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Enhancing nutrient uptake and grapefruit (*Citrus paradisi*) growth through soil application of beneficial bacteria (*Bacillus* spp.)

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The term plant growth-promoting rhizobacteria (PGPR) refers to a root-associated bacteria that possesses several benefits for soil and the plant. The increasing demand for sustainable food production necessitates a shift towards agricultural practices that mitigate adverse environmental impacts. Excessive use of chemical fertilizers and pesticides has raised concerns, prompting a surge of interest in harnessing the potential of beneficial soil microorganisms, particularly plant growth-promoting rhizobacteria. This study explores the impact of soil inoculation with PGPR treatments on grapefruit seedlings, including a commercial strain of *Bacillus velezensis*, a fresh inoculum of *Bacillus amyloliquefaciens*, a mixture of three selected citrus endophytic *Bacillus* spp. isolates, inorganic fertilizers, and combinations thereof. The aim was to test their ability to enhance growth and nutrient uptake reducing the input of chemical fertilization. Results indicated that the combination of *Bacillus velezensis* and inorganic fertilization significantly improved soil nutrient availability and enhanced plant growth, surpassing both negative (water) and positive (inorganic fertilization) controls. Shoot and root system biomass showed significant increases from *Bacillus velezensis* plus inorganic fertilization compared to other treatments. These findings provide insights into management methods that can reduce chemical inputs while promoting plant productivity. The demonstrated benefits of PGPR on grapefruit seedlings highlight a promising approach for future research and applications in sustainable citrus cultivation.

KEYWORDS

PGPR, beneficial bacteria, nutrient uptake, growth promotion, sustainable production, biofertilizer

Introduction

Developing methods to enhance agricultural sustainability is necessary for ensuring not only an adequate food supply but also for upholding global environmental protection for future generations (Nwachukwu et al., 2021; Wang J. et al., 2022). The activity of plant growth-promoting rhizobacteria (PGPR) has been documented in a wide range of strains across several different genera, including *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Hydrogenophaga*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Klebsiella*, *Microbacterium*, *Paenibacillus*, and *Azospirillum* (Grobela et al., 2015; Kumar Meena et al., 2015; Kumar et al., 2018; Numan et al., 2018; Barnawal et al., 2019). Root colonization by microbes is active (Compant et al., 2019) but it will be influenced by the root exudates from different plant species (Elsharkawy et al., 2023) reported by Jamil et al. (2022), and Antoszewski et al. (2022). Among the rhizobacteria that can colonize plants, we can find *Bacillus amyloliquefaciens* (Fan et al., 2015; Gamez et al., 2019), *Bacillus subtilis* (Blake et al., 2021; Hu et al., 2023), *Bacillus velezensis* (Shi H. et al., 2022; Sun et al., 2022) and some commercial strains of these species serve as biofertilizers and biocontrol agents. The ability of these strains to form resilient endospores can assist in ensuring their viability for the production of microbial inoculants (Tsetetsi et al., 2022) when added to the soil. As a result, its utilization in agricultural practices is rapidly expanding (Shi J. W. et al., 2022) as biostimulants for growth promotion (Senger et al., 2022), biofertilization (Bueno et al., 2022), and biocontrol agents (Chen et al., 2020).

A microbial inoculant (MI) is a category of agricultural amendments that uses live microorganisms to provide fertilizer benefits for crops (Gong et al., 2018). In numerous instances, utilizing microbial inoculants containing beneficial microorganisms instead of synthetic chemicals has raised attention for their ability to help plant growth, and offer enhanced nutrient availability while simultaneously fostering environmental benefits and augmenting soil productivity (Luo et al., 2022; Mawarda et al., 2022; Etesami et al., 2023). Global markets for PGPR are growing, especially products involving multiple live bacterial species in “consortia”. When multiple PGPRs are used together, they appear to provide additive benefits to plants compared to a single PGPR (Rosier et al., 2021).

Essentially, microbe-based biofertilizers can enhance both the correct development of plants as well as yield (Rosier et al., 2021; Shi JW et al., 2022) in several agricultural plants such as wheat (*Triticum aestivum* L.) (Zahra et al., 2023), sweet corn (*Zea mays* subsp. *mays* L.) (Akinrinlola et al., 2018), and tomato (*Lycopersicon esculentum* Mill.) (Mengistie and Awlache, 2022; Patani et al., 2023). They achieved this by augmenting the soil availability of mineral nutrients, solubilizing phosphorus, and synthesizing phytohormones, all of which contribute significantly to the overall health of plants (Kumar et al., 2011; Gohil et al., 2022).

Citrus production in Florida has been adversely affected since the first detection of the Huanglongbing disease (HLB; *Candidatus Liberibacter asiaticus*), which is incurable and leads to an eventual tree death (Dala-Paula et al., 2019). Therefore, the need for vigorous citrus rootstock seedlings is a key component for the good development of trees at young stages, to provide tolerance of

biotic and abiotic challenges as well as enhance their overall growth and yield (Vashisth et al., 2020). On the other hand, water and fertilizer play vital roles in the growth and fruit yield of citrus. The quantity of fertilizer used in citrus management is linked to both production costs and environmental concerns (Ma et al., 2022), thus nutrient management has been implemented to promote citrus trees' health in Florida where soils are poorly drained and have low fertility (Hallman et al., 2022). Based on the current disease and nutrient problems the citrus industry in Florida is facing, the objective of this study is to evaluate the role of *Bacillus* spp. soil inoculations in citriculture, particularly in its ability to stimulate and enhance plant growth and improve nutrient uptake of grapefruit seedlings.

Materials and methods

The experimental site and grapefruit seedling establishment

The experiment was conducted in a greenhouse located at the Citrus Research and Education Center (CREC), Lake Alfred, Florida (28.1026° N, 81.7120° W). ‘Duncan’ grapefruit (*Citrus Paradisi*) seedlings were propagated from disease-free seeds collected directly from the fruit grown in the CREC CUPS (Citrus Under Protective Screens; Schumann et al., 2023) facility. Seeds were rinsed three times with distilled water to remove any juice vesicles, and then soaked in distilled water to discard floating seeds. The selected seeds were dried with paper towels and the seed coats were carefully removed with a previously sanitized blade (GEM 62-0161 carbon steel extra sharp single-edge blade). Two seeds were planted into each growth media plug in germination trays of the Double Bio Dome Seed-Starting System (Park Seed, Greenwood, SC) and allowed to germinate in the greenhouse. After three weeks of completed germination, the tallest vigorous seedlings with true leaves were transferred individually into 1.67-L plastic pots filled with unsterilized substrate media of 50% Canadian peat moss with the addition of pine bark, perlite, and vermiculite. All plants were fertilized every week until the start of the experiment 18 weeks after transplanting, then fertilization was stopped and only treatments based on fertilizer (T1) and *B. velezensis* plus fertilizer (T6) continued receiving reduced fertilization every four weeks (25% of the full rate) (Table 1). Monitoring and treating for any potential pest incidence were assessed weekly.

Experimental design and treatments

A randomized complete block design with seven treatments and five replications was used, with each plot (experimental unit) composed of four potted grapefruit seedlings. The treatments (Table 2) were applied as a 0.129 L pot⁻¹ root drench and correspond to: (1) fertilization (Table 1), (2) *B. velezensis* (LALRISE VITA[®]; Lallemand Plant Care, Montreal, Canada), (3) fresh inoculum of *B. amyloliquefaciens* (commercial product Double Nickel 55[®]; Certis, Columbia, MD) where dormant

TABLE 1 Fertilizer description and nutrient concentration.

Fertilizer		Nutrient concentration
0.883 g L ⁻¹	Miracle-Gro®	Total N 24%, P ₂ O ₅ 8% K ₂ O 16% Boron (B) 0.02% Copper (Cu) 0.07% Iron (Fe) 0.15% Manganese (Mn) 0.05% Molybdenum (Mo) 0.0005% Zinc (Zn) 0.06%
0.402 g L ⁻¹	Pennington Epsom salt	Magnesium (Mg) 9.8% Sulfur (S) 12.9%
0.169 g L ⁻¹	Sequestrene™ 138	Iron (Fe) 6%

Dilute mixed fertilizer solution was applied as a root drench at 0.129 L pot⁻¹.

spores were activated in a bioreactor 24 hours before application, (4) fresh inoculum of a consortium of *B. subtilis*, *B. megaterium*, and *Bacillus* sp. (citrus endophytic isolates) (Li et al., 2021), (5) a combination of fresh inoculum of *B. amyloliquefaciens* and the consortium of three *B. subtilis* isolates to test for additional synergy with bacterial combinations, (6) *B. velezensis* with the addition of fertilization and (7) water (control) with no additional application. The fertilizer treatment consisted of three different sources described in Table 1. The fresh inoculum of *Bacillus* spp. consortia was prepared by culturing the bacterium in a nutrient broth medium with agitation at 30°C for 24 hours and adjusted to 10⁹ cells per milliliter with water.

Plant growth measurements

Tree seedling heights and diameters in two perpendicular directions were measured every month to obtain canopy volume based on the formula of Thorne et al. (2002), as well as trunk diameter measured 5 cm above the potting media. SPAD

TABLE 2 Description of treatments applied as a root drench to the pots (0.129 L pot⁻¹).

Treatments		Interval of applications
T1	Fertilization	Every four weeks
T2	<i>Bacillus velezensis</i> (LALRISE VITA®)	Every four weeks
T3	<i>Bacillus amyloliquefaciens</i> (Double Nickel®) fresh bioreactor	Every three weeks
T4	Consortia <i>Bacillus subtilis</i> isolates	Every three weeks
T5	Fresh <i>B. amyloliquefaciens</i> + <i>B. subtilis</i> consortia	Every four weeks
T6	<i>B. velezensis</i> + fertilizer	Every four weeks
T7	Control (water)	As needed*

*Water was also applied to treatments 1 to 6 as needed to replace evapotranspiration losses. The time interval of applications was designed based on recommendation labels from the bacterial products utilized.

chlorophyll index (Konika Minolta, Ramsey, NJ) was collected monthly from three leaves per tree and averaged for each 4-tree plot. At the end of the experiment (three months after treatments began), every plot of four trees was photographed and four leaves per tree were collected and sent for N, P, K, Ca, Mg, S, Zn, B, Mn, Fe, and Cu concentration analysis at the Waters Agricultural Laboratory, Camilla, GA. Tree above-ground biomass and root biomass were obtained by separating leaves, shoots, and roots for each 4-tree plot. Bagged samples were placed in a drying oven (Fisher Scientific, Pittsburgh, PA) for 48 hours at 70°C, and were then weighed to obtain dry mass per plot.

Media sampling and estimation of microbial respiration

We hypothesized that microbial respiration from the most effective rhizobacterial treatments would increase compared to the controls. Growing media samples in the rhizosphere were obtained from the excavated root systems by gentle shaking into a plastic tray, to collect samples, mix and transfer them into plastic Ziploc bags. A mixed media sample from 4 trees was collected, representing the treatment plot and processed for microbial respiration in the lab by implementing the substrate-induced respiration method (Anderson and Domsch, 1978). Empty Mason jars were weighed, then 50 grams of media from the samples was weighed into the jars. To measure the CO₂ respired, a 20 mL plastic scintillation vial was placed inside the jar with 4 mL of 1M NaOH. After 10 days of incubation in a growth chamber in dark-conditions at 35 degrees Celsius, vials were removed from the jars and capped. Jars were placed in the drying oven at 105 degrees Celsius for 48 hours to dry the media. Dry media was then weighed to calculate the percentage of gravimetric water content. The determination of CO₂ produced by microbial respiration processes was based on the titration of 1M NaOH in vials with standardized 0.1M HCl and the use of phenolphthalein as a color indicator to determine the endpoint. An additional blank jar with a vial inside and no media was included in the incubation to calculate the titration amount with zero respiration.

Calculations

To obtain shoot biomass we summed the leaf and stem biomass and then conducted an ANOVA to find the significance among treatments. Similarly, the shoot-to-root ratio was calculated by dividing shoot biomass by root biomass. Finally, the N content in leaves was obtained by multiplying the leaf N concentration and the leaf biomass. In order to test the N fertilizer equivalent saved as a result of using PGPRs, a linear regression line of mg N applied per pot versus mg N/pot accumulated in leaves was calculated for treatments T1 (fertilizer) and T7 (control). The regression equation was then used to interpolate the mg N fertilizer equivalent contributed by each PGPR treatment according to its N accumulation in the leaf biomass.

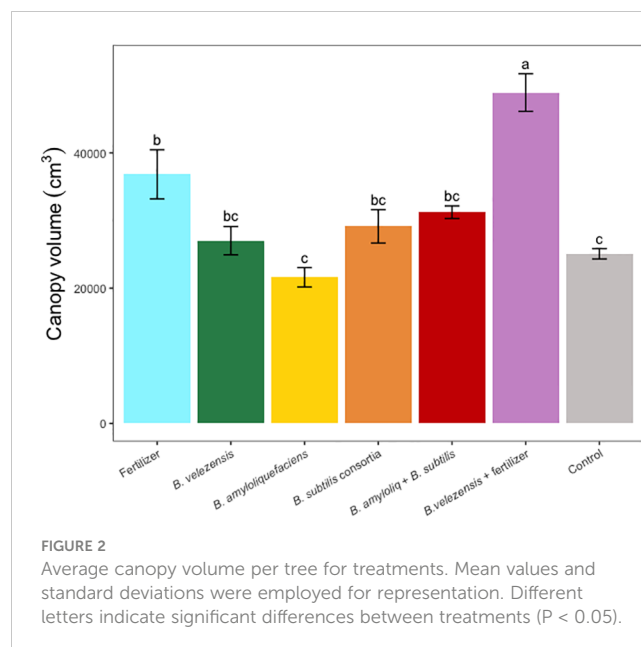
Statistics

To identify significant differences between treatments, Analysis of Variance (ANOVA) was performed using Rstudio (Rstudio Team (2020). Rstudio: Integrated Development for R. Rstudio, PBC, Boston, MA URL <http://www.rstudio.com/>.) and conducting *Post hoc* comparisons with the Tukey HSD test to find statistical differences between means using the ‘agricolae’ package (De Mendiburu, 2022). The data was tested to be normally and independently distributed, from the seven treatment combinations with 5 replications. Differences were considered statistically significant when $P \leq 0.05$.

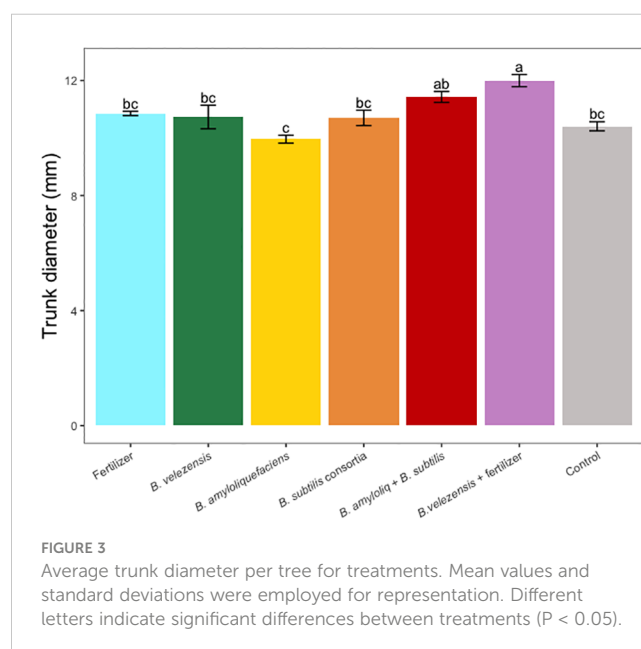
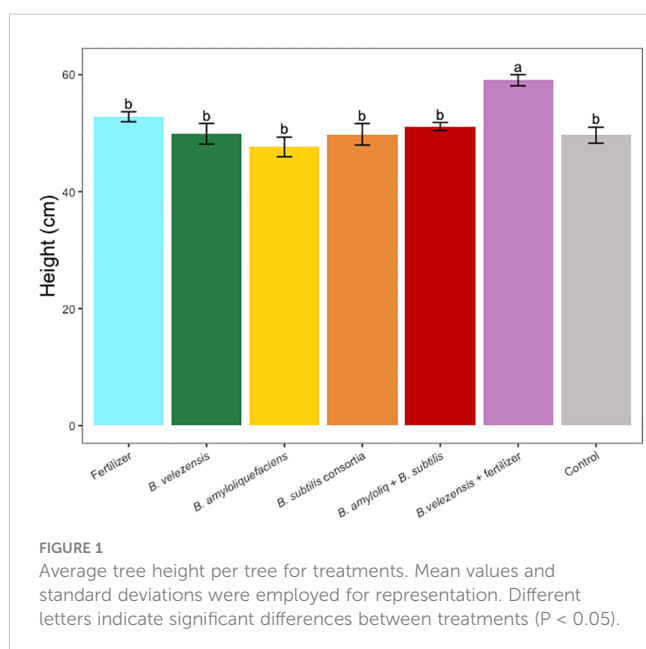
Results and discussion

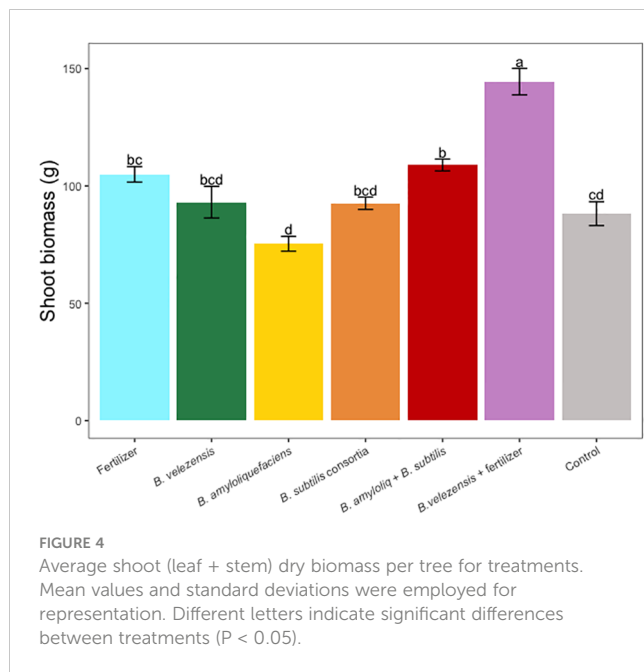
Grapefruit seedling growth

Results of grapefruit seedling growth measurements included height, canopy volume, trunk diameter, root, stem, and leaf biomass, and SPAD chlorophyll index. Tree height, canopy volume, trunk diameter, shoot biomass, root biomass, and chlorophyll index were significantly greater for plants inoculated with *B. velezensis* that also received fertilizer (Figures 1–6). The *B. subtilis* + *B. amyloliquefaciens* consortium treatment (T5) had a significantly higher shoot biomass than the untreated negative control (T7), despite the lack of any added fertilizer. The growth promotion ability may have an effect on roots (Shen et al., 2023) and can lead to an increase in water and nutrient uptake for the overall plant growth (Wang et al., 2020). In our results, Supplementary Figure 3 shows a very highly significant correlation ($p < 0.001$) between leaf N accumulated and root biomass per tree to illustrate the linkage between enhanced root biomass and N uptake. The *B. velezensis* inoculation + fertilizer treatment (T6) produced significantly more shoot biomass than any other treatment, even including T1, the fertilized positive control (Figure 4), and the *B. velezensis* inoculation (T2), suggesting synergy between the *B. velezensis* and fertilizer. The ratio of shoot/root biomass is an estimate of response to physical, chemical, and biological factors related to above-ground architecture (Lopez et al., 2023). Supplementary Figure 1 shows no significant differences among treatments suggesting that the relative changes in root and shoot growth according to treatments were approximately proportional. The *B. subtilis* + *B. amyloliquefaciens* treatment produced a significantly higher ($p < 0.001$) SPAD chlorophyll index than the control (Figure 6),



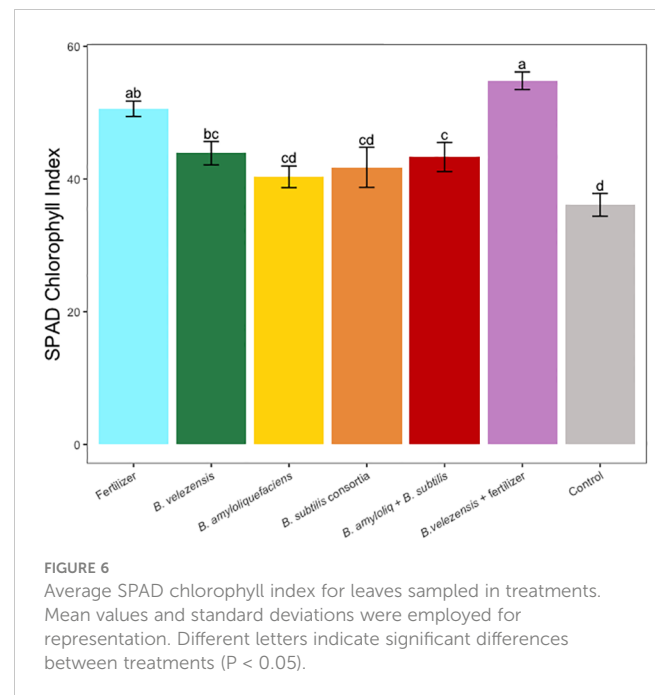
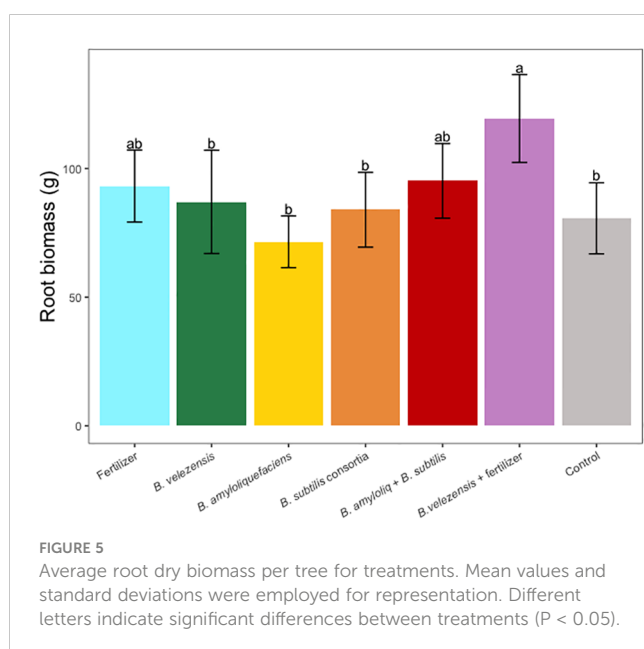
produced significantly more shoot biomass than any other treatment, even including T1, the fertilized positive control (Figure 4), and the *B. velezensis* inoculation (T2), suggesting synergy between the *B. velezensis* and fertilizer. The ratio of shoot/root biomass is an estimate of response to physical, chemical, and biological factors related to above-ground architecture (Lopez et al., 2023). Supplementary Figure 1 shows no significant differences among treatments suggesting that the relative changes in root and shoot growth according to treatments were approximately proportional. The *B. subtilis* + *B. amyloliquefaciens* treatment produced a significantly higher ($p < 0.001$) SPAD chlorophyll index than the control (Figure 6),





but the other *B. subtilis* and *B. amyloliquefaciens* treatments did not show evidence of promoting tree growth (Figures 1–6).

Soil inoculation with bacteria that promote plant growth is a promising technique for sustainable agricultural management and an effort to increase the efficiency of crops to uptake nutrient via symbiosis with microbes. In the present study, we anticipated that the applied bacteria would successfully establish around the rhizosphere in the potting media, as demonstrated in this study. However, when conducting microbial inoculation in open field soil conditions, the response of soil bacterial communities may be less consistent due to environmental variability such as tillage (Sergaki et al., 2018), pesticides, fertilizers, and sewage sludge amendments



applied in raw form (Omotayo and Babalola, 2021). These environmental factors could disrupt the establishment and proliferation of the bacteria. *Bacillus* species are well known for their plant growth-promoting potential (Gohil et al., 2022), and based on our results, we could see a marked increase in above and below-ground plant biomass with PGPR inoculation. Although the specific mechanisms by which PGPR promotes plant growth are not fully understood (Backer et al., 2018), they are thought to include auxin secretion by *B. velezensis*, systemic induced resistance, phosphate solubilization (Wang et al., 2020; Wang et al., 2022) pathogen control, and secondary metabolites (Grobelak et al., 2015; Ghazy & El-Nahrawy, 2021) to protect and promote plant growth (Wang et al., 2020).

Microbial respiration assay

We hypothesized that microbial respiration from the most effective bacterial treatments would vary compared to the controls, however there were no significant differences among the microbial respiration of the treatments (Supplementary Figure 2). The positive and negative controls (untreated and fertilized) showed respiratory activity even though they were not inoculated with bacteria, and this indicates that unsterilized potting media can harbor a wide variety of microorganisms, whether they are beneficial (Huang et al., 2012; Al-Mazroui and Al-Sadi, 2015) or pathogenic species (Al-Sa'di et al., 2008; Al-Sadi et al., 2011). The fact that in the present work all the treatments showed microbial respiration activity suggests that it could have masked the activity of added PGPR treatments. From previous studies, we can identify the lack of analysis for bacterial communities' characterization in potting media to assess changes in the activity of inoculated soil microbial communities (Al-Sadi et al., 2016; Mawarda et al., 2022).

Leaf nutrient analyses

Inoculation with *B. velezensis* together with the addition of a low (25%) fertilizer rate significantly increased leaf nitrogen (N), magnesium (Mg), and sulfur (S) concentrations compared to the other treatments of *Bacillus* species along with the positive and negative control (Table 3). Salvagiotti et al. (2009) reported a synergism in the uptake of N and S by plants, and a synergism of P and Mg uptake by plants was reported by Weih et al., 2021. However, in our study P concentration differences were not significant (Table 3) but increased levels of P uptake were observed from treatments inoculated with *B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis*. Leaf concentrations of K, Ca (Table 3), Mn, and Cu (Table 4) were also not significantly different among treatments.

Nitrogen is one of the essential nutrients for crop growth and has been widely studied (Wang et al., 2014). In our study, due to a low rate of fertilization, leaf N concentration for all treatments was below the optimum rate required for citrus crops (Obreza et al., 2020), however, leaf N concentration for the *B. velezensis* plus fertilizer treatment, significantly outperformed the rest of the treatments (Table 3). A similar pattern was observed in the SPAD chlorophyll index (Figure 6), where fertilizer treatment *B. velezensis* plus fertilizer had the highest average chlorophyll content correlated to N status in the leaf.

In the absence of fertilizer, there were non-significant differences in leaf nutrient concentrations among treatments inoculated only with bacteria (Tables 3, 4), emphasizing the importance of synergistically including fertilizer with PGPR inoculation as some previous studies found, increasing yield in tomato (*Solanum lycopersicum* L.) (Clagnan et al., 2023) and cowpea (*Vigna unguiculata* L. Walp) (Stamford et al., 2020). Surprisingly, leaf iron (Fe) concentrations were higher for the control treatment (T7; Table 4), although Fe concentration was at the optimum level for all the treatments. For the negative control (T7), it is possible that other nutrients were more limiting than Fe, resulting in a lack of biomass growth and a relative concentration of Fe in the leaves when compared to the more thrifty treatments.

Stamford et al. (2020); Clagnan et al. (2023) and Angulo et al. (2020) have examined the potential beneficial effects of microbial-based fertilizers on the rhizosphere in a competitive nutrient-limited

environment. After soil inoculation, these microorganisms contribute to nitrogen fixation, solubilization/mobilization of soil nutrients, and secretion of substances that enhance plant growth (Shahwar et al., 2023). The *B. amyloliquefaciens* + *B. subtilis* consortia (T5) treatment accumulated the most leaf N per tree for any unfertilized treatment, and significantly more than *B. amyloliquefaciens* (T3; Supplementary Figure 4). PGPRs play a key role when unavailable nutrients are present in the soil or nearby the rhizosphere (Prasad et al., 2019). From the regression line of N fertilizer applied versus leaf N accumulated ($y = 0.4015x - 169.19$), we estimated the amount of N contributed by PGPRs that was not derived by chemical N fertilizer. T6 was the highest (105 mg N/pot), followed by T5 (47 mg N/pot), T2 (16 mg N/pot), T4 (14 mg N/pot), and T3 (-23 mg N/pot). The negative value for T3 suggests microbial N immobilization. For T6, the amount of fertilizer N applied (82 mg N/pot) was subtracted from the interpolated result, leaving only the equivalent portion derived from PGPR activity (105 mg N/pot). The highest N fertilizer equivalent provided only by PGPRs was T5 (*B. amyloliquefaciens* + triple *B. subtilis* consortia), which was 57% of the amount of chemical N fertilizer supplied in treatments 1 and 6. Gohil et al., 2022 reported that *Bacillus* sp. PG-8 was able to solubilize insoluble nutrients to help the plant growth. Phosphate solubilization is most frequently attributed to *Bacillus* isolates by 80% compared to *Azotobacter*, *Pseudomonas*, and least by *Mesorhizobium* (Ahmad et al., 2008). The promotion of plant growth can be ascribed to various mechanisms, including the synthesis of plant growth-promoting hormones within the rhizosphere and the involvement in other plant growth-promoting activities (Ahmad et al., 2008). In recent years, numerous strains of *B. velezensis* have been discovered and extensively examined for their impact on plant growth, disease, and pest resistance, and the induction of systemic plant defenses, while some strains have proven to be pivotal in enhancing crop yield.

Conclusions

This study demonstrated that PGPR inoculation using *B. velezensis* plus fertilizer as a root drench significantly increased root biomass, trunk diameter, tree height, canopy volume, and

TABLE 3 The concentration of macronutrients in leaves for the different treatments.

Treatments	Macronutrients					
	N%	P%	K%	Ca%	Mg%	S%
Fertilizer	1.85 ± 0.15 ab	0.15 ± 0.02 ab	1.8 ± 0.235	2.8 ± 0.292	0.434 ± 0.039 ab	0.526 ± 0.065 a
<i>B. velezensis</i>	1.69 ± 0.13 b	0.15 ± 0.007 ab	1.86 ± 0.182	2.82 ± 0.259	0.42 ± 0.023 abc	0.412 ± 0.035 b
<i>B. amyloliquefaciens</i>	1.65 ± 0.07 b	0.15 ± 0.004 ab	1.96 ± 0.114	2.78 ± 0.192	0.418 ± 0.027 abc	0.424 ± 0.045 b
<i>B. subtilis</i> consortia	1.59 ± 0.17 b	0.13 ± 0.008 ab	1.72 ± 0.130	2.82 ± 0.228	0.384 ± 0.013 c	0.394 ± 0.041 b
<i>B. amyloliq</i> + <i>B. subtilis</i> consortia	1.73 ± 0.20 ab	0.13 ± 0.018 b	1.76 ± 0.195	2.82 ± 0.192	0.396 ± 0.028 bc	0.424 ± 0.032 b
<i>B. velezensis</i> + fertilizer	1.97 ± 0.08 a	0.16 ± 0.011 a	1.78 ± 0.083	2.94 ± 0.288	0.454 ± 0.008 a	0.536 ± 0.061 a
Control	1.59 ± 0.19 b	0.13 ± 0.008 ab	1.78 ± 0.130	2.64 ± 0.152	0.398 ± 0.028 bc	0.416 ± 0.041 b
ANOVA p	<0.001	0.0261	0.299	0.583	0.00205	<0.001

Mean values and standard deviations are shown. Different letters indicate significant differences between treatments ($P < 0.05$).

TABLE 4 The concentration of micronutrients in leaves for the different treatments.

Treatments	Micronutrients				
	B ppm	Zn ppm	Mn ppm	Fe ppm	Cu ppm
Fertilizer	67.6 ± 5.98	16.8 ± 1.79 ab	27.4 ± 2.07	132 ± 21.2 ab	4.8 ± 1.48
<i>B. velezensis</i>	66 ± 10.1	17.8 ± 3.03 ab	22.6 ± 6.80	97.8 ± 10.8 bc	4.4 ± 0.548
<i>B. amyloliquefaciens</i>	62.4 ± 5.94	19 ± 2.24 a	20.2 ± 2.05	91.6 ± 14.5 c	4 ± 1.22
<i>B. subtilis</i> consortia	61.2 ± 6.61	15.4 ± 1.14 b	22.4 ± 2.79	107 ± 20.8 abc	3.6 ± 1.14
<i>B. amyloliq</i> + <i>B. subtilis</i> consortia	67.2 ± 4.60	15.6 ± 1.67 ab	23.4 ± 8.44	126 ± 26.8 abc	4.2 ± 1.64
<i>B. velezensis</i> + fertilizer	64.8 ± 9.12	17.2 ± 1.48 ab	26.4 ± 7.47	125 ± 29.5 abc	4 ± 1.58
Control	58.2 ± 7.01	16 ± 1.41 ab	23.8 ± 5.07	140 ± 26.0 a	5.2 ± 1.64
ANOVA <i>p</i>	0.342	0.0332	0.455	0.001	0.231

Mean values and standard deviations are shown. Different letters indicate significant differences between treatments ($P < 0.05$).

SPAD chlorophyll index of grapefruit seedlings. The combination of these factors can contribute to healthier and more robust plants. The increase in canopy volume is indicative of greater foliage density, which can have a direct influence on photosynthesis, light interception, and overall productivity. In the same context, the study also shows that the chlorophyll index, an indicator of photosynthetic activity, was significantly enhanced by the *B. velezensis* inoculation and fertilizer. Furthermore, the study reveals that leaf nutrient concentrations, particularly nitrogen (N), sulfur (S), and magnesium (Mg), were significantly increased in plants treated with PGPR and fertilizer compared to other treatments.

Another key finding of this research was the necessity of sufficient fertilizer in the root zone in order to obtain the desired benefits from PGPR inoculation. Future studies designed to establish the critical nutrient levels required for optimizing the function and benefits of PGPR would help refine recommendations for their widespread use in citrus cultivation. Nursery trees grown in artificial media and drenched with PGPR could reach marketable size sooner, with less resources. The increased root biomass of PGPR-grown nursery trees would be especially valuable during the transplanting and establishment of trees in the field.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

LC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. JL: Resources, Writing – review & editing. NW: Resources, Writing – review &

editing. SS: Writing – review & editing. AS: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fhort.2024.1383013/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Average shoot/root dry biomass ratio for treatments. Mean values and standard deviations were employed for representation. Different letters indicate significant differences between treatments ($P < 0.05$).

References

- Ahmad, F., Ahmad, I., and Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol. Res.* 163, 173–181. doi: 10.1016/j.micres.2006.04.001
- Akinrinola, R. J., Yuen, G. Y., Drijber, R. A., and Adesemoye, A. O. (2018). Evaluation of *Bacillus* strains for plant growth promotion and predictability of efficacy by *in vitro* physiological traits. *Int. J. Microbiol.* 2018, 11. doi: 10.1155/2018/5686874
- Al-Mazroui, S. S., and Al-Sadi, A. M. (2015). Highly variable fungal diversity and the potential occurrence of plant pathogenic fungi in potting media, organic fertilizers and composts originating from 14 countries. *J. Plant Pathol.* 97, 6.
- Al-Sadi, A. M., Al-Said, F. A., Al-Jabri, A. H., Al-Mahmooli, I. H., Al-Hinai, A. H., and De Cock, A. W. A. M. (2011). Occurrence and characterization of fungi and oomycetes transmitted via potting mixtures and organic manures. *Crop Prot.* 30, 38–44. doi: 10.1016/j.cropro.2010.09.015
- Al-Sadi, A. M., Al-Zakwani, H. A., Nasehi, A., Al-Mazroui, S. S., and Al-Mahmooli, I. H. (2016). Analysis of bacterial communities associated with potting media. *Springerplus* 5, 1–5. doi: 10.1186/s40064-016-1729-0
- Al-Sa'di, A. M., Drenth, A., Deadman, M. L., Al-Said, F. A., Khan, I., and Aitken, E. A. B. (2008). Potential sources of *Pythium* inoculum into greenhouse soils with no previous history of cultivation. *J. Phytopathol.* 156, 502–505. doi: 10.1111/j.1439-0434.2008.01396.x
- Anderson, J. P. E., and Domsch, K. H. (1978). A physiological method for the quantitative measurement of microbial biomass in soil. *Soil Biol. Biochem.* 11, 215–221. doi: 10.1016/0038-0717(78)90099-8
- Angulo, J., Martínez-Salgado, M. M., Ortega-Blu, R., and Fincheira, P. (2020). Combined effects of chemical fertilization and microbial inoculant on nutrient use efficiency and soil quality indicators. *Scientia Agropecuaria* 11, 375–380. doi: 10.17268/sci.agropecu.2020.03.09
- Antoszewski, M., Mierek-Adamska, A., and Dąbrowska, G. B. (2022). The importance of microorganisms for sustainable agriculture—a review. *Metabolites* 12, 1100. doi: 10.3390/metabo12111000
- Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (2018). Plant growth-promoting rhizobacteria: Context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. *Front. Plant Sci.* 9, 1473. doi: 10.3389/fpls.2018.01473
- Barnawal, D., Singh, R., and Singh, R. P. (2019). “Role of plant growth promoting rhizobacteria in drought tolerance: regulating growth hormones and osmolytes,” in *PGPR amelioration in sustainable agriculture* (Cambridge, United Kingdom: Woodhead Publishing), 107–128. doi: 10.1016/B978-0-12-815879-1.00006-9
- Blake, C., Christensen, M. N., and Kovacs, A. T. (2021). Molecular aspects of plant growth promotion and protection by *Bacillus subtilis*. *Mol. Plant-Microbe Interact.* 34, 15–25. doi: 10.1094/MPMI-08-20-0225-CR
- Bueno, C. B., Dos Santos, R. M., de Souza Buzo, F., de Andrade da Silva, M. S. R., and Rigobelo, E. C. (2022). Effects of chemical fertilization and microbial inoculum on *Bacillus subtilis* colonization in soybean and maize plants. *Front. Microbiol.* 13, 901157. doi: 10.3389/fmicb.2022.901157
- Chen, K., Tian, Z., He, H., Long, C. A., and Jiang, F. (2020). *Bacillus* species as potential biocontrol agents against citrus diseases. *Biol. Control* 151, 104419. doi: 10.1016/j.biocontrol.2020.104419
- Clagnan, E., Cucina, M., De Nisi, P., Dell'Orto, M., D'Imporzano, G., Kron-Morelli, R., et al. (2023). Effects of the application of microbiologically activated bio-based fertilizers derived from manures on tomato plants and their rhizospheric communities. *Sci. Rep.* 13, 22478. doi: 10.1038/s41598-023-50166-5
- Compant, S., Samad, A., Faist, H., and Sessitsch, A. (2019). A review on the plant microbiome: Ecology, functions, and emerging trends in microbial application. *J. Adv. Res.* 19, 29–37. doi: 10.1016/j.jare.2019.03.004
- Dala-Paula, B. M., Plotto, A., Bai, J., Manthey, J. A., Baldwin, E. A., Ferrarezi, R. S., et al. (2019). Effect of huanglongbing or greening disease on orange juice quality, a review. *Front. Plant Sci.* 9, 1976. doi: 10.3389/fpls.2018.01976
- De Mendiburu, F. (2022) *agricolae: Statistical Procedures for Agricultural Research. R package version x.y.z.* Available online at: <https://CRAN.R-project.org/package=agricolae>.
- Elsharkawy, M. M., Khedr, A. A., Mehari, F., El-Kady, E. M., Alwutayd, K. M., and Behiry, S. I. (2023). Rhizobacterial colonization and management of bacterial speck pathogen in tomato by *Pseudomonas* spp. *Microorganisms* 11, 1103. doi: 10.3390/microorganisms11051103
- Etesami, H., Jeong, B. R., and Glick, B. R. (2023). Potential use of *Bacillus* spp. as an effective biostimulant against abiotic stresses in crops—A review. *Curr. Res. Biotechnol.* 5, 100128.
- Fan, B., Li, L., Chao, Y., Förstner, K., Vogel, J., Borriss, R., et al. (2015). DRNA-seq reveals genomewide TSSs and noncoding RNAs of plant beneficial rhizobacterium *Bacillus amyloliquefaciens* FZB42. *PLoS One* 10, e0142002. doi: 10.1371/journal.pone.0142002
- Gamez, R., Cardinale, M., Montes, M., Ramirez, S., Schnell, S., and Rodriguez, F. (2019). Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (Musa acuminata Colla). *Microbiol. Res.* 220, 12–20. doi: 10.1016/j.micres.2018.11.006
- Ghazy, N., and El-Nahrawy, S. (2021). Siderophore production by *Bacillus subtilis* MF497446 and *Pseudomonas koreensis* MG209738 and their efficacy in controlling *Cephalosporium maydis* in maize plant. *Arch. Microbiol.* 203, 1195–1209. doi: 10.1007/s00203-020-02113-5
- Gohil, R. B., Raval, V. H., Panchal, R. R., and Rajput, K. N. (2022). Plant Growth-Promoting Activity of *Bacillus* sp. PG-8 Isolated From Fermented Panchagavya and Its Effect on the Growth of *Arachis hypogea*. *Front. Agron.* 4, 805454.
- Gong, H., Li, J., Ma, J., Li, F., Ouyang, Z., and Gu, C. (2018). Effects of tillage practices and microbial agent applications on dry matter accumulation, yield and the soil microbial index of winter wheat in North China. *Soil Tillage Res.* 184, 235–242. doi: 10.1016/j.still.2018.07.002
- Grobela, A., Napora, A., and Kacprzak, M. (2015). Using plant growth-promoting rhizobacteria (PGPR) to improve plant growth. *Ecol. Eng.* 84, 22–28. doi: 10.1016/j.ecoleng.2015.07.019
- Hallman, L., Kadyampakeni, D., Ferrarezi, R., Wright, A., Ritenour, M., Johnson, E., et al. (2022). Impact of ground applied micronutrients on root growth and fruit yield of severely Huanglongbing-affected grapefruit trees. *Horticulturae* 8, 763. doi: 10.3390/horticulturae8090763
- Hu, G., Wang, Y., Blake, C., Nordgaard, M., Liu, X., Wang, B., et al. (2023). Parallel genetic adaptation of *Bacillus subtilis* to different plant species. *bioRxiv* 9, p001064. doi: 10.1099/mgen.0.001064
- Huang, T. P., Tzeng, D. D. S., Wong, A. C., Chen, C. H., Lu, K. M., Lee, Y. H., et al. (2012). DNA polymorphisms and biocontrol of *Bacillus* antagonistic to citrus bacterial canker with indication of the interference of phyllosphere biofilms. *PLoS ONE* 7 (7), e42124. doi: 10.1371/journal.pone.0042124
- Jamil, F., Mukhtar, H., Fouillaud, M., and Dufossé, L. (2022). Rhizosphere signaling: Insights into plant–rhizomicrobiome interactions for sustainable agronomy. *Microorganisms* 10, 899. doi: 10.3390/microorganisms10050899
- Kumar, A., Prakash, A., and Johri, B. N. (2011). “*Bacillus* as PGPR in crop ecosystem,” in *Bacteria in agrobiolgy: crop ecosystems* (Heidelberg, Germany: Springer Berlin Heidelberg), 37–59.
- Kumar, A., Singh, V. K., Tripathi, V., Singh, P. P., and Singh, A. K. (2018). “Plant growth-promoting rhizobacteria (PGPR): perspective in agriculture under biotic and abiotic stress,” in *New and future developments in microbial biotechnology and bioengineering: crop improvement through microbial biotechnology* (Amsterdam, The Netherlands: Elsevier), 333–342. doi: 10.1016/B978-0-444-63987-5.00016-5
- Kumar Meena, R., Kumar Singh, R., Pal Singh, N., Kumari Meena, S., and Singh Meena, V. (2015). Isolation of low temperature surviving plant growth - promoting rhizobacteria (PGPR) from pea (*Pisum sativum* L.) and documentation of their plant

- growth promoting traits. *Biocatalysis Agric. Biotechnol.* 4, 806–811. doi: 10.1016/j.bcab.2015.08.006
- Li, J., Pang, Z., Lee, D., and Wang, N. (2021). Biological control of citrus bacterial canker by native endophytic *Bacillus* spp. isolates. (*Abstr.*) *Phytopathol.* 111, S2.1. doi: 10.1094/PHYTO-111-10-S2.1
- Lopez, G., Ahmadi, S. H., Amelung, W., Athmann, M., Ewert, F., Gaiser, T., et al. (2023). Nutrient deficiency effects on root architecture and root-to-shoot ratio in arable crops. *Front. Plant Sci.* 13, 5385. doi: 10.3389/fpls.2022.1067498
- Luo, L., Zhao, C., Wang, E., Raza, A., and Yin, C. (2022). *Bacillus amyloliquefaciens* as an excellent agent for biofertilizer and biocontrol in agriculture: An overview for its mechanisms. *Microbiol. Res.* 259, 127016. doi: 10.1016/j.micres.2022.127016
- Ma, X., Li, F., Chen, Y., Chang, Y., Lian, X., Li, Y., et al. (2022). Effects of fertilization approaches on plant development and fertilizer use of citrus. *Plants* 11, 2547. doi: 10.3390/plants11192547
- Mawarda, P. C., Lakke, S. L., Dirk van Elsas, J., and Salles, J. F. (2022). Temporal dynamics of the soil bacterial community following *Bacillus* invasion. *iScience* 25, 529–534. doi: 10.1016/j.isci.2022.104185
- Mengistie, G. Y., and Awlachev, Z. T. (2022). Evaluation of the plant growth promotion effect of *Bacillus* species on different varieties of tomato (*Solanum lycopersicum* L.) seedlings. *Adv. Agric.* 2022, 6. doi: 10.1155/2022/1771147
- Numan, M., Bashir, S., Khan, Y., Mumtaz, R., Shinwari, Z. K., Khan, A. L., et al. (2018). Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: A review. *Microbiol. Res.* 209, 21–32. doi: 10.1016/j.micres.2018.02.003
- Nwachukwu, B. C., Ayangbenro, A. S., and Babalola, O. O. (2021). Elucidating the rhizosphere associated bacteria for environmental sustainability. *Agric. (Switzerland)* 11, 1–18. doi: 10.3390/agriculture11010075
- Obreja, T., Zekri, M., and Hanlon, E. (2020). Nutrition of Florida Citrus Trees, 3rd Edition: Chapter 4. Soil and Leaf Tissue Testing. *EDIS*. doi: 10.32473/edis-ss671-2020
- Omotayo, O. P., and Babalola, O. O. (2021). Resident rhizosphere microbiome's ecological dynamics and conservation: Towards achieving the envisioned Sustainable Development Goals, a review. *Int. Soil Water Conserv. Res.* 9, 127–142. doi: 10.1016/j.iswcr.2020.08.002
- Patani, A., Prajapati, D., Ali, D., Kalasariya, H., Yadav, V. K., Tank, J., et al. (2023). Evaluation of the growth-inducing efficacy of various *Bacillus* species on the salt-stressed tomato (*Lycopersicon esculentum* Mill.). *Front. Plant Sci.* 14, 1168155. doi: 10.3389/fpls.2023.1168155
- Prasad, M., Srinivasan, R., Chaudhary, M., Choudhary, M., and Jat, L. K. (2019). Plant growth promoting rhizobacteria (PGPR) for sustainable agriculture. *PGPR Amelioration Sustain. Agric.*, 129–157. doi: 10.1016/B978-0-12-815879-1.00007-0
- Rosier, A., Beaugerard, P. B., and Bais, H. P. (2021). Quorum Quenching Activity of the PGPR *Bacillus subtilis* UD1022 Alters Nodulation Efficiency of *Sinorhizobium meliloti* on *Medicago truncatula*. *Front. Microbiol.* 11, 596299. doi: 10.3389/fmicb.2020.596299
- Salvagiotti, F., Castellarin, J. M., Miralles, D. J., and Pedrol, H. M. (2009). Sulfur fertilization improves nitrogen use efficiency in wheat by increasing nitrogen uptake. *Field Crops Res.* 113, 170–177. doi: 10.1016/j.fcr.2009.05.003
- Schumann, A., Singerman, A., Ritenour, M., Qureshi, J., and Alferez, F. (2023). 2023–2024 Florida Citrus Production Guide: Citrus under Protective Screen (CUPS) Production Systems: CPG ch. 21, H51304/CMG19, rev. 5/2023 (University of Florida, USA: EDIS). doi: 10.32473/edis-hs1304-2023
- Senger, M., Moresco, E., Dalbosco, M., Santin, R., Inderbitzin, P., and Barrocas, E. N. (2022). Methods to quantify *Bacillus simplex*-based inoculant and its effect as a seed treatment on field-grown corn and soybean in Brazil. *J. Seed Sci.* 44, 13. doi: 10.1590/2317-1545v44i263329
- Sergaki, C., Lagunas, B., Lidbury, I., Gifford, M. L., and Schäfer, P. (2018). Challenges and approaches in microbiome research: from fundamental to applied. *Front. Plant Sci.* 9, 1205. doi: 10.3389/fpls.2018.01205
- Shahwar, D., Mushtaq, Z., Mushtaq, H., Alqarawi, A. A., Park, Y., Alshahrani, T. S., et al. (2023). Role of microbial inoculants as biofertilizers for improving crop productivity: a review. *Heliyon* 9 (2), e16134. doi: 10.1016/j.heliyon.2023.e16134
- Shen, Y., Yang, H., Lin, Z., Chu, L., Pan, X., Wang, Y., et al. (2023). Screening of compound-formulated *Bacillus* and its effect on plant growth promotion. *Front. Plant Sci.* 14, 1174583. doi: 10.3389/fpls.2023.1174583
- Shi, J. W., Lu, L. X., Shi, H. M., and Ye, J. R. (2022). Effects of plant growth-promoting rhizobacteria on the growth and soil microbial community of *Carya illinoensis*. *Curr. Microbiol.* 79, 352. doi: 10.1007/s00284-022-03027-9
- Shi, H., Lu, L., Ye, J., and Shi, L. (2022). Effects of two *Bacillus velezensis* microbial inoculants on the growth and rhizosphere soil environment of *Prunus davidiana*. *Int. J. Mol. Sci.* 23, 13639. doi: 10.3390/ijms232113639
- Stamford, N. P., da Silva, E. V. N., da Silva Oliveira, W., dos Santos Martins, M., Moraes, A. S., de Barros, J. A., et al. (2020). Benefits of microbial fertilizer in wastespecific interaction with textile sludges on cowpea in a Brazilian Ultisol and on wastes toxicity. *Environ. Technol. Innovation* 18, 100756. doi: 10.1016/j.eti.2020.100756
- Sun, X., Xu, Z., Xie, J., Hesselberg-Thomsen, V., Tan, T., Zheng, D., et al. (2022). *Bacillus velezensis* stimulates resident rhizosphere *Pseudomonas stutzeri* for plant health through metabolic interactions. *ISME J.* 16, 774–787. doi: 10.1038/s41396-021-01125-3
- Thorne, M. S., Skinner, Q. D., Smith, M. A., Rodgers, J. D., Laycock, W. A., and Cerekci, S. A. (2002). Evaluation of a technique for measuring canopy volume of shrubs. *Rangeland Ecol. Management/Journal Range Manage. Arch.* 55, 235–241.
- Tsotetsi, T., Nephali, L., Malebe, M., and Tugizimana, F. (2022). *Bacillus* for plant growth promotion and stress resilience: What have we learned? *Plants* 11, 2482. doi: 10.3390/plants11192482
- Vashisth, T., Chun, C., and Ozores Hampton, M. (2020). Florida citrus nursery trends and strategies to enhance production of field-transplant ready citrus plants. *Horticulturae* 6, 8. doi: 10.3390/horticulturae6010008
- Wang, J., Qu, F., Liang, J., Yang, M., and Hu, X. (2022). *Bacillus velezensis* SX13 promoted cucumber growth and production by accelerating the absorption of nutrients and increasing plant photosynthetic metabolism. *Scientia Hort.* 301, 111151. doi: 10.1016/j.scienta.2022.111151
- Wang, Y., Wang, D., Shi, P., and Omasa, K. (2014). Estimating rice chlorophyll content and leaf nitrogen concentration with a digital still color camera under natural light. *Plant Methods* 10, 1–11. doi: 10.1186/1746-4811-10-36
- Wang, C., Zhao, D., Qi, G., Mao, Z., Hu, X., Du, B., et al. (2020). Effects of *Bacillus velezensis* FKM10 for Promoting the Growth of *Malus hupehensis* Rehd. *Inhibiting Fusarium verticillioides*. *Front. Microbiol.* 10, 2889. doi: 10.3389/fmicb.2019.02889
- Wang, H. W., Zhu, Y. X., Xu, M., Cai, X. Y., and Tian, F. (2022). Co-application of spent mushroom substrate and PGPR alleviates tomato continuous cropping obstacle by regulating soil microbial properties. *Rhizosphere* 23, 100563. doi: 10.1016/j.rhisph.2022.100563
- Weih, M., Liu, H., Colombi, T., Keller, T., Jäck, O., Vallenback, P., et al. (2021). Evidence for magnesium-phosphorus synergism and co-limitation of grain yield in wheat agriculture. *Sci. Rep.* 11, 9012. doi: 10.1038/s41598-021-88588-8
- Zahra, S. T., Tariq, M., Abdullah, M., Azeem, F., and Ashraf, M. A. (2023). Dominance of *Bacillus* species in the wheat (*Triticum aestivum* L.) rhizosphere and their plant growth promoting potential under salt stress conditions. *PeerJ* 11, e14621.