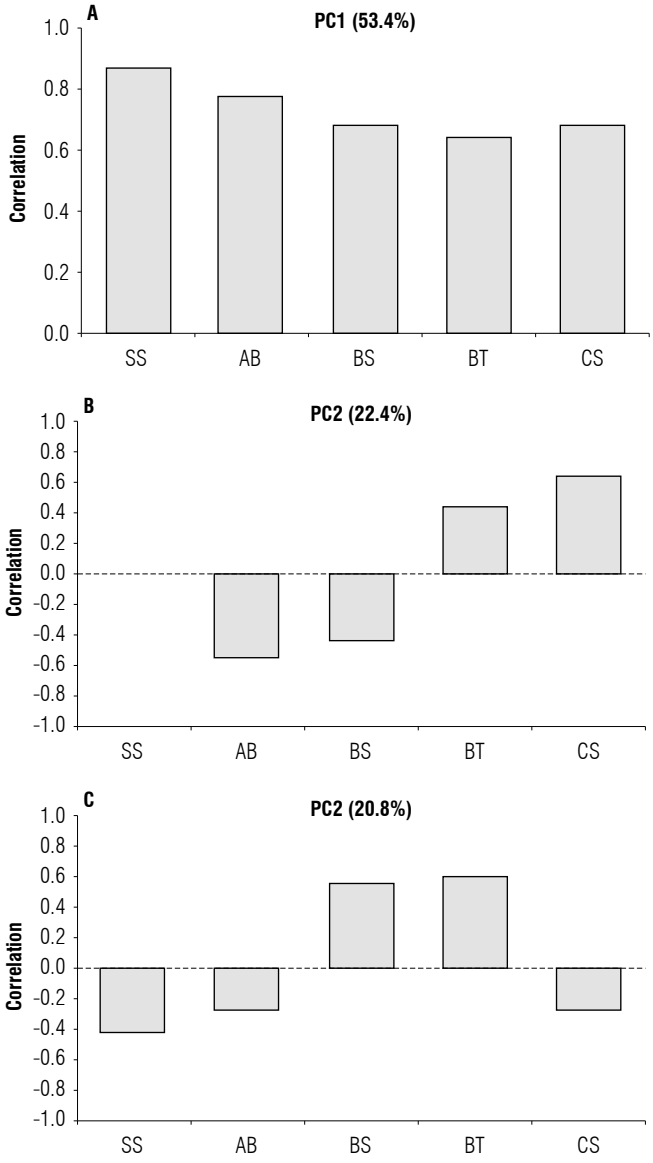

FIGURE 3. Coefficient of variation of variables correlated with the three main components A) PC1, B) PC2, and C) PC3 in bacteria *Saccharopolyspora spinosa* (SS), *Azospirillum brasilense* (AB), *Bacillus subtilis* (BS), *Bacillus thuringiensis* (BT), and *Chromobacterium subtsugae* (CS).

TABLE 3. Scores of the effects of treatments on the three principal components.

Treatment	Dose	PC1	PC2	PC3
Atrazine	0 L ha^{-1}	-2.06	0.05	0.04
Atrazine	1 L ha^{-1}	-2.19	-0.45	-0.37
Atrazine	2 L ha^{-1}	1.99	-1.23	-0.25
Atrazine	3 L ha^{-1}	0.66	1.99	1.12
Atrazine	4 L ha^{-1}	2.14	1.00	-1.66
Glyphosate	0 L ha^{-1}	-1.66	0.10	0.51
Glyphosate	1 L ha^{-1}	-0.11	-0.53	-1.11
Glyphosate	2 L ha^{-1}	1.65	-0.57	1.85
Glyphosate	3 L ha^{-1}	-0.61	0.97	-0.35
Glyphosate	4 L ha^{-1}	0.19	-1.34	0.22

The PCA results were plotted on a biplot chart (Fig. 4). The doses of atrazine 2 L, 3 L, and 4 L ha^{-1} and glyphosate 2 L ha^{-1} were responsible for the largest growth inhibition halos. We also observed that doses below 1 L ha^{-1} of atrazine and glyphosate obtained the best responses, thus significantly influencing bacterial growth.

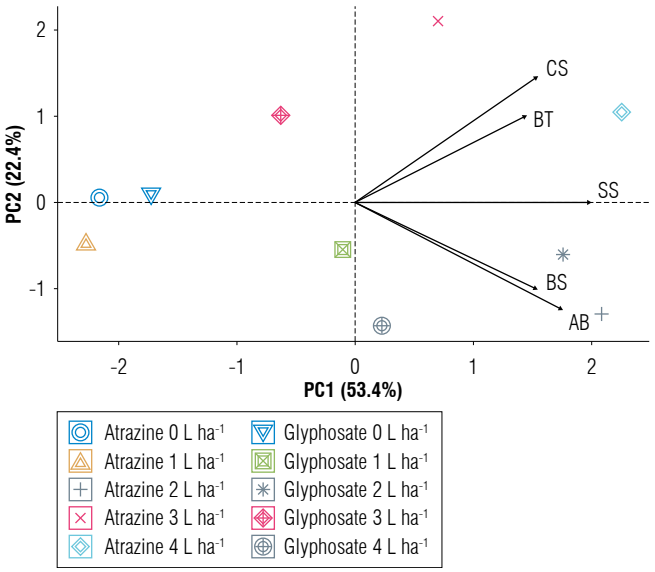

FIGURE 4. PC1 x PC2 biplot of the variable responses of doses and sources of herbicides for bacterial growth inhibition. *Bacillus thuringiensis* (BT), *Bacillus subtilis* (BS), *Azospirillum brasilense* (AB), *Chromobacterium subtsugae* (CS) and *Saccharopolyspora spinosa* (SS).

Chromobacterium subtsugae and *B. thuringiensis* were more influenced by the atrazine treatments at doses 3 L and 4 L ha^{-1}, while the *A. brasilense*, *B. subtilis*, and *S. spinosa* were more inhibited in terms of their growth at the doses of 2 L ha^{-1} atrazine and 2 L ha^{-1} glyphosate. It is noteworthy that when using lower dosages than these, the inhibition of microbial growth became lower, thus not affecting the final development of the bacteria.

Discussion

Changes in bacterial growth were observed. The linear effect of atrazine doses on *A. brasilense, S. spinosa, B. thuringiensis*, and *C. subtsugae* is related to the mechanism of action of the herbicide and tolerance of bacteria to the product. Atrazine causes membrane rupture, dehydration, and disintegration of cells and organelles through the oxidation of lipids and proteins (Oliveira Jr., 2011).

Inhibition of bacterial growth due to the use of atrazine may be related to a lower absorption of nutrients present in the culture medium, causing stress to the bacteria. Thus, part of the energy available for the development of bacteria is lost to

the maintenance of cellular and biochemical mechanisms, affecting their growth (Schimel *et al*., 2007).

Bacillus subtilis can metabolize very high concentrations of atrazine. In some cases, there is a description of urea formation from biuret or allophanate that can be cleaved by the urease enzyme releasing CO_2 and $2NH_3$, besides the rapid degradation of cyanuric acid which can serve as nitrogen source for bacteria (Wang *et al*., 2014).

Glyphosate application affected the growth of bacteria. In *A. brasilense,* it followed the same behavior of atrazine, increasing the effect on the inhibition halo with higher herbicide doses. This showed that the bacterium did not show tolerance to either of the two molecules.

Among the tested doses of glyphosate, the most harmful was 2 L ha^{-1} (Fig. 4), causing a greater inhibition halo, an effect linked to the ability of glyphosate to acidify the medium, decrease cell density, and provide unfavorable conditions for bacterial growth (Manogaran *et al*., 2017).

The reduction of bacterial growth at doses above 2 L ha^{-1} is directly linked to the composition of glyphosate. This herbicide is an organophosphate consisting of carbon-phosphorus bonds that allow its easy degradation by a select group of microorganisms that use phosphorus from glyphosate degradation for their development. Additionally, other bacteria have the ability to adapt to the stress that the herbicide can cause, not compromising their development.

The primary and predominant metabolites of microbial degradation in glyphosate are glyoxylate and aminomethyl phosphonic acid (AMPA) that turn into water, carbon dioxide, and phosphate (Carranza *et al*., 2019). The AMPA metabolite can later be transformed into phosphate and methylamine by the action of a C-P lyase and/or into phosphate and formaldehyde by the combined action of a transaminase and a phosphonatase (Carranza *et al*., 2019; Artigas *et al*., 2020). Unlike the AMPA pathway, some bacteria such as *Bacillus* sp., *Pseudomonas* sp. and others, can metabolize glyphosate into sarcosine using this component as a growth nutrient (Fan *et al*., 2012).

The ability of bacteria to use glyphosate as a source of phosphorus for the synthesis of their cellular components is determined by the presence of a C-P lyase enzyme system that breaks the C-P bond to form non-toxic components, like sarcosine (*N*-methylglycine) and orthophosphate (Kryuchkova *et al*., 2014). However, the uptake of glyphosate by bacterial cells and its subsequent degradation by the C-P-lyase pathway are induced only when other sources of P are scarce (Fitzgibbon & Braymer, 1988).

Bacteria that can use glyphosate as a source of phosphorus are associated with adaptation directed by a genetic mutation in which the isolate uses the herbicide for its cell propagation (Dibua *et al*., 2015). This result was similar to that found in the present study in which the bacteria *B. subtilis*, *B. thuringiensis* and *C. subtsugae* showed a lower interference of the herbicide in the growth of bacteria at doses of 3 L ha^{-1} and 4 L ha^{-1} compared to the lowest doses of 1 L ha^{-1} and 2 L ha^{-1}. This suggests that the bacteria used the herbicide as the only source of phosphorus present in the culture medium for its growth.

Regardless of the glyphosate concentration, this herbicide can cause harmful effects on *B. thuringiensis*, causing a detrimental effect on its development and formation of colonies (Agostini *et al*., 2013).

Conclusions

The herbicides glyphosate and atrazine affect the development of the studied bacteria. However, atrazine has an increasing relationship between doses and inhibition of bacterial growth. Regardless of the herbicide, the higher the dose, the greater the growth inhibition halo for bacteria *B. thuringiensis*, *C. subtsugae*, *S. spinosa*, and *A. brasilense*. The bacterium *B. subtilis* can degrade high doses of atrazine in the medium, demonstrated by the smaller halo of bacterial growth.

In general, the glyphosate dose that most affected the development of bacteria was 2 L ha^{-1}; higher doses affected the development of bacteria less. According to the package leaflet of the herbicide, the most frequently recommended doses range from 0.5 to 2 L ha^{-1}, coinciding with the most harmful dose of the product in the present study. Thus, the data presented in this paper provide relevant information regarding the use of bacteria in agriculture and the effects that agricultural pesticides may cause in their development, which may help to support decisions made by growers.

Author's contributions

DIOAF, RCL and AFCJ formulated the research goals and aims. DIOAF, RCL, AFCJ and ALLM developed the methodology. DIOAF, EAR and RCL verified the experiments and reproducibility of results. DIOAF and EAR applied the statistical techniques. DIOAF and RCL conducted the experiments. AFCJ provided the materials. DIOAF

and RCL managed the activities to annotate and maintain research data. DIOAF and EAR prepared the initial draft. AFCJ performed the critical review of the manuscript. DIOAF, EAR and RCL prepared and presented data. DIOAF, RCL, AFCJ and ALLM oversaw and led the research activity. AFCJ managed and coordinated the research activity and obtained the financial support for the project.

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SCIENTIFIC NOTE

Pre-sowing application of combinations of burndown and pre-emergent herbicides for *Conyza* spp. control in soybean

Aplicación en la pre-siembra de mezclas de herbicidas desecantes y pre-emergentes para el control de *Conyza* spp. en la soya

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ABSTRACT

Together, *Conyza bonariensis*, *C. canadensis* and *C. sumatrensis* show 105 reported cases of biotypes resistant to herbicides like glyphosate, paraquat, and acetolactate synthase (ALS) inhibitors. The application of pre-emergent herbicides combined with burndown herbicides is believed to be effective in controlling *Conyza* spp. during soybean pre-sowing management. The objective of this study was to evaluate the efficacy of sulfentrazone/diuron, imazethapyr/flumioxazin, and diclosulam in mixtures with diquat, paraquat, or glufosinate on the control of *Conyza* spp. Two field experiments were conducted in a randomized block design with four replicates. Treatments consisted of the application of pre-emergent plus burndown herbicides, besides the weedy control treatment (without application), for a total of 10 treatments. The control of *Conyza* spp. was evaluated at 7, 14, 21, 28, and 35 d after herbicide application, and symptoms of injury in soybean plants were evaluated at 14, 21, 28, and 35 d after herbicide application. The herbicides sulfentrazone/diuron, imazethapyr/flumioxazin, and diclosulam in combination with burndown herbicides diquat, paraquat, or glufosinate were effective in controlling *Conyza* spp. in the pre-sowing management of soybean, highlighting good options for pre- and post-emergent herbicide rotations. Mixtures with diclosulam showed a higher potential for injury to soybean plants than sulfentrazone/diuron and imazethapyr/flumioxazin.

Key words: diclosulam, flumioxazin, glufosinate, *Glycine max*, imazethapyr, sulfentrazone, weeds.

RESUMEN

Juntas, *Conyza bonariensis*, *C. canadensis* y *C. sumatrensis* presentan 105 casos reportados de biotipos resistentes a herbicidas tales como glifosato, paraquat y los inhibidores de la acetolactato sintasa (ALS). Se cree que la aplicación de herbicidas pre-emergentes en mezclas con desecantes es efectiva para controlar *Conyza* spp. en el manejo pre-siembra de la soya. El objetivo de este estudio fue evaluar la eficacia de sulfentrazona/diuron, imazetapir/flumioxazin y diclosulam, en mezcla con diquat, paraquat o glufosinato, en el control de *Conyza* spp. Se realizaron dos experimentos de campo en diseño de bloques al azar con cuatro repeticiones. Los tratamientos consistieron en la aplicación de herbicidas pre-emergentes en mezclas con desecantes, además del tratamiento control (sin aplicación), para un total de 10 tratamientos. El control de *Conyza* spp. se evaluó a los 7, 14, 21, 28 y 35 d después de la aplicación del herbicida, y se evaluaron los síntomas de daño en las plantas de soya a los 14, 21, 28 y 35 d después de la aplicación del herbicida. Los herbicidas sulfentrazona/diuron, imazetapir/flumioxazin y diclosulam en mezclas con diquat, paraquat o glufosinato, fueron efectivos para controlar *Conyza* spp. en la pre-siembra de la soya, destacando buenas opciones para la rotación de herbicidas antes y después de la emergencia. Las mezclas con diclosulam mostraron un mayor potencial de daño a las plantas de soya que sulfentrazona/diuron y imazetapir/flumioxazin.

Palabras clave: diclosulam, flumioxazin, glufosinato, *Glycine max*, imazetapir, sulfentrazona, malezas.

Introduction

Glyphosate has become the most widely used herbicide in grain crops with the adoption of glyphosate-tolerant crops, such as Roundup Ready® soybean. This intensive use leads to the selection of herbicide-resistant weed biotypes. There are currently 53 weed species with cases of glyphosate-resistant biotypes worldwide (Heap, 2021).

The weeds hairy fleabane (*Conyza bonariensis*), horseweed (*C. canadensis*), and Sumatran fleabane (*C. sumatrensis*) are among the main weeds found worldwide (Trainer *et*

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al., 2005). They are annual herbaceous plants with high seed production and are found in various agricultural environments, such as grain crops (Lorenzi, 2014). Altogether, they have 105 reported cases of biotypes that are resistant to herbicides, such as glyphosate, paraquat, and acetolactate synthase (ALS) inhibitors (Heap, 2021). Recent studies showed that there are biotypes of *C. sumatrensis* with resistance to paraquat (Zobiole *et al.*, 2019) or 2,4-D (Queiroz *et al.*, 2020), and multiple resistance to glyphosate, chlorimuron, and paraquat (Albrecht, Pereira, *et al.*, 2020).

Thus, it is necessary to use other herbicides, such as pre-emergent herbicides, and different modes of action that are effective in controlling hard-to-control weeds, whether herbicide-tolerant or resistant (Mueller *et al.*, 2014). Studies highlight the efficacy of pre-emergent herbicides in controlling *Conyza* spp. in soybeans. These include flumioxazin (Zimmer *et al.*, 2018), diclosulam (Braz *et al.*, 2017), diuron (Moreira *et al.*, 2010), sulfentrazone (Zimmer *et al.*, 2018), and imazethapyr (Hedges *et al.*, 2019).

In addition to the use of pre-emergent herbicides for the effective management of *Conyza* spp., burndown herbicides should be used in combination in situations with the presence of these weeds prior to soybean sowing. Among these herbicides, diquat (Weaver *et al.*, 2004), paraquat, and glufosinate (Zobiole *et al.*, 2018) stand out.

The application of pre-emergent herbicides combined with burndown herbicides is believed to be effective in controlling *Conyza* spp. in the pre-sowing management of soybean. Therefore, the present study aimed at evaluating the efficacy of pre-emergent herbicides sulfentrazone/diuron, imazethapyr/flumioxazin, and diclosulam in combination with burndown herbicides diquat, paraquat, or glufosinate on the control of *Conyza* spp.

Materials and methods

Two field experiments were carried out in the city of Palotina, state of Parana, Brazil, at coordinates 24°20'44.54" S, 53°51'50.93" W (experiment 1) and 24°20'48.89" S, 53°51'37.58" W (experiment 2) during the 2018-19 growing season.

For both experiments, the soil was classified as clay texture, with 63% clay, 19% silt, and 15% sand. The climate of the region is temperate humid with hot summers (Cfa), according to the Köppen classification (Aparecido *et al.*, 2016), and the weather conditions for the experimental period are illustrated in Figure 1. The area in experiment 1 was previously cultivated with maize and in experiment 2 with wheat. Both areas were infested with *Conyza* spp. plants up to 15 cm high and with 16 leaves. No flowering plants were observed at the time of application, and plant density was 17 and 20 *Conyza* plants m^{-2} in experiments 1 and 2, respectively.

The treatments consisted of different herbicide applications in a randomized block design with four replicates (Tab. 1). Application of treatments occurred on October 15, 2018 with sowing of soybean cultivar Monsoy® 5947 IPRO (Monsanto Co. do Brasil, São Paulo, SP, Brazil) on the same day, immediately after application. Herbicides were applied using a CO_2 pressurized backpack sprayer (Pulverizador Pesquisa - Herbicat Ltda, Catanduva, SP, Brazil) equipped with six AIXR 110.015 nozzles at a pressure of 2.5 kgf cm^{-2}

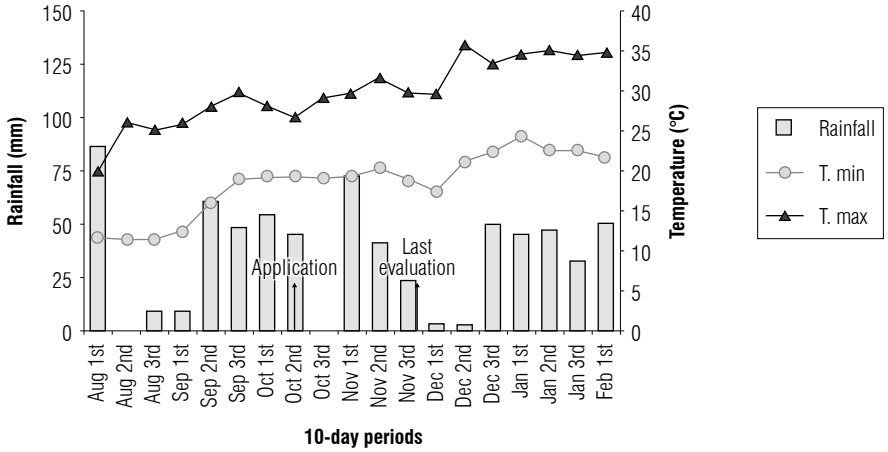

FIGURE 1. Rainfall and minimum and maximum temperature for the experimental sites. 2018/19 season, Palotina, PR, Brazil.

and a speed of 3.6 km/h, providing an application volume of 150 L ha^{-1}. For experiment 1, the climatic conditions during the application were as follow: temperature of 27.3ºC, relative air humidity of 61.5%, and wind speed of 6.4 km/h. For experiment 2, the conditions were as follow: temperature of 29.5ºC, relative air humidity of 60.2%, and wind speed of 7.0 km/h.

The experimental units were composed of 3 x 5 m parcels; soybean sowing was carried out after the application of the treatments with 13 seeds m^{-1}. Six soybean lines were sown in each parcel with a spacing of 0.45 m. The evaluations were carried out in the useful area of the parcel, discarding the external lines and the first and last meters.

The control of *Conyza* spp. plants was assessed at 7, 14, 21, 28, and 35 d after herbicide application (DAA). Injury symptoms in soybean plants were assessed at 14, 21, 28 and 35 DAA. These assessments were carried out through visual analysis at each experimental unit considering significantly visible symptoms in 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). The treatment without application (without herbicide effect) was used as a reference for evaluations, always with a score of 0, whether for weed control or injuries to soybean plants, as in other studies (Braz *et al*., 2017; Chahal & Jhala, 2019; Guerra *et al*., 2020).

Data were tested by analysis of variance and F-test ($P \leq 0.05$) according to Pimentel-Gomes and Garcia (2002). The means of the treatments were compared by the Tukey test ($P \leq 0.05$) using the Sisvar 5.6 program (Ferreira, 2011).

Results

In experiment 1, all herbicide treatments were effective in controlling *Conyza* spp., with values higher than or equal to 83.8% at 7 DAA for all treatments. No differences were detected between herbicide treatments, and all of them were superior to the weedy control treatment (without application) throughout all evaluations. It is worth noting the control for all combinations at 35 DAA with values of at least 94.3% (Tab. 2).

As observed for experiment 1 (area previously cultivated with maize), in experiment 2 (area previously cultivated with wheat) herbicide treatments were effective in controlling *Conyza* spp. with no differences between combinations from 21 to 35 DAA. Some differences were observed between herbicide treatments at 7 and 14 DAA; however, all exhibited weed control of at least 89.0%. At 35 DAA, a control of *Conyza* spp. of at least 94.8% was observed for all herbicide treatments (Tab. 3).

For experiment 1, at 14 DAA, no differences were detected between treatments regarding injury symptoms in soybean plants. Treatments with diclosulam caused stronger symptoms, up to 10.3% at 21 DAA; these were superior to almost all other treatments from 21 to 35 DAA. For the treatments composed of the application of sulfentrazone/diuron and imazethapyr/flumioxazin, injury symptoms were 3.8% to 4.3% and 2.3% to 2.5%, respectively at 35 DAA (Tab. 4).

TABLE 1. Herbicide mixture treatments applied in pre-sowing of soybean to control *Conyza* spp. 2018/19 season, Palotina, PR, Brazil.

Treatments	Trade names	Rate*
Weedy control (without application)	-	-
Sulfentrazone/diuron + paraquat	Stone® + Gramoxone® 200	245/490 + 400
Sulfentrazone/diuron + diquat	Stone® + Reglone®	245/490 + 400
Sulfentrazone/diuron + glufosinate	Stone® + Finale®	245/490 + 500
Imazethapyr/flumioxazin + paraquat	Zhetamaxx® + Gramoxone® 200	100/50 + 400
Imazethapyr/flumioxazin + diquat	Zhetamaxx® + Reglone®	100/50 + 400
Imazethapyr/flumioxazin + glufosinate	Zhetamaxx® + Finale®	100/50 + 500
Diclosulam + paraquat	Spider® 840 WG + Gramoxone® 200	25 + 400
Diclosulam + diquat	Spider® 840 WG + Reglone®	25 + 400
Diclosulam + glufosinate	Spider® 840 WG + Finale®	25 + 500

*Rates at g of acid equivalent (ae) ha^{-1} for imazethapyr and at g of active ingredient (ai) ha^{-1} for the other herbicides. Addition of 0.5% mineral oil to all treatments.

TABLE 2. Control (%) of *Conyza* spp. from 7 to 35 d after application of herbicide mixtures (DAA). 2018/19 season, Palotina, PR, Brazil (experiment 1).

Treatments	7 DAA*	14 DAA*	21 DAA*	28DAA*	35 DAA*
Weedy control (without application)	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
Sulfentrazone/diuron + paraquat	84.5 a	89.5 a	92.5 a	95.8 a	96.3 a
Sulfentrazone/diuron + diquat	85.0 a	91.5 a	95.8 a	96.0 a	96.8 a
Sulfentrazone/diuron + glufosinate	84.0 a	93.8 a	95.8 a	96.3 a	96.5 a
Imazethapyr/flumioxazin + paraquat	85.8 a	88.0 a	94.0 a	95.5 a	95.0 a
Imazethapyr/flumioxazin + diquat	85.3 a	91.3 a	93.0 a	94.0 a	94.3 a
Imazethapyr/flumioxazin + glufosinate	86.0 a	94.3 a	95.5 a	96.3 a	95.5 a
Diclosulam + paraquat	84.3 a	91.3 a	94.5 a	96.5 a	96.3 a
Diclosulam + diquat	89.0 a	94.0 a	96.3 a	97.3 a	97.0 a
Diclosulam + glufosinate	83.8 a	93.3 a	96.8 a	96.8 a	96.8 a
Mean	76.8	82.7	85.4	86.4	86.4
CV (%)	2.9	4.1	4.0	3.8	3.2

*Means followed by the same letter in the line do not differ from each other according to the Tukey test at the 5% probability level. CV - coefficient of variation.

TABLE 3. Control (%) of *Conyza* spp. from 7 to 35 d after application of herbicide mixtures (DAA). 2018/19 season, Palotina, PR, Brazil (experiment 2).

Treatmentts	7 DAA*	14 DAA*	21 DAA*	28 DAA*	35 DAA*
Weedy control (without application)	0.0 c	0.0 d	0.0 b	0.0 b	0.0 b
Sulfentrazone/diuron + paraquat	90.0 ab	91.5 bc	95.3 a	97.0 a	96.3 a
Sulfentrazone/diuron + diquat	92.8 ab	94.0 abc	97.0 a	97.0 a	96.8 a
Sulfentrazone/diuron + glufosinate	91.5 ab	96.0 a	96.3 a	98.5 a	98.3 a
Imazethapyr/flumioxazin + paraquat	92.0 ab	92.5 abc	94.3 a	95.8 a	94.8 a
Imazethapyr/flumioxazin + diquat	91.3 ab	90.5 c	95.0 a	97.0 a	96.8 a
Imazethapyr/flumioxazin + glufosinate	91.5 ab	93.5 abc	95.0 a	96.0 a	97.3 a
Diclosulam + paraquat	91.5 ab	96.0 a	96.3 a	96.5 a	96.5 a
Diclosulam + diquat	93.8 a	94.5 ab	96.5 a	96.8 a	96.8 a
Diclosulam + glufosinate	89.0 b	95.3 ab	96.8 a	97.3 a	96.8 a
Mean	82.3	84.4	86.2	87.2	87.1
CV (%)	2.1	1.9	3.0	2.1	2.4

*Means followed by the same letter in the line do not differ from each other according to the Tukey test at the 5% probability level. CV - coefficient of variation.

TABLE 4. Crop injury (%) of soybean plants from 14 to 35 d after application of herbicide mixtures (DAA). 2018/19 season, Palotina, PR, Brazil (experiment 1).

Treatments	14 DAA ^{ns}	21 DAA*	28 DAA*	35 DAA*
Weedy control (without application)	0.0	0.0 a	0.0 a	0.0 a
Sulfentrazone/diuron + paraquat	4.3	7.3 bc	6.5 d	4.3 d
Sulfentrazone/diuron + diquat	3.0	6.3 b	6.0 cd	3.8 bcd
Sulfentrazone/diuron + glufosinate	3.0	7.0 bc	6.3 d	4.0 cd
Imazethapyr/flumioxazin + paraquat	3.3	5.3 b	4.3 b	2.3 b
Imazethapyr/flumioxazin + diquat	3.5	6.0 b	4.8 bc	2.5 bc
Imazethapyr/flumioxazin + glufosinate	3.0	6.0 b	4.5 b	2.5 bc
Diclosulam + paraquat	2.8	9.3 cd	8.5 e	6.5 e
Diclosulam + diquat	3.0	9.3 cd	8.5 e	6.3 e
Diclosulam + glufosinate	4.5	10.3 d	8.8 e	6.5 e
Mean	3.0	6.7	5.8	3.9
CV (%)	21.2	14.8	9.2	18.2

*Means followed by the same letter in the line do not differ from each other according to the Tukey test at the 5% probability level. ns - not significant, means do not differ from each other by the F-test at the 5% probability level. CV - coefficient of variation.

TABLE 5. Crop injury (%) of soybean plants from 14 to 35 d after application of herbicide mixtures (DAA) during the 2018/19 season, Palotina, PR, Brazil (experiment 2).

Treatments	14 DAA*	21 DAA*	28 DAA*	35 DAA*
Weedy control (without application)	0.0 a	0.0 a	0.0 a	0.0 a
Sulfentrazone/diuron + paraquat	2.8 bc	7.5 d	7.8 c	4.5 cde
Sulfentrazone/diuron + diquat	2.3 bc	7.0 d	7.8 c	5.0 def
Sulfentrazone/diuron + glufosinate	2.5 bc	7.0 cd	7.8 c	4.3 cd
Imazethapyr/flumioxazin + paraquat	1.5 b	4.3 b	4.0 b	2.0 ab
Imazethapyr/flumioxazin + diquat	2.3 bc	5.3 bc	5.0 b	2.8 bc
Imazethapyr/flumioxazin + glufosinate	2.0 bc	5.0 b	5.5 b	2.8 bc
Diclosulam + paraquat	2.3 bc	7.8 d	9.0 c	6.5 ef
Diclosulam + diquat	2.3 bc	4.8 b	5.3 b	3.3 bcd
Diclosulam + glufosinate	3.0 c	7.8 d	9.0 c	6.8 f
Mean	2.1	5.7	6.1	3.8
CV (%)	25.7	14.0	14.3	23.0

*Means followed by the same letter in the line do not differ from each other according to the Tukey test at the 5% probability level. CV - coefficient of variation.

Table 5 shows the injury symptoms in soybean plants due to herbicide application for experiment 2. Differences between treatments were observed in all evaluations. Symptoms were up to 9% (at 28 DAA), while at the last assessment (35 DAA) they ranged from 2.0% to 6.8%, in general with greater potential for injury to the application of diclosulam.

Discussion

In this study, the pre-sowing application of sulfentrazone/diuron (premix formulation) in combination with diquat, paraquat, or glufosinate was effective in controlling *Conyza* spp. Similar results were observed by Zimmer *et al*. (2018), where pre-sowing application of sulfentrazone (195 g ai ha^{-1}) in a mixture with halauxifen (5 g ai ha^{-1}) + glyphosate (1,120 g ae ha^{-1}) + cloransulam (25 g ai ha^{-1}) provided 94% control of *C. canadensis* at 35 d after application. Sulfentrazone was effective in different management programs to control *Amaranthus tuberculatus* (Schryver *et al*., 2017) and other weeds. Sulfentrazone has a variable half-life (34-116 d) according to soil moisture and temperature and is less persistent under conditions of higher humidity and higher temperature (40ºC); these aspects interfere with its residual period (Brum *et al*., 2013). Additionally, sulfentrazone has a spectrum of action on eudicotyledonous weeds and some grasses (Rodrigues & Almeida, 2018). These studies, and the results of the present study, indicate the effectiveness of sulfentrazone in different mixtures on the control of weeds.

The application of diuron with mixtures was effective in controlling *Conyza* spp. in the present study. In other research, a control of 91% of *C. bonariensis* at 30 DAA was observed with the application of diuron (200 g ai ha^{-1}) at mixtures (Paula *et al*., 2011). Other studies also highlight the effectiveness of diuron in controlling *Conyza* spp. in different combinations (Lamego *et al*., 2013; Santos *et al*., 2015). Diuron is an herbicide that is especially effective for the control of eudicotyledons and some grasses. In the present study, diuron was effective in controlling *Conyza* spp. in a premixture with sulfentrazone. The half-life can vary from 40 to 91 d and is generally more persistent in soils with higher levels of clay and organic matter (Rocha *et al*., 2013). This characteristic helps to explain the results of this study.

The pre-sowing application of imazethapyr/flumioxazin (premix formulation) was also effective for controlling *Conyza* spp. in this study. Similar results were observed by Albrecht, Albrecht, *et al*. (2020), who found that this premix formulation obtained a control of about 80% of *Conyza* spp. up to 35 DAA. Imazethapyr has a soil half-life varying from 36 to 98 d (Marinho *et al*., 2019), and flumioxazin from 13 to 18 d (Ferrell & Vencill, 2003), according to the edaphoclimatic conditions. The persistence of imazethapyr helps to explain the effectiveness of this mixture over time, especially in the emergence of plants.

Pre-sowing application of imazethapyr (100 g ae ha^{-1}) in a mixture with glyphosate, dicamba, and saflufenacil provided 93% control of *C. canadensis* 12 weeks after application (Hedges *et al*., 2019). This herbicide, in mixture with saflufenacil, is also effective in controlling *Abutilon theophrasti*, *Amaranthus retroflexus,* and *Chenopodium album* (Underwood *et al*., 2017). Pre-sowing application of flumioxazin (76 g ai ha^{-1}) in combination with halauxifen (5 g ai ha^{-1}) + glyphosate (1,120 g ae ha^{-1}) + cloransulam (25 g ai ha^{-1}), following glyphosate application (1,120 g ae ha^{-1}) in post-emergence soybean, provided 96% control of *C. canadensis* at 35 d after the first application (Zimmer

et al., 2018). The results of the present study corroborated these findings, indicating the effectiveness of imazethapyr/flumioxazin in different chemical control programs.

In the present study, diclosulam was also effective in controlling *Conyza* spp. in combination with diquat, paraquat, or glufosinate. In general, no significant differences were observed between the management adopted for the control of *Conyza* spp. in initial post-emergence (plants up to 15 cm high) and pre-emergence. This highlights the importance of rotation of mechanisms of action not only in post-emergence, but also in pre-emergence.

Diclosulam is one of the most widely used herbicides in soybean pre-sowing for controlling *Conyza* spp. and other weeds. Krenchinski *et al.* (2019) report control of up to 97.25% of *Conyza* spp. for diclosulam applied at pre-sowing in soybean, plus halauxifen + glyphosate. Other studies also report the control of *Conyza* spp. with the application of diclosulam in different combinations (Braz *et al*., 2017; Zobiole *et al*., 2018). This herbicide has a soil half-life varying from 16 to 87 d, according to the edaphoclimatic conditions (Lavorenti *et al*., 2003) and provides a broad-spectrum control (Rodrigues & Almeida, 2018).

The results of this study indicated that glufosinate may be an alternative in the management of *Conyza* spp. as a tool for preventing resistance to herbicides. In this sense, it is worth mentioning the recent cases of a *C. sumatrensis* biotype reported to be resistant to paraquat (Zobiole *et al*., 2019) or with multiple resistance to glyphosate, chlorimuron, and paraquat (Albrecht, Pereira, *et al*., 2020) in the state of Parana, Brazil. Paraquat is a photosystem I inhibitor herbicide with the same mechanism of action as that of diquat. These two herbicides are among the main desiccants for pre-sowing application in soybean.

Several studies also highlight the efficacy of glufosinate in controlling *Conyza* spp. (Oliveira Neto *et al*., 2010; Tahmasebi *et al*., 2018; Zobiole *et al*., 2018; Albrecht, Albrecht, *et al*., 2020). This herbicide is also effective in controlling other weeds, such as *Amaranthus* spp. (Hay *et al*., 2019), *Spermacoce latifolia* and *Richardia brasiliensis* (Gallon *et al*., 2019). Glufosinate can be used in pre-sowing, as in the present study, and in post-emergence of tolerant transgenic cultivars (soybean, maize, and cotton with *pat* or *bar* genes).

Regarding symptoms of injury, diclosulam showed higher phytotoxic potential, especially in experiment 1, with symptoms of up to 10.3%. For the other pre-emergent herbicides, minor injuries were observed in the soybean plants. Different studies also report injury symptoms in soybean with the application of diclosulam (Osipe *et al*., 2014; Braz *et al*., 2017). However, plants recover from the symptoms with no effect on the agronomic performance, and this proves the selectivity of the herbicide.

Belfry *et al.* (2016) found symptoms of 8% and 2% injury for pre-sowing application of flumioxazin and sulfentrazone, respectively. Belfry *et al.* (2015) also observed symptoms of 8% and 4% injury for pre-sowing application of flumioxazin and imazethapyr, respectively. Braswell *et al.* (2015) observed up to 15% soybean injury for pre-sowing application of diuron. For these herbicides, injury symptoms up to a maximum of 5% were observed in soybean plants at the end of the evaluations. These results confirm the potential selectivity of pre-emergent herbicides for soybeans.

Conclusions

Based on the results of this study we can conclude that it is important to rotate both post-emergent and pre-emergent herbicides since there have been several reports of resistance to glyphosate and ALS inhibitors that are widely used in pre-emergence. We would like to highlight the equivalence of the different herbicide combinations in the control of *Conyza* spp. in this study, after growing maize (experiment 1) or wheat (experiment 2).

The herbicides sulfentrazone/diuron, imazethapyr/flumioxazin, and diclosulam in mixtures with burndown herbicides (diquat, paraquat or glufosinate) were effective in controlling *Conyza* spp. at pre-sowing of soybean. The choice of pre-emergent or burndown herbicides must consider the history of use of the area, the weed community, and the cost of management, among other factors. Regardless of the choice, pre-emergent herbicides in combinations with burndown herbicides are important tools for the effective management of weeds.

This study fills a gap in the research since studies with the premixes sulfentrazone/diuron and imazethapyr/flumioxazin are not easily found in the literature in contrast to other products. Glyphosate was not used in this study, which characterizes the management adopted as an alternative to this herbicide.

Author's contributions

AJPA, LPA, SNRA and AAMB designed and conceptualized the experiments; WOS, JBL and MTYD carried out the experiments, and AFMS contributed to the data analysis and wrote the article. All authors reviewed the manuscript.

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SCIENTIFIC NOTE

Propagation of macadamia (*Macadamia integrifolia* Maiden & Betche) by cuttings

Propagación de macadamia (*Macadamia integrifolia* Maiden & Betche) por estacas

Vitor Hugo Domingos da Silva[1], Mariana Nunes Vieira de Melo[1], Gabriel Constantino Blain[2], Marcos José Perdoná[3], and Graciela da Rocha Sobierajski[4*]

ABSTRACT

Macadamia trees require a long period for flowering when propagated by seeds. To anticipate the yield, maintain cultivar characteristics, and increase the homogeneity of nut quality, it is essential to establish orchards with grafted trees. Although semi-hardwood cutting propagation tests have been performed in Brazil, no method has been shown to be appropriate at a large scale due to the difficulties in implementation of techniques or the high cost. Establishing an effective and affordable protocol may provide great improvement to this productive chain since it will meet the demand of nurseries and stimulate the expansion of orchards. The aim of this study was to develop a protocol for macadamia semi-hardwood cutting propagation. Six cultivars (HAES 344, HAES 816, HAES 660, IAC 4-12B, IAC 9-20, and IAC 4-20) were evaluated and selected because of their ample cultivation in Brazil. The propagation material was collected for four months (October and November 2018, and February and March 2019). The semi-hardwood cuttings were also soaked in indole butyric acid (IBA) at three different concentrations (0, 5, or 10 g L^{-1}) and in the commercial product Clonex®. The experiment was arranged in a two-way completely randomized design with four replicates and data were analyzed by R software. The cultivar IAC 4-12B showed the highest index for root development (37.0%). The treatments did not show significant differences between IBA doses and Clonex® for root development (IBA 10 g L^{-1} - 31.5%, Clonex® - 29.4%, and IBA 5 g L^{-1} - 27.4%). November was the best cutting season for root development of semi-hardwood cuttings (10.0%).

Key words: indole butyric acid, rooting, cultural practices.

RESUMEN

Los árboles de macadamia requieren un largo período para empezar la floración cuando se propagan por semillas. Para anticipar el rendimiento, mantener las características del cultivar y aumentar la homogeneidad de la calidad de las nueces, es esencial establecer huertos con árboles injertados. Aunque se han realizado ensayos de propagación de esta planta por estacas semileñosas en Brasil, ningún método ha demostrado ser el apropiado a gran escala debido a las dificultades en la implementación de las técnicas o al alto costo. El establecimiento de un protocolo eficiente y accesible puede proporcionar una gran mejora a esta cadena productiva ya que satisfará la demanda de viveros y estimulará la expansión de los huertos. El objetivo de este estudio fue desarrollar un protocolo para la propagación de macadamia por estacas semileñosas. Seis cultivares (HAES 344, HAES 816, HAES 660, IAC 4-12B, IAC 9-20, e IAC 4-20) fueron evaluados y seleccionados por su amplio cultivo en Brasil. El material de propagación se recolectó durante cuatro meses (octubre y noviembre de 2018 y febrero y marzo de 2019). Las estacas semileñosas se sumergieron en ácido indolbutírico (AIB) en tres concentraciones diferentes (0, 5, o 10 g L^{-1}), y en el producto comercial Clonex®. El experimento se realizó siguiendo un diseño bidireccional con cuatro repeticiones y los datos fueron analizados por el software R. El cultivar IAC 4-12B mostró el índice más alto para el desarrollo de las raíces (37.0%). Los tratamientos no mostraron diferencias significativas entre las dosis de AIB y Clonex® para el desarrollo de las raíces (AIB 10 g L^{-1} - 31.5%, Clonex® - 29.4%, AIB 5 g L^{-1} - 27.4%). Noviembre fue la mejor temporada de recolección para el enraizamiento de las estacas semileñosas (10.0%).

Palabras clave: ácido indolbutírico, enraizamiento, prácticas culturales.

Introduction

The macadamia (*Macadamia integrifolia* Maiden & Betche) is a nut tree belonging to the Proteaceae family, native to tropical and subtropical Australian forests (Peace *et al.*, 2005). It was introduced into Brazil in 1931 (Dierberger & Marino Netto, 1985) and in 1940 the Agronomic Institute began the only macadamia breeding program in the

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country (Sobierajski *et al*., 2006). Orchards are generally established by using grafted trees to anticipate yield, maintain cultivar characteristics, and increase the homogeneity of nut quality (Melo *et al.*, 2019). The technique usually used for vegetative propagation is grafting (Campo-Dall'Orto *et al*., 1988). It takes 18 months for the seedling to be ready for planting in the orchard (Russell *et al*., 2016). In previous studies, the rate of grafting success reached 80% (Campo-Dall'Orto *et al*., 1983). However, this rate is lower than 50% in commercial nurseries. These factors increase the cost of seedlings and reduce their availability to growers making it difficult to expand the culture in Brazil (Melo *et al*., 2019).

The macadamia tree produces a nut of great acceptance and high value in the international market (Penoni *et al*., 2011; Maro *et al*., 2012). In recent years, there has been an increase in the demand for seedlings of macadamia in Brazil (Perdoná & Soratto 2015; 2016).

The propagation by semi-hardwood cuttings can reduce the time and costs of seedling production (Bell, 1993). Therefore, establishing an effective and affordable protocol of seedling production by semi-hardwood cuttings will provide an advance for macadamia in Brazil, stimulating orchard expansion.

Pereira *et al.* (1987) studied the rate of rooting in cuttings of five cultivars from two seasons. The authors observed significant differences for cultivars and seasons but no interactions between them. The emission of roots in cuttings of macadamia is the major difficulty for vegetative propagation. Therefore, the use of synthetic growth regulators, such as auxin can be an alternative to overcoming this problem (Garbelini, 2009). Another option is the use of plant growth regulators, e.g., indole butyric acid (IBA) that, when compared to a synthetic auxin, shows lower sensitivity to biological degradation (Fachinello *et al*., 2005). These plant growth regulators also stimulate root growth (Alvarenga, 1990). However, the ideal IBA concentration for cutting immersion varies among different species and cultivars (Hartmann *et al*., 2002), and it is still unknown for macadamia. On such a background, the aim of this study was to identify the macadamia cultivars with the best rooting performance of semi-hardwood cuttings and test different IBA concentrations and seasons for seedling production by semi-hardwood cuttings.

Materials and methods

Six macadamia cultivars were propagated by semi-hardwood cuttings. The percentage of survival, callus formation, and rooting were evaluated. The cultivars were selected for their characteristics of yield and nut quality. The cultivars HAES 344, HAES 816, and HAES 660 were developed by the Hawaii Agricultural Experiment Station (HAES) and the Agronomic Institute (IAC) developed the cultivars IAC 4-12B, IAC 9-20, and IAC 4-20.

The semi-hardwood cuttings were collected from 12-year-old trees in Dois Córregos, São Paulo, Brazil, for four months (October and November 2018, and February and March 2019). The materials were cut from the medium treetop with 15-20 cm of length and 3-5 cm of diameter; three leaves were kept for all cuttings, as recommended by Russell *et al.* (2016). The semi-hardwood cuttings were soaked in IBA (0, 5, or 10 g L^{-1}) (NEON®, Suzano, Brazil) for 15 min, followed by planting in a propagation tube (120 ml) filled with substrate (peat-vermiculite-limestone, Carolina Soil®, Pardinho, Brazil). In addition, the tips of cuttings collected in November, February and March were also soaked in Clonex® (IBA 3 g L^{-1}, for 15 min) (Growth Technology, Taunton, UK). To prevent the cuttings from drying out, they were planted on the same day of collection in a mist-house.

The humidity in the mist-house was maintained using a controller (Fascitec NTI 12 - AC, São Bernardo do Campo, Brazil) for intermittent watering and to avoid accumulation of water on the leaves. The experimental design was a two-way completely randomized design with four replicates and five cuttings by plot/month, of which 20 cuttings were the control treatment (distilled water). Thus, 1,800 cuttings were evaluated.

After 120 d, the semi-hardwood cuttings were removed from the tubes, washed, and evaluated for survival, callus, and root development. The data were transformed by the BoxCox method (Box & Cox, 1964) to meet the normality assumption. The data were analyzed using the R software (R Development Core Team, 2019), and the cluster analyses and threshold were calculated using the R-package 'factoextra' (Kassambara & Mundt, 2017).

Results and discussion

From the 1,800 semi-hardwood cuttings, 9.8% showed callus, 5.7% developed roots, and 84.5% did not survive. This high mortality caused the data to be unbalanced, which prevented the use of an analysis of variance. The statistical analysis was performed by descriptive and multivariate analysis.

The best season for removing semi-hardwood cuttings for the presence of callus was October 2018 (22.8%), considering all cultivars and doses of IBA and Clonex® (Fig. 1A). However, the best results for root development were observed for those plants collected in November 2018 (10.0%; Fig. 1B). The lowest semi-hardwood cutting mortality was observed when the removals occurred in October 2018 (71.9%), while the highest rates occurred in February 2019 (90.2%) and March 2019 (92.3%; Fig. 1C).

The control treatment (without IBA application) showed the highest rate for the presence of callus (42.4%), considering all cultivars and collecting seasons (Fig. 2A). However, this treatment showed the lowest rooting rate (11.8%). The other treatments showed no statistical differences for root development with 31.4% for 10 g L^{-1} IBA, 29.4% for Clonex®, and 27.4% for 5 g L^{-1} IBA (Fig. 2B). The mortality was statistically lower in semi-hardwood cuttings treated with Clonex® (19.5%) than with the other doses of IBA (Fig. 2C).

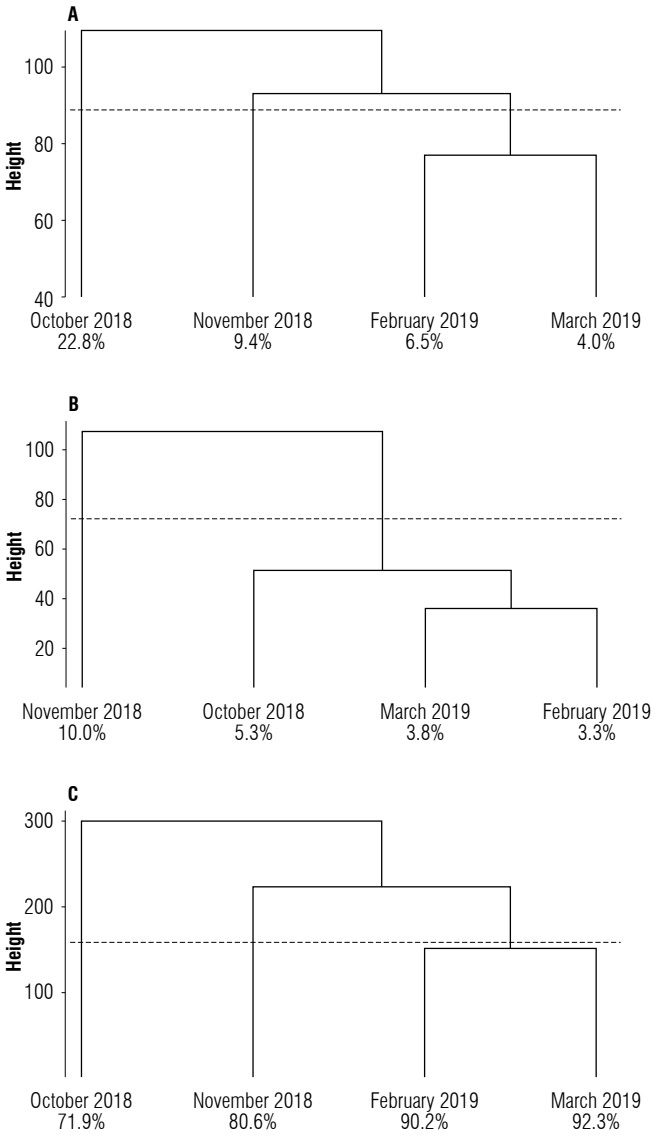

FIGURE 1. Similarity matrix (Euclidean distance) among collection seasons of semi-hardwood cuttings of *Macadamia integrifolia*, considering all cultivars and doses of IBA and Clonex®. The similarity matrix considered the following parameters: A) presence of callus, B) root development, and C) mortality. The grouping method (cluster analyses) was based on the K-means algorithm (Kassambara & Mundt, 2017).

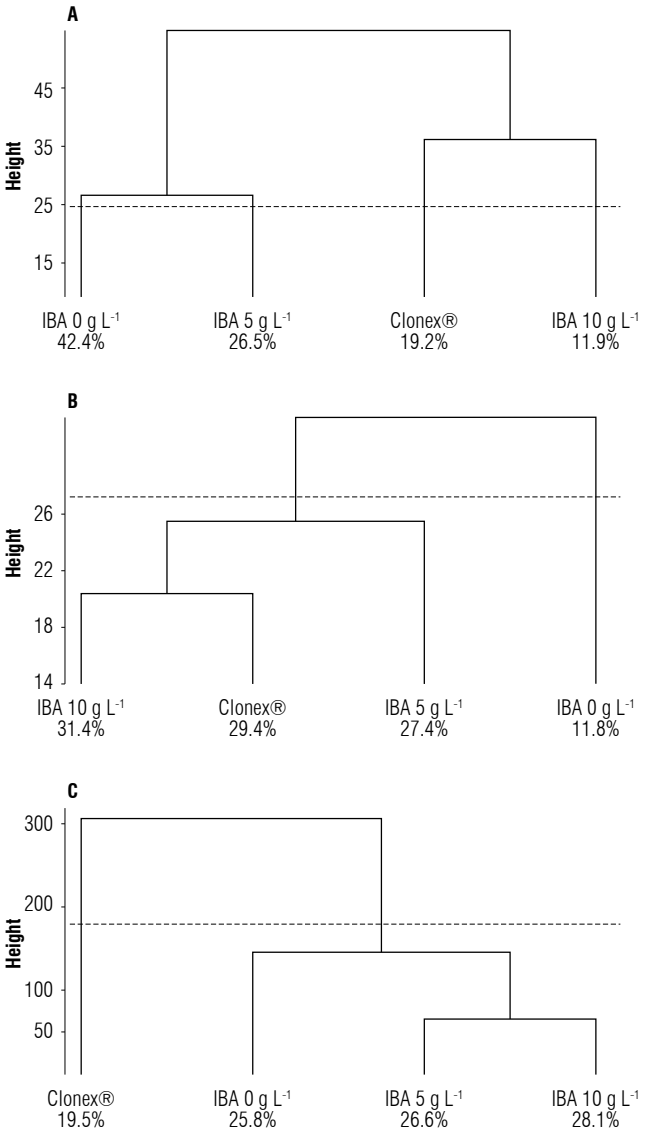

FIGURE 2. Similarity matrix (Euclidean distance) among doses of indole butyric acid (IBA) and Clonex® for semi-hardwood cuttings of *Macadamia integrifolia* treatment, considering all cultivars and collecting seasons. The similarity matrix considered the following parameters: A) presence of callus, B) root development, and C) mortality. The grouping method (cluster analyses) was based on the K-means algorithm (Kassambara & Mundt, 2017).

The cultivar HAES 660 showed the highest presence of callus (35.6%) considering all seasons of collection and doses of IBA and Clonex® (Fig. 3A). However, only 12.0% of the semi-hardwood cuttings of this cultivar effectively developed roots (Fig. 3B). Regarding root development, the cultivar IAC 4-12B showed the highest rate (37.0%), followed by the second group (formed by 'IAC 9-20' and 'IAC 4-20') that showed the second-highest performance (Fig. 3B). The mortality rates by cultivar were similar to each other, but the cultivars HAES 660 (14.8%) and IAC 4-12B (15.6%) showed the lowest values (Fig. 3C).

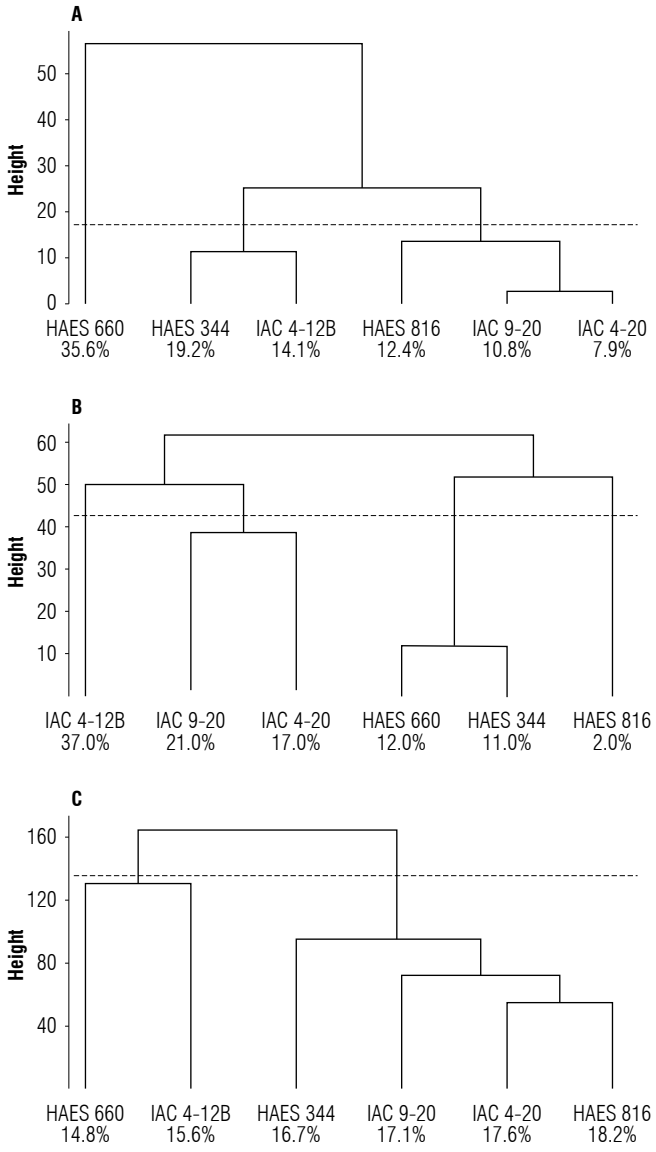

FIGURE 3. Similarity matrix (Euclidean distance) among cultivars of *Macadamia integrifolia* 'IAC 4-12B', 'IAC 9-20', 'IAC 4-20', 'HAES 344', 'HAES 660' and 'HAES 816', considering all collecting seasons and doses of IBA and Clonex®. The similarity matrix considered the following parameters: A) presence of callus, B) root development, and C) mortality. The grouping method (cluster analyses) was based on the K-means algorithm (Kassambara & Mundt, 2017).

The descriptive analysis regarding the cultivars in all IBA doses and cutting seasons showed 'HAES 660' as the best cultivar regarding the presence of callus (60%), also exhibiting the lowest mortality (40%). These rates were observed for those plants collected in October 2018 and subjected to the control treatment (Tab. 1). The callus is formed by new meristematic cells near the phloem (Hartmann *et al.*, 2002). The presence of calluses in species of hard rooting can precede root emission (Bitencourt *et al.*, 2010). However, the development of callus and roots can be regarded as independent events. For instance, Singh and Ansari (2014) observe high rates for callus development in *Dalbergia latifolia* (91.5%) and *Gmelina arborea* (75.0%) in the control treatment, without these calluses evolving into roots (3.1 and 0%, respectively). The authors suggest that the presence of callus in treatments without hormones is a reaction of the plant to restore the injured cutting and not a prerequisite for root induction. These two events may simultaneously occur, because both processes involve cell division and depend on favorable conditions (Preti *et al.*, 2012).

Regarding root development, 'IAC 4-20' showed the highest rate of rooting (30%) when collected in November 2018 and subjected to treatment with IBA at a dose of 5 g L^{-1} (Tab. 1). For this variable, all cultivars developed by the Agronomic Institute showed higher results than those found for the Hawaiian cultivars. Cultivar IAC 4-12B was the most stable with 20, 20, 15 and 25% of rooting in November 2018 (IBA doses 0, 5, 10 g L^{-1}, and Clonex®, respectively; Tab. 1). This cultivar also showed high values of rooting in the February collection season (20% - Clonex®) and March 2019 (25% for IBA dose of 10 g L^{-1} and 20% for Clonex®; Tab. 1). Cultivar IAC 9-20 also showed a high rooting rate (20%) in the October 2018 collection season in semi-hardwood cuttings treated with 10 g L^{-1} of IBA (Tab. 1). Russell *et al.* (2016) observe a rooting rate of 44% in *M. integrifolia* cultivars. Entelmann *et al.* (2014) obtain rates of 22.9% of rooting when studying the cultivar Aloha 10-14 with an IBA concentration of 3 g L^{-1}.

According to our data, the capability of the cultivars for rootstocks for the presence of callus, rooting, and survival were classified as nine distinct groups by the cluster analyses (Fig. 4). Cluster 3 was formed by 'IAC 4-20' (IBA at a dose of 5 g L^{-1}), 'IAC 4-12B' (IBA at a dose of 5 g L^{-1}) and 'IAC 4-20' (control treatment). These cultivars showed the highest rooting rates and moderate presence of callus. Cluster 7 was formed by the cultivars with lowest mortality rates ('IAC 9-20' - control treatment and 'HAES 660' - IBA at doses of 5 and 10 g L^{-1}). The cultivars grouped into cluster

TABLE 1. Percentage of semi-hardwood cuttings with presence of callus (Callus), root development (Root) and mortality (Mort.) in macadamia (*Macadamia integrifolia*) cultivars collected in four seasons and treated with indole butyric acid (IBA; g L^{-1}) and Clonex®.

Cultivar	Dose IBA	October 2018			November 2018			February 2019			March 2019		
		Callus	Root	Mort.	Callus	Root	Mort.	Callus	Root	Mort.	Callus	Root	Mort.
HAES 344	0	50	0	50	10	15	75	0	0	100	5	0	95
	5	50	0	50	5	0	95	0	0	100	5	0	95
	10	10	5	85	0	15	85	0	5	95	0	5	95
	Clonex®	NA	NA	NA	15	10	75	0	0	100	20	0	80
HAES 660	0	60	0	40	30	0	70	0	0	100	5	0	95
	5	25	15	60	25	10	65	20	5	75	15	0	85
	10	30	10	60	25	1	70	15	0	85	0	0	100
	Clonex®	NA	NA	NA	20	5	75	35	5	60	10	5	85
HAES 816	0	15	0	85	5	0	95	0	0	100	0	0	100
	5	25	5	70	5	0	95	10	0	90	0	0	100
	10	15	5	80	0	0	100	0	0	100	0	0	100
	Clonex®	NA	NA	NA	10	0	95	20	0	80	5	0	95
IAC 4-12B	0	20	0	80	30	20	50	10	0	90	15	5	80
	5	20	15	65	5	20	75	0	5	95	0	5	95
	10	0	0	100	0	15	85	0	10	90	0	25	75
	Clonex®	NA	NA	NA	10	25	65	5	20	75	10	20	70
IAC 4-20	0	25	0	75	15	10	75	10	0	90	0	0	100
	5	20	0	80	0	30	70	0	5	95	0	0	100
	10	0	10	90	0	10	90	0	5	95	0	0	100
	Clonex®	NA	NA	NA	0	15	85	0	0	100	0	0	100
IAC 9-20	0	40	0	60	5	10	85	20	0	80	5	0	95
	5	0	10	90	5	5	90	0	10	90	0	0	100
	10	5	20	75	5	5	90	0	0	100	0	10	90
	Clonex®	NA	NA	NA	0	10	90	10	10	80	0	15	85

Clonex® - commercial product; NA - not evaluated.

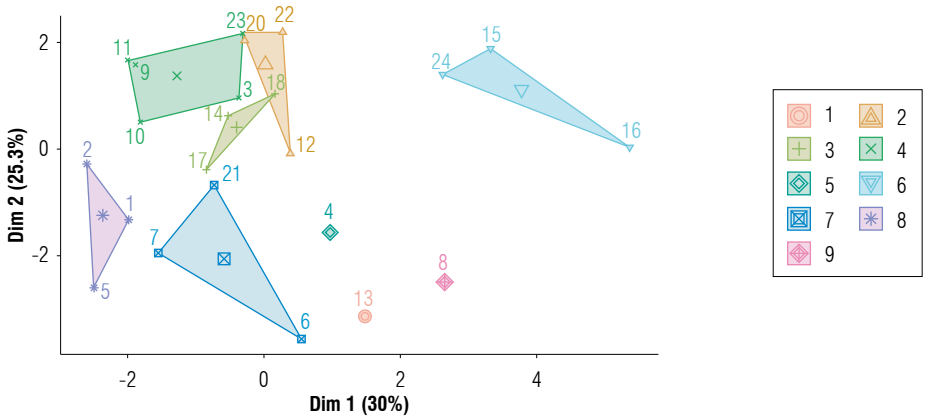

FIGURE 4. Grouping by multivariate features (cultivars, indole butyric acid, and collecting seasons) of semi-hardwood cuttings of *Macadamia integrifolia*. Cultivars (dose of IBA - g L^{-1}): 1 - HAES 344 (0), 2 - HAES 344 (5), 3 - HAES 344 (10), 4 - HAES 344 (Clonex®), 5 - HAES 660 (0), 6 - HAES 660 (5), 7 - HAES 660 (10), 8 - HAES 660 (Clonex®), 9 - HAES 816 (0), 10 - HAES 816 (5), 11 - HAES 816 (10), 12 - HAES 816 (Clonex®), 13 - IAC 4-12 B (0), 14 - IAC 4-12 B (5), 15 - IAC 4-12 B (10), 16 - IAC 4-12 B (Clonex®), 17 - IAC 4-20 (0), 18 - IAC 4-20 (5), 19 - IAC 4-20 (10), 20 - IAC 4-20 (Clonex®), 21 - IAC 9-20 (0), 22 - IAC 9-20 (5), 23 - IAC 9-20 (10), and 24 - IAC 9-20 (Clonex®). The threshold was obtained by the K-means algorithm (Kassambara & Mundt, 2017).

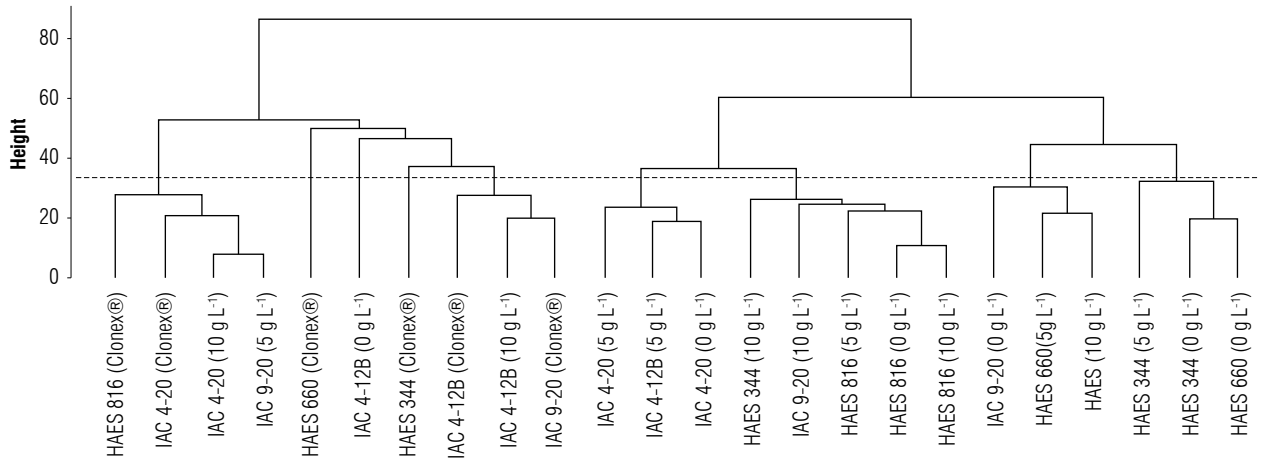

FIGURE 5. Similarity matrix (Euclidean distance) among *Macadamia integrifolia* cultivars HAES 816, HAES 660, HAES 344, IAC 9-20, IAC 4-20, and IAC 4-12 B subjected to treatment with indole butyric acid (0 g L^{-1}, 5 g L^{-1} and 10 g L^{-1}) and Clonex® and collected in different seasons. The threshold was obtained by the K-means algorithm (Kassambara & Mundt, 2017).

8 ('HAES 344' - control treatment and IBA at a dose of 5 g L^{-1}, and 'HAES 660' - control treatment) showed a high rate of presence of callus; however, these cultivars did not develop roots. These clusters are in agreement with the dendrogram obtained by the similarity matrix (Euclidean distance) (Fig. 5).

The high mortality rates observed in this experiment can be related to diseases caused by high environmental moisture, probably due to the identified presence of *Cladosporium* sp. on the leaves of the semi-hardwood cuttings. High mortality rates (46.2%) were also observed by Russell *et al.* (2016) evaluating the rooting ability of 32 macadamia cultivars in Australia. These authors found fungi from *Nectria* spp., seriously affecting *M. integrifolia*.

Conclusions

Considering all the cultivars evaluated in this study, the macadamia cultivar IAC 4-12B showed the highest rooting. This cultivar, along with HAES 660, showed the lowest mortality rate. Therefore, it can be concluded that IAC 4-12B is an interesting option for macadamia producers.

Although there were no statistical differences between indole butyric acid and Clonex® for root development, this last treatment showed the lowest mortality.

Under the conditions of Brazil, November showed the highest rooting values for semi-hardwood cuttings, and October showed the highest values for the presence of callus and low mortality for semi-hardwood cuttings.

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Author's contributions

GRS and MJP formulated the overarching research goals and aims. GRS and GCB implemented the computer code and supporting algorithms. GRS and GCB applied the statistical and computational techniques to analyze the study data. VHDS, MNVM and MJP conducted the research and performed the experiments and data collection. GRS and MJP provided the study materials, reagents, and computing resources for analysis. GRS, VHDS and MNVM managed the activities to annotate and maintain research data for initial use and later re-use. GRS, GCB, and MJP prepared the published work and specifically wrote the initial draft. GRS and GCB carried out the critical review, commentary and revision of 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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Basic information about the use of APA for the list of references is available here: https://apastyle.apa.org/style-grammar-guidelines/references. In order to illustrate these standards, authors can check some examples about how to create each item of the list of references, keeping in mind the type of publication cited as follows (click on each option to open APA web information):

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Published dissertation or thesis references

Example: Franco, C. V. (2012). *Efecto de la colchicina sobre el número cromosómico, número de cloroplastos y características morfológicas del fruto en ecotipos de uchuva (*Physalis peruviana *L.) Colombia, Kenia y Perú* [Undergraduate thesis, Universidad Francisco de Paula Santander]. UFPS Library. http://alejandria.ufps.edu.co/descargas/tesis/1610259.pdf

Whole book

Example: Suescún, L., Sánchez, E., Gómez, M., García-Arias, F. L., & Núñez Zarantes, V. M. (2011). *Producción de plantas genéticamente puras de uchuva*. Editorial Kimpres Ltda.

Edited book chapter

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