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FOLIAR AND ROOT INOCULATION OF LETTUCE PLANTS WITH BACTERIA ISOLATED FROM MELIPONIDS IN COMBINATION WITH ARBUSCULAR MYCORRHIZAL FUNGI AND PHOSPHORUS APPLICATION

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ABSTRACT

This research evaluated the plant growth-promoting effects of the inoculation of bacterial strains isolated from meliponids (stingless bees) in lettuce. Two bioassays were performed under greenhouse conditions. In the first bioassay, eleven bacterial strains previously isolated from meliponids were inoculated through leaves or roots of lettuce seedlings; greenness index (SPAD units), leaf area, and dry weight (aerial, root and total) were evaluated using a completely randomized design, and the two bacteria with the highest plant-promoting capacity were selected. In the second bioassay, the selected strains were inoculated in combination with arbuscular mycorrhizal fungi (AMF; *Claroideoglomus claroideum* and *Funneliformis mosseae*) and three doses of phosphate fertilization (11, 22 and 44 μg P mL^{-1}); fresh weight, leaf area, dry weight (aerial, root and total), and mycorrhizal colonization were assessed using a 4x2x3 factorial design. In the first bioassay, the results showed that the evaluated bacteria had positive effects on all variables, with *Pantoea anthophila* and *Serratia nematodiphila* being the most prominent strains as plant growth promoters. In the second bioassay, significant differences were observed between the inoculated plants and the control, particularly in dry weight and leaf area. AMF-inoculation did not show significant differences compared to the bacterial combination. In addition, the application of 22 μg P mL^{-1} with the combined inoculation of *Pantoea anthophila* and *Serratia nematodiphila* had a significant impact on plant growth. Overall, the use of bacteria isolated from stingless bees enhances plant growth and, combined with AMF and/or low or medium doses of P-fertilization, constitutes a good strategy to produce lettuce plants.

Keywords: meliponids, stingless bees, *Pantoea anthophila, Serratia nematodiphila*, phosphorus.

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INTRODUCTION

Stingless bees (meliponids) are eusocial insects that live in colonies in tropical and subtropical regions throughout the world, being one of the few social groups recorded in America (Yurrita et al., 2017). Since ancient times, dating back to the Mayan civilization, meliponid bees have been considered important pollinators and honey producers, and thus they have acquired ecological, cultural and economic relevance (Ayala et al., 2013). The traditional identification methods of these insects are based on their behavior, sensory perception, morphological characteristics and functionality or utility (Zamudio and Hilgert, 2012). The study of this group of bees, like those of the *Apis melifera* species, is based on the quality of honey in terms of nutritional properties (vitamins and minerals) and antioxidant capacity, offering significant medicinal benefits (Cauich et al., 2015). Specifically, honey from stingless bees may have different properties when compared to that produced by *A. melifera*, since they have greater exploration areas and floristic diversity for foraging (Álvarez-Suarez et al., 2018).

In nature, there are complex relationships among several groups of organisms. As an example, insects and beneficial microorganisms establish important mutualistic relationships. For instance, insects may benefit bacteria by providing adequate conditions for their growth and survival, either outside or inside bee bodies, while bacteria help insects by improving nutritional status, contributing to physiological functions, and providing protection against pathogens (Zheng et al., 2018; Arias 2019; Khan et al., 2020), in turn enhancing insect capacity to adapt to new environments.

The benefits of bacteria for plants are related to biochemical properties such as nitrogen fixation and phosphate solubilization, which is reflected in increases on greenness index, chlorophyll synthesis, photosynthetic activity and leaf growth (García et al., 2019; Wu et al., 2019). On the other hand, arbuscular mycorrhizal fungi (AMF) are root symbionts of terrestrial plants (Diagne et al., 2020). The AMF provide multiple benefits as plant growth promoters and are feasible alternative for improving plant production due to benefits related to nutrient uptake, protection against pathogens, and resistance to drought and other stress conditions (Khaliq et al., 2022), as well as enhancing metabolic activity in mycorrhizal plants (Cela, 2022).

Lettuce (*Lactuca sativa* L.) is one of the most consumed vegetables, produced intensively all over the world. This plant species tolerate a wide temperature range (-12 °C to 35 °C), with an optimal temperature for growth of 18 °C (Lamichhane et al., 2018). As the edible part is the leaf, lettuce is typically harvested during its vegetative stage, which has an important economic value in the market (Medina et al., 2021). In fact, this plant species has important nutritional properties, including low calories, phenolic compounds, vitamins, and minerals, and is a fundamental part of the human diet due to its health benefits (Shi et al., 2022).

Previous research has proven the efficacy of combining beneficial microorganisms such as plant growth-promoting bacteria (PGPB) and AMF to promote plant growth of lettuce (Laurindo and Cruz, 2024; Gutiérrez et al., 2019). Nevertheless, there is limited information about the use of bacteria isolated from insects, particularly from stingless bees, in combination with AMF, in the production of lettuce plants. Therefore, this research evaluated the growth-promoting effects of the combined utilization of bacteria isolated from meliponids (stingless bees) and AMF, with the application of three doses of phosphate fertilization in nutrient solution on lettuce plants grown under greenhouse conditions.

MATERIALS AND METHODS

Bioassay 1. Evaluation and screening of bacterial strains isolated from meliponids as growth promoters of lettuce seedlings

The experiment was carried out from September to October 2020 under greenhouse conditions, with a maximum temperature of 25 °C, and a relative humidity of 75 %. The substrate used was a mixture of vermiculite and perlite (1:1 v/v) previously autoclaved at 120 °C for 2 h during two consecutive days.

The bacterial strains utilized in the experiment were selected from the microbial collection of strains isolated from body surface, digestive tract, and nest of meliponid bees (*Scaptotrigona pectoralis, Frieseomelitta nigra, and Melipona bechii*) from Campeche, Mexico (Baqueiro 2020; Ferrera-Rodriguez et al., unpublished data). Of these, eleven bacterial strains were selected based on previous characterization of important physiological activities for agriculture, and biochemical tests aimed to detect their potential as PGPB in accordance with the Bergey's Manual (Claus and Berkeley 1984). Selected strains were *Pantoea anthophila* (S38; Genbank access code MN733038), *P. anthophila* (T38; Genbank access code MN733052), *P. anthophila* (T83; Genbank access code MN733062), *P. anthophila* (T84; Genbank access code MN733063), *Bacillus* sp. (T69; Genbank access code MN733066), and

Pseudomonas psychrotolerans (T82; Genbank access code MN733061), which were isolated from the digestive tract of *S. pectoralis*. In addition, *Bacillus* sp. (T70; Genbank access code MN733057) and *Sinorhodobacter* sp. (T31; Genbank access code MN733050), were isolated from the body surface of *F. nigra*. Strains isolated from *M. bechii* corresponded to *Serratia nematodiphila* (15; Genbank access code MW857195) obtained from the digestive tract, whereas *Staphylococcus arlettae* (18; Genbank access code MW857196) and *Staphylococcus kloosii* (27; Genbank access code MW857197) were isolated from the bee nest.

Seed germination was performed by using an inert substrate composed of vermiculite and perlite in proportions (1:1 v/v) previously autoclaved for 2 h at 120 ºC for two consecutive days. Plastic seedbeds with 180 cavities were disinfected with 70% alcohol and filled up to three-quarters of their total capacity with the substrate. Subsequently, 5 seeds of lettuce variety Parrish Island (90% germination), previously disinfected with 5% sodium hypochlorite for 30 s and rinsed with sterile distilled water, were sown, and then covered with the same substrate.

Bacterial inocula were prepared after each bacterial strain was reactivated in liquid medium Luria Bertani (Lb) (Birruga et al., 2020). Later, they were placed on Petri dishes with Lb solid medium for further distinction and characterization of pure bacterial colonies. For the preparation of each bacterial inoculum, 30 mL of nutrient broth (Merck, Mexico) were set in Falcon tubes, and sterilized at 120 °C for 18 min. A colony of the reactivated bacterial strain was transferred into the corresponding tube, and vortexed. Subsequently, all tubes were stirred (160 rpm) at 28 °C for 3 days, until obtaining a bacterial population of $7x10^8$ colony forming units (CFU) estimated by means of the serial dilution method. Furthermore, an aliquot of 30 µL of each bacterial suspension was placed in new tubes with sterile nutrient broth medium; these tubes were maintained under agitation (160 rpm) at 28 °C for 3 days. Once the bacterial concentration of $7x10^8$ CFU was reached, the tubes were centrifuged at 7000 rpm for 10 min to eliminate the liquid medium. The remaining bacterial pellet was re-suspended with sterile water for preparing each bacterial inoculum by inducing the homogenization with vortex and maintaining a bacterial population of $7x10^8$ cells mL^{-1} for further plant inoculation.

Eight-day-old seedlings, grown in 10-cavity trays per treatment, were inoculated when presenting two true leaves, and only one seedling was kept per each seedbed cavity. Based on the purpose of this bioassay, two different types of bacterial inoculation were carried out: 1) root inoculation, by applying 1 mL of each bacterial suspension directly at the stem base, and 2) foliar inoculation, by spraying each bacterial strain on leaves avoiding cross-bacterial contamination. For foliar inoculation, each inoculum was applied with an atomizer, previously disinfected with 5% sodium hypochlorite. A volume of 5 mL of bacterial suspension was placed in each atomizer and homogenized. The inoculum was carefully sprayed onto all the leaves of the plant at a height of 10 cm from the leaves. This step was repeated for each plant for the corresponding treatment.

After 25 days of bacterial inoculation, greenness index (SPAD readings) was measured in leaves (Spadmeter, Minolta, SPAD-502, Japan). Subsequently, plants were harvested to measure leaf area (Leaf area meter, LI 3000, Inc. Lincoln, NE, USA). Then, leaves were placed in paper bags and dried at 70 °C for 72 h, and their dry weight was estimated with an analytical scale.

For this bioassay, a completely randomized experimental design was used, in which 24 treatments with 10 repetitions were established. Eleven treatments corresponding to each bacterial strain and a control (without bacterial inoculation) were evaluated. Normality of data was assessed using the Shapiro-Wilk test ($P < 0.05$). Data were analyzed using an analysis of variance and means were compared using the Tukey's test (α=0.05). All statistical analyses were performed by using SAS program.

Bioassay 2. Effects of the selected bacterial strains isolated from meliponids, combined with arbuscular mycorrhizal fungi and three doses of phosphorus fertilization, on lettuce plants

The experiment was carried out under greenhouse conditions from November to December 2020, with a maximum and minimum temperature of 17 °C and 10 °C, respectively, and an average relative humidity of 75 %. The substrate consisted of a mixture of vermiculite and perlite (1:1 v/v) previously autoclaved for 2 h at 120 °C, for two consecutive days.

From the previous bioassay, the two bacterial strains that showed the highest capacity to enhance growth and development in lettuce plants were selected. The strains *Pantoea anthophila* (T38) and *Serratia nematodiphila* (15) were selected for foliar and root inoculations, respectively. These bacteria were combined with the inoculation of an arbuscular mycorrhizal consortium composed of the species *Claroideoglomus claroideum* and *Funneliformis mosseae*, which has previously been evaluated as a growth promoter of horticultural species (Angulo-Castro et al., 2018; Carballar

et al., 2018). The treatments inoculated with all these microorganisms were also combined with three doses of phosphorus (P) fertilization (11, 22 and 44 μg mL^{-1}), applied with the Long Ashton nutrient solution (Hewitt, 1966).

For seed germination, an inert substrate composed of vermiculite and perlite was utilized (1:1 v/v). The substrate was previously autoclaved for 2 h at 120 ºC for two consecutive days. Plastic seedbeds with 180 cavities were utilized and previously disinfected with 70% alcohol. Thus, 24 seedbeds of 10 cavities each were established, applying two mycorrhizal inoculation conditions: 1) with the AMF consortium, and 2) without AMF-inoculation.

For the treatments with AMF consortium inoculation, 5 g of the inoculum were applied; this inoculum was previously propagated in river sand using alfalfa (*Medicago sativa*) as a trap plant. The AMF inoculum had 200 spores per 5 g, and root fragments with 35 % of total colonization. On the AMF inoculum, 10 lettuce seeds, previously disinfected with 5 % sodium hypochlorite and rinsed with sterile distilled water, were sown and then covered with another layer of the sterile substrate. For the treatments without AMF inoculation, the seedbed cavities were simply filled up to three quarters of their total capacity, and then 10 lettuce seeds were sown, and covered with another layer of sterile substrate.

The inoculum of each bacterial strain was prepared as described in bioassay 1, and adjusted for applying $6x10^8$ bacterial cells mL^{-1} to the corresponding treatments. Bacterial inoculation was performed after 8 days of seed germination when seedlings showed two true leaves. Therefore, only two lettuce seedlings were kept per cavity after 5 days of inoculation. As in the previous bioassay and according to the selected bacterial strain, root and foliar inoculations were carried out, respectively. Thus, inoculation of *S. nematodiphila* was applied in roots of 10 seedlings with 1 mL of the bacterial inoculum, whereas the inoculation of *P. anthophila* was applied by spraying the inoculum on leaves of 10 seedlings at the corresponding treatments.

After 20 days of bacterial inoculation, seedlings were transplanted from seedbeds to plastic pots containing 500 g of the substrate previously described. Transplanting was carefully performed to avoid the loss of AMF-inoculum in root balls from those seedbeds in which the AMF consortium was inoculated. After 24 h of transplanting, a second bacterial inoculation was performed according to its form of inoculation in the corresponding treatments by applying the same amount of inoculum as accounted at the first bacterial inoculation.

Additionally, the three doses of P were applied by using Long Ashton nutrient solution, which was modified to apply 11, 22, and 44 μg P mL^{-1}. The P-fertilization was applied three days after bacterial inoculation.

After 35 days of bacterial inoculation, greenness index (SPAD readings) was measured. Subsequently, plants were harvested for determining fresh weight, dry weight (leaves, root and total), leaf area and root mycorrhizal colonization. The greenness index was measured using a Spadmeter (Minolta, SPAD-502, Japan) by taking three readings from mature leaves of each plant, and considering 8 plants per treatment (Singh et al., 2019).

Fresh weight of shoots was measured with a portable scale, and the leaf area was determined by using a leaf area meter (LI 3000, Inc. Lincoln, NE, USA). Then, leaves and roots were placed in paper bags, and dried at 70 ° C for 72 h, to determining shoot and total dry weight.

A three factorial experiment (4x2x3) was established by considering 4 levels of bacterial inoculation (without inoculation, *P. anthophila*, *S. nematodiphila*, and their combination), 2 levels of mycorrhizal inoculation (with and without AMF), and three levels of P (11, 22, and 44 μg P mL^{-1}), yielding 24 treatments in total with 8 replicates each, and distributed in a completely randomized experimental design. Normality of data was assessed using the Shapiro-Wilk test ($P < 0.05$). Furthermore, data were analyzed with the SAS program using analysis of variance and means comparison test (Tukey, α=0.05).

RESULTS

Bioassay 1. Evaluation and screening of bacterial strains isolated from meliponids as growth promoters of lettuce seedlings

Greenness index was significantly enhanced due to bacterial inoculation of lettuce plants when compared to the control (Fig. 1A-B), recording a 52% increase regardless of the type of inoculation. The highest greenness index values were recorded with *Pantoea anthophila* (T38) (Fig. 1A) and *Sinorhodobacter* sp. (T31) for foliar and root inoculations, respectively (Fig. 1B).

All treatments with bacterial inoculation on leaves showed significant differences (Tukey, α=0.05) in the leaf area in comparison to the control. The value achieved by inoculating *P. anthophila* (T38) was significantly higher when compared to the rest of the inoculated treatments and to the control (Fig. 1C). In regards to root inoculation, *Serratia nematodiphila* (15) significantly increased leaf area (Tukey, α=0.05) as compared to plants

inoculated with *Sinorhodobacter* sp. (T31) or control plants (Fig. 1D).

Regarding shoot dry weight, foliar inoculation with *P. anthophila* (T38) recorded a significantly higher values (Tukey, α=0.05) when compared to those observed with the inoculation of *Sinorhodobacter* sp. (T31) or control plants (Table 1). For root inoculation, all bacteria showed significant differences (Tukey, α=0.05) as compared to the control. The highest value was obtained by inoculating *Serratia nematodiphila* (15), whereas the lowest values were recorded in plants inoculated with *Sinorhodobacter* sp. (T31) and control plants (Table 1).

The highest root dry weight was obtained by inoculating *Staphylococcus arlettae* (18) on leaves, showing significant differences (Tukey, α=0.05) with plants inoculated with *Serratia nematodiphila* (15) and *Bacillus* sp. (T69). However, all bacterial strains showed significant differences in comparison to the control (Table 1). Regarding root inoculation, plants inoculated with *Pseudomonas psychrotolerans* (T82) significantly increased (Tukey, α=0.05) root dry weight with respect to control plants (Table 1).

The foliar inoculation of *P. anthophila* (T38) resulted in significantly (Tukey, α=0.05) higher total dry weight when compared to plants inoculated with *Serratia nematodiphila* (15), *Bacillus* sp. (T69), *P. anthophila* (T83) and *Sinorhodobacter* sp. (T31), and to control plants (Table 1). Conversely, the root inoculation of all bacterial strains resulted in significantly (Tukey, α=0.05) enhanced total dry weight of plants in comparison to control plants. In this case, plants inoculated with *Serratia nematodiphila* (15) had the highest total dry weight (Table 1).

Bioassay 2. Effects of the selected bacterial strains isolated from meliponids, combined with arbuscular mycorrhizal fungi and three doses of phosphorus fertilization, on lettuce plants

The main effects of individual factors are shown in Table 2. Regarding bacterial inoculation, the single inoculation of *S. nematodiphila* or in combination with *P. anthophila* induced the most significant plant growth responses (Tukey, α=0.05), while the root mycorrhizal colonization was significantly enhanced by inoculating both bacteria in plants (Table 2). On the contrary, the AMF-inoculation had significant increases (Tukey, α=0.05) on leaf and total dry weight (Table 2). In the case of P-fertilization, the application of 22 μg P mL^{-1} resulted in significant increases (Tukey, α=0.05) in all plant growth parameters. Conversely, root mycorrhizal colonization was significantly higher (Tukey, α=0.05) with the application of 44 μg P mL^{-1} (Table 2).

The results obtained due to the combination

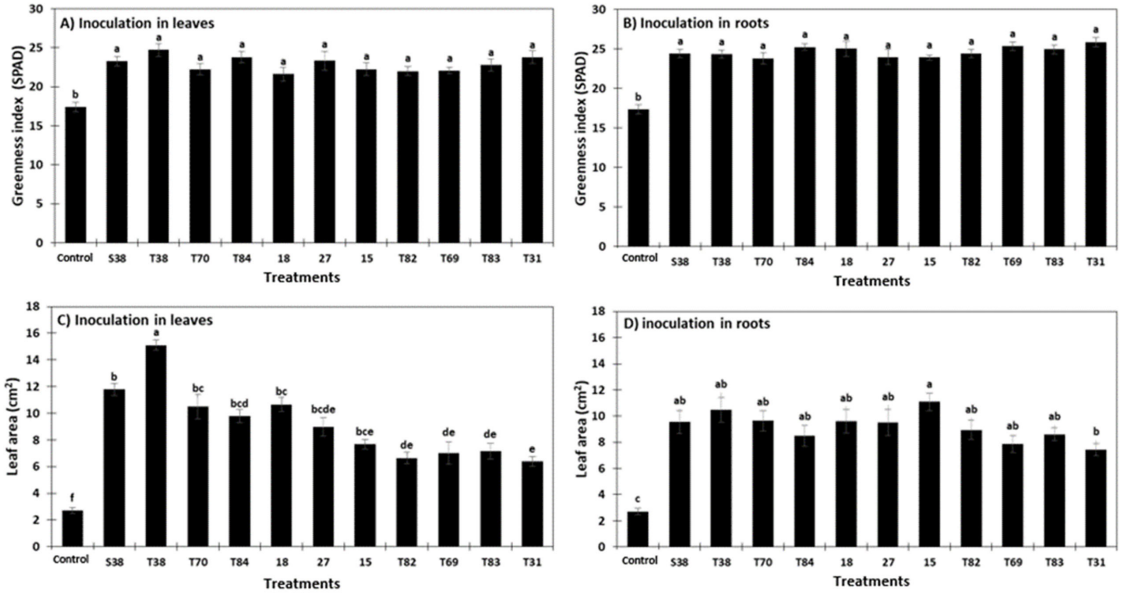

Fig 1. Greenness index (SPAD readings) (A-B) and leaf area (C-D) of lettuce plants (Parrish Island variety) inoculated on leaves (A and C) or in roots (B and D), with eleven bacteria isolated from meliponids (stingless bees), after 25 days of experimentation. Same letters above bars indicate no significant differences (Tukey, α=0.05). Means ± standard error. n=10.

Table 1. Effects of eleven bacterial strains isolated from meliponids (stingless bees), inoculated in leaves or roots, on the dry weight of lettuce plants (Parrish Island variety), after 25 days of experimentation.

	Dry weight (g)					
	Bacterial inoculation in leaves			Bacterial inoculation in roots		
Treatments	**Shoots**	**Roots**	**Total**	**Shoots**	**Roots**	**Total**
Control	0.008 g ± 0.0006	0.002 e ± 0.0001	0.013 e ± 0.0005	0.008 c ± 0.0006	0.002 b ± 0.0001	0.013 b ± 0.0005
S38	0.026 ab ± 0.0006	0.005 bcd ± 0.0003	0.031 ab ± 0.0007	0.018 ab ± 0.0018	0.006 a ± 0.0003	0.025 a ± 0.0024
T38	0.027 a ± 0.0019	0.007 ab ± 0.0005	0.034 a ± 0.0022	0.019 ab ± 0.0017	0.006 a ± 0.0002	0.026 a ± 0.0016
T70	0.022 abc ± 0.0013	0.005 bcd ± 0.0006	0.027 abc ± 0.0016	0.021 ab ± 0.0018	0.006 ab ± 0.0005	0.027 a ± 0.0021
T84	0.022 abcd ± 0.0011	0.005 abcd ± 0.0005	0.027 abc ± 0.0013	0.017 ab ± 0.0011	0.004 ab ± 0.0006	0.021 a ± 0.0016
18	0.021 bcde ± 0.0011	0.008 a ± 0.0006	0.029 ab ± 0.0014	0.021 ab ± 0.0015	0.006 ab ± 0.0004	0.027 a ± 0.0017
27	0.018 cdef ± 0.0015	0.006 abc ± 0.0007	0.025 bcd ± 0.0019	0.019 ab ± 0.0015	0.006 ab ± 0.0007	0.025 a ± 0.0021
15	0.015 ef ± 0.0006	0.004 ed ± 0.0004	0.019 de ± 0.0008	0.022 a ± 0.0014	0.006 a ± 0.0004	0.029 a ± 0.0018
T82	0.016 def ± 0.0008	0.004 bcde ± 0.0005	0.021 cd ± 0.0012	0.018 ab ± 0.0013	0.007 a ± 0.0004	0.026 a ± 0.0019
T69	0.016 ef ± 0.0013	0.003 ed ± 0.0004	0.019 de ± 0.0016	0.017 ab ± 0.0011	0.005 ab ± 0.0007	0.023 a ± 0.0017
T83	0.015 ef ± 0.0013	0.004 cde ± 0.0006	0.019 de ± 0.0018	0.018 ab ± 0.0008	0.005 ab ± 0.0007	0.024 a ± 0.0014
T31	0.014 f ± 0.009	0.005 bcd ± 0.0006	0.019 de ± 0.0011	0.016 b ± 0.0011	0.006 a ± 0.0008	0.023 a ± 0.0016
Significance	0.0001	0.0001	0.0001	0.0001	0.0998	0.0001
CV	20.94	32.15	19.40	20.34	43.19	23.13
MSD	0.0058	0.0026	0.007	0.0064	0.0039	0.0085

Same letters in the same column are not significantly different (Tukey, α=0.05). Means ± standard error. n= 10. CV= Coefficient of variation. MSD= Minimum Significant Difference. Bacterial codes description: *Pantoea anthophila* (S38), *P. anthophila* (T38), *Bacillus* sp. (T70), *P. anthophila* (T84), *Staphylococcus arlettae* (18), *Staphylococcus kloosii* (27), *Serratia nematodiphila* (15), *Pseudomonas psychrotolerans* (T82), *Bacillus* sp. (T69), *P. anthophila* (T83), and *Sinorhodobacter* sp. (T31).

Table 2. Main effects of bacterial inoculation, inoculation of arbuscular mycorrhizal fungi (AMF) and phosphorus (P) fertilization, as independent factors, on the growth and root mycorrhizal colonization of lettuce plants, after 35 days of experimentation.

Independent factors and levels	Shoot fresh weight (g)	Leaf dry weight (g)	Root dry weight (g)	Total dry weight (g)	Greenness index (SPAD)	Leaf area (cm^2)	Mycorrhizal colonization (%)
Bacterial inoculation							
Control	3.24 b	0.190 c	0.04 c	0.229 c	26.62 b	77.06 c	19.31 b
Serratia nematodiphila	5.41 a	0.302 a	0.05 ab	0.357 a	31.34 a	130.4 ab	19.58 ab
Pantoea anthophila	5.12 a	0.261 b	0.05 b	0.311 b	30.92 a	120.17 b	20.62 ab
Both bacteria	5.56 a	0.304 a	0.05 a	0.360 a	31.05 a	134.96 a	21.33 a
Significance	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Coefficient of variation	19.28	25.01	23.28	22.29	10.62	20.48	19.01
Minimum Significant Difference	0.4	0.03	0.006	0.03	1.6	12.4	3.3
AMF inoculation							
AMF	4.92 a	0.27 a	0.05 a	0.32 a	29.71 a	116.47 a	40.42 a
Non-AMF	4.75 a	0.25 b	0.04 a	0.31 b	30.25 a	114.87 a	0 b
Significance	NS	NS	NS	NS	NS	NS	NS
Coefficient of variation	19.28	25.01	23.28	22.29	10.62	20.48	19.01
Minimum Significant Difference	0.2	0.02	0.003	0.02	0.9	6.7	1.8
P-fertilization (μg P mL^{-1})							
11	4.08 c	0.21 c	0.04 b	0.25 c	27.88 b	102.56 b	19.62 b
22	5.46 a	0.31 a	0.05 a	0.36 a	31.26 a	124.91 a	19.14 b
44	4.96 b	0.27 b	0.04 b	0.32 b	30.82 a	119.52 a	21.87 a
Significance	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Coefficient of variation	19.28	25.01	23.28	22.29	10.62	20.48	19.01
Minimum Significant Difference	0.38	0.2	0.004	0.02	1.33	9.85	2.6

Same letters in the same column are statistically equal (Tukey, α=0.05). n= 48 for bacterial inoculation; n=96 for AMF application, and n=64 for P-application.

of three factors (yielding 24 treatments) are described in Fig. 2. By applying 11 µg P mL^{-1}, the inoculation of all microorganisms (alone or in combination) showed higher leaf greenness index (Tukey, α=0.05) with respect to plants without bacteria or AMF inoculation (Table 3). In the dose of 22 µg P mL^{-1}, the highest greenness index was achieved with the single inoculation of *S. nematodiphila*. However, no significant differences were observed with the other treatments (Table 3). In the case of 44 µg P mL^{-1}, the highest greenness index was obtained with the single inoculation of *P. anthophila*, which showed significant differences (Tukey, α=0.05) in plants without bacterial inoculation either with or without AMF (Table 3).

The fresh weight of shoots had significant increases (Tukey, α=0.05) with the application of 11 µg P mL^{-1} and with the inoculation of all microorganisms (alone or combined) with respect to plants without microbial inoculation (Fig. 3A). In 22 µg P mL^{-1}, the single inoculation of *S. nematodiphila* significantly enhanced fresh weight (Tukey, α=0.05) when compared to plants without bacterial inoculation, with or without AMF. Nevertheless, no significant differences were observed among treatments inoculated with the microorganisms (Fig. 3B). For 44 µg P mL^{-1}, the bacterial inoculation without AMF significantly increased (Tukey, α=0.05) the fresh weight in comparison to plants without microbial inoculation, whereas no significant differences were observed among the remaining treatments inoculated with the microorganisms (Fig. 3C).

Leaf dry weight was significantly increased (Tukey, α=0.05) due to the bacterial inoculation (alone or combined with AMF) with the application of 11 µg P mL^{-1} when compared to plants without bacterial inoculation and without AMF (Table 3). In the case of plants fertilized with 22 µg P mL^{-1}, the single inoculation of *S. nematodiphila* significantly increased (Tukey, α=0.05) leaf dry weight in comparison to plants either without microbial inoculation or inoculated with *P. anthophila* (Table 3). For 44 µg P mL^{-1}, all microorganisms (alone or in combination)

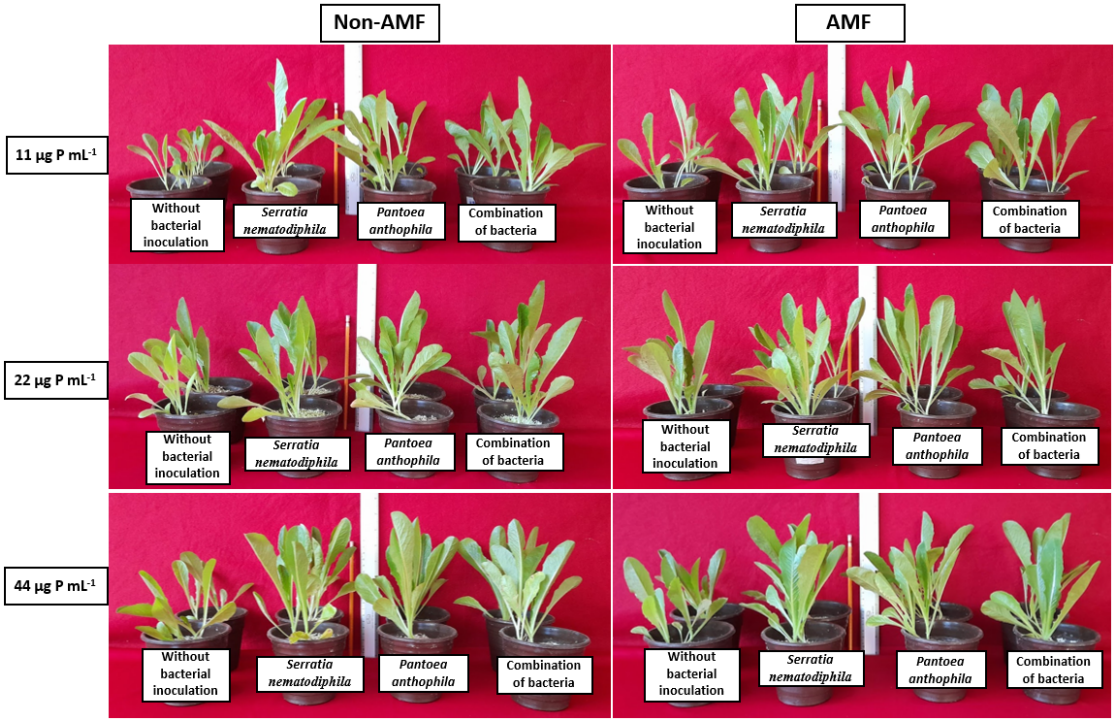

Fig 2. Aspects of lettuce plant growth achieved in bioassay 2. Inoculation of both selected bacteria (*Pantoea anthophila* and *Serratia nematodiphila*) isolated from stingless bees, arbuscular mycorrhizal fungi (*Claroideoglomus claroideum* and *Funneliformis mosseae*), and three doses of phosphate fertilization (11, 22 and 44 µg P mL^{-1}) under greenhouse conditions, with a maximum temperature of 35 ° C and a minimum of 10 ° C, and a relative humidity of 80%, after 20 days of growth.

Table 3. Effects of two bacterial strains (*Serratia nematodiphila* 15 and *Pantoea anthophila* T38) isolated from meliponids in combination with arbuscular mycorrhizal fungi (AMF) and three doses of phosphorus (P) fertilization, on dry weight, greenness index, and mycorrhizal colonization of lettuce plants, after 35 days of experimentation.

		Bacterial inoculation	Shoot dry weight (g)	Root dry weight (g)	Total dry weight (g)	Greenness index (SPAD units)	AMF colonization (%)
11 µg P mL^{-1}	Non-AMF	Control	0.07 g ± 0.007	0.03 ef ± 0.003	0.11 e ± 0.006	22.1 e ± 0.8	0 ± 0
		S. nematodiphila	0.22 ef ± 0.007	0.04 bcde ± 0.003	0.27 d ± 0.009	28.3 abcd ± 1	0 ± 0
		P. anthophila	0.22 def ± 0.006	0.04 bcde ± 0.002	0.27 d ± 0.007	28.3 bcd ± 1	0 ± 0
		Both bacteria	0.25 cde ± 0.01	0.06 ab ± 0.003	0.31cd ± 0.006	29.4 abcd ± 1	0 ± 0
	AMF	Control	0.11 g ± 0.01	0.03 def ± 0.004	0.14 e ± 0.01	25 cde ± 1	51.0 d ± 1.8
		S. nematodiphila	0.25 cde ± 0.02	0.05 bcde ± 0.003	0.31cd ± 0.002	30 abcd ± 1.3	62.8 cd ± 3.2
		P.anthophila	0.25 cde ± 0.01	0.05 abc ± 0.002	0.31cd ± 0.008	29.7 abcd ± 0.8	70.2 ab ± 1.8
		Both bacteria	0.25 cde ± 0.01	0.05 bcd ± 0.002	0.32 cd ± 0.01	29.9 abcd ± 1.8	76.3 a ± 2.8
22 µg P mL^{-1}	Non-AMF	Control	0.27 bcde ± 0.01	0.05 bcde ± 0.002	0.32bcd ± 0.01	29.1 abcd ± 0.9	0 ± 0
		S. nematodiphila	0.44 a ± 0.02	0.07 a ± 0.005	0.51 a ± 0.01	34.1 a ± 0.6	0 ± 0
		P.anthophila	0.23 de ± 0.008	0.04 bcde ± 0.002	0.28 d ± 0.01	31.7 abc ± 1.1	0 ± 0
		Both bacteria	0.31 bcde ± 0.02	0.05 abc ± 0.002	0.36bcd ± 0.02	32.5 abc ± 0.5	0 ± 0
	AMF	Control	0.24 de ± 0.01	0.05 bcde ± 0.001	0.29 d ± 0.01	28.3 abcd ± 1.1	65.1 bc ± 4.3
		S. nematodiphila	0.31 bcde ± 0.02	0.06 ab ± 0.004	0.36bcd ± 0.02	30.4 abcd ± 1.2	63.1 bc ± 4.6
		P.anthophila	0.31 bcde ± 0.02	0.06 ab ± 0.004	0.36bcd ± 0.02	32.1 abc ± 0.7	58.7 cd ± 2.8
		Both bacteria	0.37 ab ± 0.02	0.06 ab ± 0.002	0.43 ab ± 0.02	31.6 abc ± 0.8	67.0 abc ± 2.6
44 µg P mL^{-1}	Non-AMF	Control	0.13 fg ± 0.009	0.02 f ± 0.001	0.15 e ± 0.01	27.75 cde ± 0.9	0 ± 0
		S. nematodiphila	0.26 cde ± 0.01	0.04 bcde ± 0.002	0.31 cd ± 0.02	32.2 abc ± 0.6	0 ± 0
		P. anthophila	0.26 cde ± 0.01	0.04 bcde ± 0.002	0.31cd ± 0.009	33.7 ab ± 1.1	0 ± 0
		Both bacteria	0.34 abc ± 0.01	0.05 ab ± 0.002	0.41 bc ± 0.01	33.6 ab ± 0.5	0 ± 0
	AMF	Control	0.31 bcde ± 0.01	0.03 cdef ± 0.002	0.35bcd ± 0.01	27.3 cde ± 1.1	76.0 a ± 2.5
		S. nematodiphila	0.33 bcd ± 0.02	0.04 bcde ± 0.003	0.37bcd ± 0.01	32.8 abc ± 1.2	68.7 abc ± 1
		P. anthophila	0.28 bcde ± 0.01	0.04 bcde ± 0.003	0.33bcd ± 0.02	29.8 abcd ± 0.7	76.2 a ± 2.3
		Both bacteria	0.28 bcde ± 0.02	0.04 bcde ± 0.002	0.33bcd ± 0.02	29.2 abcd ± 0.8	69.1 abc ± 1.5
		Significance	0.0001	0.0001	0.0001	0.0001	0.0001
		Coefficient of variation	21.26	22.36	18.66	10.52	16.91
		Minimum Significant Difference	0.1	0.02	0.1	5.8	10.4

Same letters in the same column are not significantly different (Tukey, α=0.05). Means ± standard error. n=8

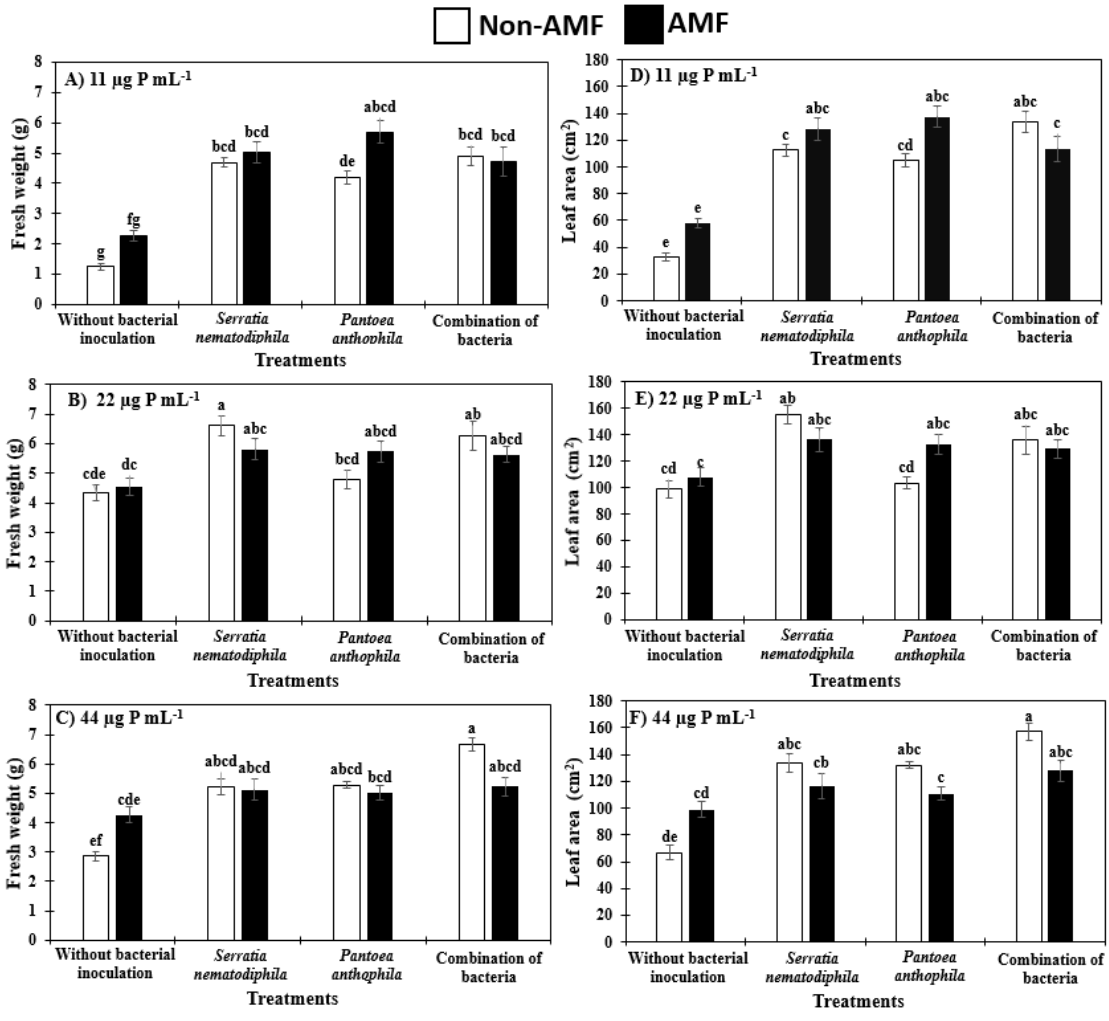

Fig 3. Fresh weight of shoots (A-C) and leaf area (D-F) of lettuce plants (Parrish Island variety) inoculated with two bacteria isolated from meliponids (stingless bees) or an arbuscular mycorrhizal consortium (AMF), and with the application of three doses of phosphorus (P) in Long Ashton nutrient solution, after 35 days of experimentation. Same letters above bars (for each plant growth parameter) indicate no significant differences (Tukey, α=0.05). Means ± standard error. n=8.

significantly increased (Tukey, α=0.05) leaf dry weight compared to plants without microbial inoculation (Table 3).

In terms of root dry weight, all microorganisms (alone or in combination) with the application of 11 µg P mL^{-1} had significant increases (Tukey, α=0.05) with respect to treatments without microbial inoculation (Table 3). However, the inoculation of both bacteria without AMF showed the greatest root dry weight (Table 3). In plants fertilized with 22 µg P mL^{-1}, root dry weight was significantly increased (Tukey, α=0.05) by inoculating *S. nematodiphila* without AMF with respect to the remaining treatments (Table 3). In the application of 44 µg P mL^{-1}, the combination of both bacteria without AMF had significant increases (Tukey, α=0.05) when compared to plants without microbial inoculation. However, no significant differences were found among the remaining treatments (Table 3).

Total dry weight was significantly higher (Tukey, α=0.05) with the bacterial inoculation (alone or in combination with AMF) in plants fertilized with 11 µg P mL^{-1} when compared to those plants without microbial inoculation (Table 3). In plants with 22 µg P mL^{-1}, the highest total dry weight was obtained with the single inoculation of *S. nematodiphila* without AMF,

which showed significant differences with the rest of the treatments (Tukey, α=0.05), except for the treatment with the consortium of both bacteria combined with AMF (Table 3). In plants fertilized with 44 µg P mL^{-1}, the single or combined inoculation with all beneficial microorganisms showed significant increases in total dry weight with respect to control plants and without AMF (Table 3).

Leaf area was significantly increased (Tukey, α=0.05) due to bacterial inoculation alone or in combination with AMF, and with the application of 11 µg P mL^{-1} when compared to plants without microbial inoculation (Fig. 3D). Fertilization with 22 µg P mL^{-1} had significant effects on leaf area (Tukey, α=0.05) mainly due to the single inoculation of *S. nematodiphila* in comparison to plants with the single inoculation of *P. anthophila* or to those treatments without bacterial inoculation but combined or not with AMF (Fig. 3E). In plants fertilized with 44 µg P mL^{-1}, the significant largest leaf area (Tukey, α=0.05) was achieved in the treatment inoculated with both bacteria without AMF, in comparison to treatments without microbial inoculation, or those with the single bacterial inoculation in combination with AMF (Fig. 3F).

Regarding mycorrhizal colonization in roots, the AMF plants inoculated with both bacteria at 11 µg P mL^{-1} showed significantly higher percentage of colonization (Tukey, α=0.05) when compared to AMF plants without bacterial inoculation (Table 3). In contrast, AMF plants fertilized with 22 µg P mL^{-1} did not show significant differences among treatments in terms of mycorrhizal colonization (Table 3). No significant differences were detected among bacterial treatments in plants fertilized with 44 µg P mL^{-1}, but the mycorrhizal colonization reached the highest values when compared to fertilization with 11 or 22 µg P mL^{-1}, particularly with the single inoculation of AMF or with the inoculation of *P. anthophila* (Table 3). No mycorrhizal inoculation was detected in roots of plants without AMF inoculation (Table 3).

DISCUSSION

There is evidence that beneficial microorganisms influence plant metabolism and contribute to plant growth and protection against pathogens (Harman et al., 2021; Koza et al., 2022). In lettuce, several studies have described the growth-promoting effects of different bacterial genera (Laurindo and Cruz, 2024; Gutiérrez et al., 2019), but the inoculation of plants with bacteria isolated from meliponids has been scarcely studied. All the evaluated bacteria (11 strains) had beneficial effects on plants regardless the inoculation method (via roots or leaves).

Benefits from bacterial inoculations, like improving greenness index, may be partially explained due to the ability of microorganisms to enhance plant nutrient absorption, which is also reflected in enhanced leaf growth and chlorophyll concentration (Wu et al., 2019). Nitrogen fixation and phosphate solubilization are important biochemical properties that exert direct effects on the synthesis of chlorophyll, and on the increase of photosynthetic activity of plants (García et al., 2019; Maharana et al., 2022), thus enhancing plant growth (Blanco et al., 2018; Latef et al., 2020), as observed in the present research. As described in the Bergey´s manual (Claus and Berkeley 1984), in terms of physiological activity, *Pantoea* sp., *Bacillus* sp. and *Pseudomonas* sp., are able to produce indole acetic acid (IAA); *Pantoea* sp., *Pseudomonas* sp. and *Serratia* sp., produce siderophores; *Pantoea* sp., *Bacillus* sp., *Sinorhodobacter* sp. and *Staphylococus* sp. may release the enzyme catalase; and *Pantoea* sp. may also benefit plant growth under saline soils. These bacteria may induce physiological and structural changes on plants through specific mechanisms such as phytohormone production (e.g., IAA production, ACC- deaminase activity, nitrogenase activity), and phosphate solubilization (El-Saadony et al., 2022; Fukami et al., 2017), resulting in significant plant growth increases (Çakmakçi et al., 2020) as observed under our experimental conditions.

In the case of lettuce, beneficial results have been reported in plant growth when inoculated with bacteria (Díaz et al., 2023). Previous research reported beneficial effects of bacteria on plants subjected to water stress or to soil contamination (Devarajan et al., 2021; Morales-Guzman et al., 2020). However, no previous studies have addressed the beneficial effects of bacteria isolated from meliponids on the growth of horticultural plants like lettuce. Furthermore, bacterial communities are found in different plant organs, either externally (epiphytic) or internally (endophytic), or in rhizosphere (Wen et al., 2023). Like the rhizosphere, the so-called phyllosphere, harbors microbial communities and relationships that enhance plant growth by improving nutrient uptake and inducing tolerance to attack by microbial phytopathogens (Legein et al., 2020). In this sense, the positive effect of bacteria applied on leaves may be attributed to phytohormone production (Md-Gulzar y Mazumder, 2023; Gayathry et al., 2024).

Beneficial bacteria provide plants with various benefits. For instance, Egamberdieva (2008) demonstrated that *P. antophila* enhanced growth and nutrient uptake in wheat and pea plants under saline conditions; some species

of *Pantoea* sp. have shown inhibitory activity against bacterial and fungal phytopathogens (Krishnappa et al., 2024; Verma et al., 2018). Furthermore, there is evidence that *P. agglomerans* enhances growth and vigor of mulberry plants and promotes plant protection against bacterial blight (Xie et al., 2017). In cotton, however, Tufail et al. (2020) revealed that the foliar inoculation of *P. agglomerans* and *P. anthophila* may cause blight disease. In the present study, the inoculation of the four bacterial strains belonging to the *Pantoea* genus resulted in short-term beneficial effects on in lettuce plant growth, without showing any disease symptoms.

The obtained results showed that *Serratia nematodiphila* also had significant effects on plant growth. Recent studies confirmed that this bacterium acts as a plant growth promoter due to the expression of physiological mechanisms such as auxin production, P solubilization, and nitrogen fixation (Jagtap et al., 2023). In addition, this bacterium may also inhibit the growth of fungal phytopathogens, like *Fusarium* sp. and *Alternaria solani*, by producing and releasing cellulases or siderophores (Bellotti et al., 2023; Mendez et al., 2021). Recent studies indicated that *Serratia nematodiphila* alleviates toxicity of heavy metals (Yousaf et al., 2024) and promotes the adaptation of plants to stress factors (Kulkova et al., 2024).

In the case of *Bacillus* sp., this bacterial genus has been reported as PGPB since some strains are characterized as N-fixers, P-solubilizers, and indole-producers (Sibponkrung et al., 2020; Yousuf et al., 2020). Moreover, these physiological characteristics are also reported for bacteria like *Pseudomonas* sp., which have the capacity to release siderophores (Kang et al., 2020). In regards to *Sinorhodobacter* sp., there is scarce information about its physiological activities, but there is evidence that this bacterium may enhance plant growth by improving Fe uptake (Yang et al., 2013). Regarding *Staphylococcus* sp., it promotes plant growth by participating in P solubilization, IAA production and other metabolic processes (Sitlaothaworn et al., 2022; Taj et al., 2024).

Results from the second bioassay showed that the single bacterial inoculation, as well as their combination with AMF-inoculation or P-fertilization, improved most of plant growth parameters. These benefits are due to the physiological activities detected in all the tested bacterial strains, by which lettuce plants showed better growth responses when compared to plants without bacterial inoculation. Thus, some bacteria are associated to nutrient uptake improvement (P and N, mainly), which has an important influence on chlorophyll synthesis (Ren et al., 2019; Romero et al., 2021; Wu et al., 2019). This may partly explain the positive effects of bacterial strains on the leaf greenness index, and consequently on plant growth, under our experimental conditions.

Overall, AMF inoculation did not show significant differences when compared to non-AMF plants, but increased total dry weight, which agrees with previous studies reporting the positive effects of AMF on other horticultural plants (Angulo-Castro et al., 2018; Emmanuel and Babaloba, 2020; Carballar-Hernandez et al., 2018; Yang et al., 2020). However, AMF did not significantly improve plant growth under our short-term (35 days) experimental conditions.

Our results denoted that the application of the three doses of P resulted in significant differences among treatments. In general, plant growth was significantly enhanced by applying 22 μg P mL^{-1}, indicating that the P-application directly influences plant growth (Al-Mharib et al., 2019; Gao et al., 2020; Li et al., 2020). In the present study, the combination of P-fertilization and bacterial strains significantly increased plant growth, which concurs with findings reported by Wu et al. (2019). Growth-promoting effects were observed when some bacteria were inoculated either individually or in combination (Neelipally et al., 2020). Nevertheless, our study showed significant effects on the growth of lettuce plants inoculated with bacteria previously isolated from stingless bees, which were compatible with three doses of P.

Bacterial inoculation in combination with AMF and 22 μg P mL^{-1} improved plant growth responses, which is attributed to the ability of both microbial groups to take advantage of the applied P as described by Wahid et al. (2020) and Nacoon et al. (2021). Overall, the combined application of microorganisms may promote important effects on plants when compared to individual inoculations (Aini et al., 2019; Pandino et al., 2024). Some reports have demonstrated that *S. marcescens* combined with AMF under soil contamination, increased plant biomass, antioxidant activity, and degradation of organic contaminants (Morales-Guzmán et al., 2020). On the contrary, some reports have confirmed negative effects of *S. marcescens* by causing the cucurbit yellow vine disease (Rodriguez et al., 2023).

The root mycorrhizal colonization was significantly enhanced due to the inoculation of both bacteria and by the application of 44 μg P mL^{-1} when compared to plants without bacterial inoculation, and with the application of the other two P doses. The combined inoculation of bacteria and AMF may act synergistically

and promote plant growth by improving the nutritional status (Aini et al., 2019; Raklami et al., 2019). In some cases, bacteria may favor the mycorrhizal colonization in roots (Aalipour et al., 2021; Ibiang et al., 2020), which highlights the importance of potential outcomes derived from microbial interactions on target plants.

Some studies have demonstrated that the combined application of P-fertilization and AMF may improve P uptake and, consequently, plant growth enhancements (Begum et al., 2020; Cela et al., 2022). The results of the present study indicate that AMF had limited effects on lettuce plants, but these fungi were able to colonize the root system, while this mycorrhizal colonization was significantly stimulated by the application of 44 μg P mL^{-1}. This finding does not agree with previous reports indicating that the potential benefits of AMF on plant growth and AMF colonization are affected by the application of high doses of P (Lin et al., 2020; Nahar et al., 2020). The negative effects of P on mycorrhizal colonization depend on fungal genotype, plant mycorrhizal dependency, and experimental conditions in which plants are grown.

As previously mentioned, rhizosphere bacteria have several mechanisms by which plants may result benefited, such as nitrogen fixation, phosphate solubilization (Da-cunha et al., 2024; Xu et al., 2019), production of phytohormones like IAA, ACC-deaminase, cytokinins, and gibberellins (Hindersah et al., 2020), and inducing resistance to abiotic factors (Kaleh et al., 2024). Phyllosphere bacteria have similar properties that allow them to survive on leaves, but may also reduce the incidence of pathogens and stimulate plant growth through the production of phytohormones, auxins and cytokinins (Nascimento et al., 2019; Saghafi et al., 2020). In addition, these bacteria stimulate the production of secondary metabolites and siderophores (Helfrich et al., 2018; Kron et al., 2020), and hydrolytic enzymes (Allonsius et al., 2019), and release specific molecules for inhibiting or by competing with phytopathogens for space and nutrients (Legein et al., 2020; Li et al., 2022).

Bacteria isolated from meliponids enhanced lettuce plant growth under greenhouse conditions. The combination of bacteria and AMF induced positive effects when compared to their individual inoculation. Furthermore, foliar inoculation of bacteria led to similar or better results compared to root inoculation. Moreover, the applied P-doses resulted in beneficial effects on plant growth. Although the tested bacteria were not isolated from rhizosphere, they presented characteristics that make them promising for their application under greenhouse conditions.

A study conducted by Pronk et al. (2022) revealed that a wide range of rhizosphere bacteria considered as plant growth promoters, are not only associated with plants but also with insects. Bacteria isolated from insects have important biochemical properties similar to those inhabiting the rhizosphere. In canola and tomato plants, bacteria isolated from moth larvae were capable of solubilizing inorganic phosphates, fixing atmospheric nitrogen, and producing phytohormones, thus acting as plant growth promoters (Indiragandhi et al., 2008). Furthermore, the application of bacteria like *Dyella*, isolated from the insect *Hyalesthes obsoletus*, resulted in significant reduction of the severity of *Phytoplasma* bacterium disease in grapevine plants (Iasur et al., 2018). Similarly, *Paenibacillus polymyxa* isolated from the larval food of melipona bees (*Melipona scutellaris*) produced compounds such as (L)-(−)-3-phenyllactic acid and fusaricidins that exert antibiotic effects towards pathogenic bacteria that cause bee larval death. Therefore, larvae of melipona bees have symbiotic associations with beneficial bacteria from the beginning of their life cycle, ensuring both health and nutrition of the entire colony (Menegatti et al., 2018). In the present study, bacteria isolated from meliponids exerted beneficial effects as plant growth promoters, thus denoting their potential biotechnological applications for horticultural purposes.

CONCLUSIONS

Inoculation of lettuce plants with bacteria isolated from meliponids (stingless bees) resulted in beneficial effects on plant growth, demonstrating their biological effectiveness as bacterial inoculants. In the first bioassay, either foliar or root inoculation of all bacterial strains improved plant growth. The most prominent bacterial strains were *P. anthophila* inoculated on leaves, and *S. nematodiphila* inoculated in roots.

The second bioassay demonstrated that the combination of two bacteria (*P. anthophila* and *S. nematodiphila*) with AMF (*Claroideoglomus clarideum* and *Funneliformis mosseae*) exerted plant-growth promoting effects, especially when combined with 22 μg P mL^{-1}. Overall, the application of either 11 μg P mL^{-1} or 22 μg P mL^{-1} enhanced the effectiveness of bacterial inoculation in most of the evaluated plant parameters. The single inoculation of AMF did not induce beneficial effects on plants, but its combination with both bacteria resulted in improved plant growth.

Overall, bacteria isolated from meliponids in combination with AMF and moreover, with P fertilization, improved the growth of lettuce plants under greenhouse conditions. This research also demonstrates that stingless bees harbor beneficial microorganisms that may be biotechnologically managed and applied to horticultural plants at initial stages of growth.

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Declarations:

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material

The data that support the findings of this study are available from the Orlando Catalán-Barrera and Alejandro Alarcón upon reasonable request.

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