

Temporal application of *Bacillus* strains enhances growth and nutritional status of micropropagated banana plantlets

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Abstract

Background: Banana (*Musa* spp.) is a crop of high socioeconomic importance in tropical regions, and its productivity depends on the availability of high-quality seedlings, particularly those produced via micropropagation. However, the high costs involved and the low acclimatization rate constitute major limitations of this technique. In this context, the use of plant growth-promoting rhizobacteria (PGPR), such as species of the genus *Bacillus*, has emerged as a promising alternative due to their ability to synthesize phytohormones, solubilize nutrients, and induce stress tolerance. This study evaluated the effect of different temporal application strategies of *Bacillus* strains on micropropagated seedlings of the cultivar Prata Catarina, using a completely randomized design in a 2×4 factorial arrangement. The assessments included functional traits (indole-3-acetic acid, soluble phosphorus, and siderophore production), biomass accumulation, and mineral nutrition at 60 days after transplanting. The results showed that both isolates produced functional metabolites associated with growth promotion and that temporal application, particularly of isolate LPPC159, led to significant increases in plant growth and in the accumulation of N, P, K, and Ca. Early applications favored N and K accumulation, whereas later applications were more effective for Ca and P, demonstrating that the timing of inoculation decisively influences plant response. Thus, the inoculation of *Bacillus* at strategic moments represents a sustainable alternative to optimize banana seedling production, with the potential to replace or complement conventional fertilization practices.

Introduction

Banana (*Musa* spp.) is a crop of substantial economic and social importance, particularly in tropical and subtropical regions, where it serves both as a staple food and a key source of income (Maseko et al. 2024 ; Santos et al. 2025). The expansion and sustainability of banana cultivation are closely tied to the availability of high-quality seedlings, especially those produced through micropropagation (Rodrigues et al. 2022). However, despite the advantages of micropropagation, challenges such as high production costs and limited acclimatization success still constrain the widespread use of this technique. To overcome these limitations, the use of plant growth-promoting rhizobacteria (PGPR) has emerged as a promising strategy (Bouremani et al. 2023).

Among PGPR, *Bacillus* spp. stand out due to their ability to form endospores, survive under a wide range of environmental conditions, and produce bioactive compounds that enhance plant growth and resilience (Azeem et al. 2023; Khoso et al. 2024). These bacteria are known to facilitate nutrient solubilization, produce phytohormones such as indole-3-acetic acid (IAA), and induce systemic tolerance to environmental stressors (Etesami & Glick 2024; Yang et al. 2024). Consequently, the application of *Bacillus* strains during plantlets production holds potential to improve the physiological quality and robustness of banana plants, and this may reflect on productivity.

Despite the demonstrated benefits of *Bacillus*-based inoculants, the timing of their application is one of the most important steps in the context of micropropagated plantlets. These microorganisms can be

applied during the explant establishment, multiplication, rooting, and acclimatization phases (Guru et al., 2026), providing significant benefits during transplantation (Rodríguez-Romero et al., 2005; Rajamanickam et al., 2018). In this last phase, the presence of PGPRs is essential because transplantation occurs under stressful conditions, such as temperature, which can compromise its quality. Dantas et al. (2025) found that a strain of *Bacillus* sp. derived from the rhizosphere of banana plants, used in transplanting seedlings during the acclimatization phase and with reapplications every 15 days, combined with shading under thermal stress conditions, provided gains in pseudostem diameter, fresh mass, dry mass, and nutrient accumulation in micropropagated banana seedlings.

These temporal dynamics of plant-microbe interactions suggest that the stage at which microbial inoculants are introduced, whether during *in vitro* rooting, at transplantation, or at different points throughout acclimatization, may critically influence colonization success and the magnitude of plant responses (Ke & Levine 2021). Optimizing the timing of biological control agent (BCA) inoculation could therefore enhance the efficacy of beneficial microorganisms and provide a low-cost, environmentally friendly tool to support sustainable seedling production systems (Kong et al. 2025). In addition, the use of PGPRs originating from the rhizosphere of the host being tested can enhance their effects (Rodrigues et al., 2022; 2023), as was found in the present study.

In this context, the present study aims to investigate the influence of different temporal application strategies of *Bacillus* strains on the performance of banana seedlings. The specific objectives were: (i) to evaluate how the timing of *Bacillus* application affects morphological parameters; (ii) to determine the impact on root development; (iii) to identify the most effective application stage to maximize plant growth promotion; and (iv) what are the main mechanisms of action involved in the plant-microorganism interaction.

Our hypothesis is that the temporal application of *Bacillus* strains significantly influences the plant performance, with early or sequential applications, initiated during or after transplantation, resulting in better growth and nutritional status, since some *Bacillus* species are known for their ability to promote plant growth and enhance stress tolerance. By addressing the timing of microbial inoculation, this study aims to contribute to the refinement of bioinoculant use in banana propagation systems and to the broader development of microbial-based strategies in sustainable agriculture.

Material and Methods

Study site, experimental design, and treatments

The experiment was conducted in a greenhouse under controlled environmental conditions in Fortaleza, Ceará State, Brazil (Fig. 1).

A completely randomized experimental design was adopted, arranged in a 2 × 4 factorial scheme. The first factor corresponded to two application times of the treatments (time 1: at transplanting; time 2: at transplanting + reapplication at 30 days), and the second factor consisted of four treatments: control,

chemical fertilization (slow-release fertilizer, 14-14-14), and inoculation with two *Bacillus* strains (RAB9: *Bacillus pumilus* and LPPC159: *Bacillus* sp.). Each treatment included four replicates, totaling 32 experimental units.

The two application times were based on Rodrigues et al. (2022). In that study, bacterial strains were applied only at seedling transplantation. The results suggested that additional applications during the 60-day acclimatization period could further enhance seedling growth. Therefore, in the present study, bacterial strains were applied at transplantation and reapplied 30 days later.

Origin, production, and application of *Bacillus* strains

The strains RAB9 (*Bacillus pumilus*) and LPPC159 (*Bacillus* sp.) were obtained from the working collection of the Phytopathology Laboratory at Embrapa. The inocula were prepared in NYD culture medium (glucose: 10 mL, yeast extract: 5 g, sterile distilled water: 1000 mL) under orbital shaking at 150 rpm and 30°C for 24 hours. The biomass was centrifuged at 3,600 rpm for 15 min and washed three times with saline solution (0.9% NaCl).

The inoculum dose was defined based on preliminary greenhouse standardization assays and protocols commonly used for plant growth-promoting rhizobacteria (PGPR). A suspension containing 1×10^9 CFU mL⁻¹ was applied at 6.25 mL per 4 L of soil (1.56 mL L⁻¹), a rate considered sufficient to ensure effective rhizosphere colonization during early plant establishment. This dose was chosen to provide uniform bacterial distribution without affecting soil moisture or physicochemical properties and is consistent with rates commonly used in greenhouse studies with *Bacillus*-based inoculants. It was also selected to represent a practical inoculation strategy for nursery production systems.

In vitro assay: detection of growth-related mechanisms

To characterize the growth-promotion mechanisms exhibited by isolates LPPC159 and RAB9, the following assays were performed.

For the determination and quantification of indole-3-acetic acid (IAA) production, a loopful of a bacterial colony was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of NYDA broth, previously sterilized at 121°C for 15 min. The culture was incubated for 24 h in an orbital shaking incubator at 200 rpm and 28°C. Subsequently, a volume corresponding to 10% of the colonized medium (5 mL) was transferred to a 250 mL Erlenmeyer flask containing 45 mL of NYDA broth, either in the absence or presence of the precursor L-tryptophan. For treatments supplemented with the precursor, 5 mL of an L-tryptophan solution (100 µg mL⁻¹) was added per 50 mL of liquid medium. After an additional 24 h incubation under the same temperature and agitation conditions, the cultures were centrifuged at 6,000 rpm for 7 min, and the supernatant was collected for analysis. IAA quantification was performed using the colorimetric method described by Gordon and Weber (1951), in which 2 mL of the supernatant was mixed with 1 mL of Salkowski reagent. The reaction mixture was incubated in the dark at 28°C for 25 min to allow the development of a pink coloration. Absorbance was then measured at 530 nm using a

spectrophotometer. IAA concentrations ($\mu\text{g mL}^{-1}$) were calculated based on a standard curve prepared with known concentrations of synthetic IAA ranging from 0 to $70 \mu\text{g mL}^{-1}$. The IAA concentration in the samples was determined from the corresponding absorbance values.

Siderophore production by the strains was quantified using the universal method described by Schwyn and Neilands (1987). Initially, 50 mL of NYDA medium was dispensed into 250 mL Erlenmeyer flasks and sterilized by autoclaving at 121°C for 15 min. After cooling, a loopful of a bacterial colony was inoculated into the medium. After 24 h of incubation in an orbital shaking incubator at 200 rpm and 28°C , a volume corresponding to 10% of the colonized medium (5 mL) was transferred to 250 mL Erlenmeyer flasks containing 45 mL of NYDA broth. All flasks were further incubated for 24 h under the same temperature and agitation conditions. At the end of the incubation period, the cultures were centrifuged at 6,000 rpm to separate the bacterial pellet, and the supernatant was collected. An aliquot of 2 mL of the supernatant was mixed with 2 mL of chrome azurol S (CAS) solution in glass tubes. The reaction mixture was incubated for 1 h at room temperature and protected from light. Absorbance was then measured at 630 nm using a spectrophotometer (Cary 50).

Siderophore production was expressed as percentage units (%) according to the equation proposed by Payne (1988):

$$\text{Units (\%)} = ((A_b - A_a) \times 100) / A_b \text{ Eq. 1}$$

Where: A_b : Absorbance of the control and, A_a : Absorbance of the sample

The phosphate-solubilizing ability of the bacterial strains was evaluated in modified Kado liquid medium. Briefly, 100 mL of the medium were dispensed into 250 mL Erlenmeyer flasks, autoclaved at 121°C for 15 min, and inoculated with a single bacterial colony. Cultures were incubated for 24 h at 28°C under orbital shaking (200 rpm). Subsequently, 5 mL (10%, v/v) of the pre-culture were transferred to flasks containing 45 mL of fresh modified Kado medium and incubated under the same conditions for an additional 24 h. After incubation, cultures were centrifuged at 6,000 rpm for 7 min, and soluble phosphorus (P) in the supernatant was quantified using the colorimetric method of Murphy and Riley (1962), subtracting the P concentration measured in the uninoculated control (medium + phosphate). For P determination, 0.5 mL of the supernatant were mixed with 5 mL of distilled water and 2.5 mL of vanadomolybdate reagent (Malavolta et al., 1989). After 20 min of reaction in the dark, absorbance was measured at 420 nm using a spectrophotometer. Phosphorus concentrations were calculated from a $\text{Ca}_3(\text{PO}_4)_2$ standard curve ($8\text{--}160 \mu\text{g L}^{-1}$).

Greenhouse in vivo experiment

Micropropagated banana plantlets (cv. Prata Catarina) were transplanted into 4 L plastic pots containing a 1:1 mixture of soil and a commercial peat-based substrate. The mixture was previously sterilized in an autoclave at 121°C for 60 minutes under a pressure of 1 atm. After 24 hours, the sterilization process was repeated to ensure complete elimination of microorganisms.

The experimental units were randomly distributed within the greenhouse, and the pots were rotated weekly throughout the experimental period to minimize potential positional effects and spatial gradients in light and temperature. Irrigation was performed daily to maintain the substrate near field capacity. The application of the treatments (bacterial biomass) was carried out in accordance with what is described in the first item of the methodology.

Evaluations were carried out 60 days after transplanting, measuring plant height (cm), number of leaves, pseudostem diameter (mm), and root length (cm). After these measurements, plants were separated into leaves, pseudostems, and roots, which were washed sequentially with tap water, 3% (v:v) hydrochloric acid (HCl) solution, and deionized water. The plant material was then blotted dry with absorbent paper and weighed to obtain fresh shoot and root biomass (g). Subsequently, the vegetative tissues were placed in paper bags and dried in a forced-air oven at 65°C until reaching constant weight. After drying, the dry masses of leaves, pseudostems, total shoot, and roots (g) were determined.

The dried samples were ground using a Wiley mill, sieved through a 1 mm mesh, and stored in appropriate containers for subsequent analysis. To assess the nutritional status of the banana plantlets, samples were subjected to nitric-perchloric digestion for quantification of phosphorus, potassium, calcium, magnesium, and sulfur contents, and to sulfuric digestion for nitrogen determination, as described by Miyazawa et al. (2009). Finally, nutrient accumulation in leaves was calculated based on the dry matter and nutrient concentrations obtained from foliar samples.

Statistical treatment of data

The assumptions of normality and homogeneity of variances were assessed. Normality of the residuals was evaluated using the Shapiro–Wilk test, while homogeneity of variances was assessed using Levene’s test. The data regarding the number of leaves and the root length were transformed by the square root using $\sqrt{(x + 1)}$ before statistical analysis, since the original data did not meet the assumption of normality. Subsequently, the data were subjected to analysis of variance (ANOVA) and, when significant effects were detected, the treatment means were analyzed using the LSD test with a probability level of 5%.

Results

Seedling height was not significantly affected by the timing of treatment application (F value = 1.46) (Table 1). In contrast, the treatments had a significant effect on the height of banana plantlets (F value = 59.44) (Table 1). The treatments inoculated with the LPPC159 (24.44 cm) and RAB9 (22.19 cm) strains showed the greatest heights, not differing from each other, but differing from the fertilized treatments (17.54 cm) and the control (12.39 cm).

Table 1

Summary of the analysis of variance for height (cm), diameter (mm), number of leaves, and root length (cm) of banana plants as affected by treatments and application times.

		Height	Diameter	Number of leaves	Root length
		cm	mm		cm
Times	1	18.72 ± 1.09	12.09 ± 0.38	2.76 ± 0.02	4.97 ± 0.18
	2	19.56 ± 1.47	11.92 ± 0.60	2.61 ± 0.03	4.63 ± 0.14
Treatments	Control	12.39 ± 0.48	9.86 ± 0.42	2.64 ± 0.06	4.17 ± 0.23
	Fertilizer	17.53 ± 0.70	11.63 ± 0.46	2.70 ± 0.03	4.74 ± 0.15
	RAB9	22.19 ± 1.03	12.91 ± 0.75	2.70 ± 0.05	5.14 ± 0.15
	LPPC159	24.44 ± 0.74	13.63 ± 0.22	2.72 ± 0.03	5.16 ± 0.17
F test					
Times		1.46 ^{ns}	0.12 ^{ns}	24.20 ^{**}	3.83 ^{ns}
Treatments		59.44 ^{**}	11.30 ^{**}	1.06 ^{ns}	7.34 ^{**}
Times x treatments		2.80 ^{ns}	1.88 ^{ns}	1.72 ^{ns}	2.28 ^{ns}
SED		1.38	0.97	0.06	0.34
LSD		2.86	2.00	0.13	0.70
DF		24	24	24	24
C.V. (%)		10.23	11.43	3.23	10.02
^{ns} , * and ** indicate non-significant, significant at 5%, and significant at 1%, respectively.					
SED: Standard error of the difference between two means; LSD: Least significant difference between two means at the 5% level; DF: Degrees of freedom and CV: Coefficient of variation.					
Means transformed as $\sqrt{(x + 1)}$: Number of leaves and root length.					

The timing of treatment application did not significantly influence the pseudostem diameter of banana plantlets (F value = 0.12) (Table 1). In contrast, the different treatments caused statistically significant variations in pseudostem diameter (F value = 11.30) (Table 1). Strains LPPC159 (13.63 mm) and RAB9 (12.91 mm) produced the largest average diameters, not differing from fertilized seedlings (11.63 mm). However, minimal significant differences were observed between the strains and the control plants (9.86 mm).

The number of leaves on banana seedlings was significantly affected by the timing of application (F value = 24.20), but not by the treatments alone (F value = 1.06) (Table 1). In general, time 1 (2.76) resulted in a higher number of leaves than time 2 (2.61).

The root length of the banana seedlings was not influenced by the timing of the application (F value = 3.83), but it was influenced by the treatments (F value = 7.34). The LPPC159 and RAB9 strains provided the greatest root lengths (5.16 and 5.14 cm, respectively), but did not differ from those that were fertilized (4.74 cm). Minimal significant differences were observed in seedlings inoculated with the strains when compared to control plants (4.17 cm) (Table 1).

The bacterial strain in this study, RAB9 produced higher levels of indoleacetic acid (p value 0.0006), whereas LPPC159 showed greater siderophore production and phosphorus solubilization, which may differentially contribute to seedling growth promotion (p value < 0.0001, and 0.0002, respectively) (Table 2).

Table 2			
Indoleacetic acid production, siderophore, and soluble phosphorus synthesis by <i>Bacillus</i> strains.			
Strains	Indoleacetic acid	Siderophores	Soluble phosphorus
	µg mL ⁻¹	%	µg mL ⁻¹
RAB9	4.00 a	0.00 b	40.31 b
LPPC159	1.19 b	49.48 a	71.31 a
F test			
Strains	44.08 ^{**}	1195.61 ^{**}	67.79 ^{**}
CV (%)	23.08	8.17	9.53
ns, * and **: not significant, significant at 5% and 1% probability levels, respectively. Means followed by the same letter in the column do not differ from each other according to Tukey's test at the 5% probability level.			

In this study, the timing of *Bacillus* strain application significantly affected root dry matter (F value = 43.34) and total biomass production (F value = 4.35) of banana seedlings, without substantially impacting the aerial parts (F value = 3.95) (Table 3, and Fig. 2). Application time 2 (application at transplanting and reapplication 30 days later) resulted in the highest root dry matter content (1.60 g). Treatment with the LPPC159 strain resulted in the greatest gains in total biomass of micropropagated banana seedlings (4.31 g).

Table 3

Influence of the temporal application of *Bacillus* strains on dry matter production of banana plantlets.

		Shoot	Roots	Total
		g/plant		
Times	1	2.03 ± 0.22	0.70 ± 0.10	2.73 ± 0.28
	2	1.66 ± 0.15	1.60 ± 0.22	3.27 ± 0.36
Treatments	Control	1.04 ± 0.09	0.43 ± 0.10	1.48 ± 0.17
	Fertilizer	1.82 ± 0.21	0.98 ± 0.23	2.81 ± 0.24
	RAB9	1.92 ± 0.21	1.48 ± 0.29	3.41 ± 0.44
	LPPC159	2.58 ± 0.24	1.72 ± 0.28	4.31 ± 0.18
		F test		
Times		3.95 ^{ns}	43.34 ^{**}	4.35 [*]
Treatments		11.51 ^{**}	17.37 ^{**}	21.68 ^{**}
Times x Treatments		1.04 ^{ns}	4.91 ^{**}	1.86 ^{ns}
SED		0.37	0.27	0.51
LSD		0.77	0.57	1.05
DF		24	24	24
C.V. (%)		28.49	33.46	23.99
ns, * and ** indicate non-significant, significant at 5%, and significant at 1%, respectively.				
SED: Standard error of the difference between two means; LSD: Least significant difference between two means at the 5% level; DF: Degrees of freedom and CV: Coefficient of variation.				

The results demonstrate that the temporal application of *Bacillus* strains selectively influenced macronutrient accumulation in banana seedlings, significantly increasing N, Ca, K, and P (F value = 13.71, 6.37, 24.09 and 12.52, respectively) contents without affecting Mg and S (F value = 2.74 and 1.57) (Table 4, and Fig. 3). All treatments, including those with fertilizer, showed significant effects, highlighting the potential of *Bacillus* as an alternative or complement to conventional nutrient management (Table 4). Nitrogen accumulation increased from 24.55 mg plant⁻¹ in the control treatment to 56.42 mg plant⁻¹ in LPPC159, corresponding to a 130% increase. Calcium accumulation in strains LPPC159 and RAB9 was similar (44.49 and 37.19 mg plant⁻¹, respectively), but showed increases of 203 and 153%, respectively, when compared to control plants. Potassium accumulation in strains LPPC159 and RAB9 was similar (50.15 and 49.15 mg plant⁻¹, respectively), but showed increases of 47 and 44%,

respectively, when compared to control plants. However, phosphorus accumulation was greater in the LPPC159 strain (4.64 mg plant⁻¹), showing an increase of 174% when compared to the control plants.

Table 4
Influence of the temporal application of *Bacillus* strains on macronutrient accumulation in banana seedlings.

		N	Ca	Mg	K	S	P
		mg/plant					
Times	Time 1	47.50 ± 3.80	26.93 ± 3.35	36.60 ± 5.25	55.37 ± 3.79	5.21 ± 0.48	2.90 ± 0.36
	Time 2	34.81 ± 3.71	36.91 ± 4.41	43.18 ± 4.82	34.57 ± 2.38	5.88 ± 0.69	3.93 ± 0.32
Treatments	Control	24.55 ± 3.69	14.68 ± 1.81	16.55 ± 2.72	34.11 ± 4.71	2.71 ± 0.39	1.90 ± 0.24
	Fertilizer	37.91 ± 3.47	31.33 ± 3.36	35.28 ± 3.39	46.48 ± 4.33	5.38 ± 0.48	3.41 ± 0.26
	RAB9	45.75 ± 4.70	37.18 ± 5.52	44.80 ± 6.42	50.15 ± 5.65	6.62 ± 0.83	3.71 ± 0.57
	LPPC159	56.42 ± 4.36	44.48 ± 5.63	62.95 ± 1.86	49.15 ± 7.15	7.48 ± 0.41	4.63 ± 0.26
		F test					
Times		13.71 ^{**}	6.37 [*]	2.74 ^{ns}	24.09 ^{**}	1.57 ^{ns}	12.52 ^{**}
Treatments		15.33 ^{**}	10.28 ^{**}	23.66 ^{**}	3.05 [*]	15.22 ^{**}	15.22 ^{**}
Times x Treatments		0.70 ^{ns}	1.32 ^{ns}	0.45 ^{ns}	0.10 ^{ns}	1.71 ^{ns}	3.91 [*]
SED		6.86	7.91	7.96	8.47	1.07	0.59
LSD		14.15	16.32	16.42	17.48	2.20	1.22
DF		24	24	24	24	24	24
CV (%)		23.56	35.05	28.18	26.64	27.16	24.13
ns, * and ** indicate non-significant, significant at 5%, and significant at 1%, respectively.							
SED: Standard error of the difference between two means; LSD: Least significant difference between two means at the 5% level; DF: Degrees of freedom and CV: Coefficient of variation.							

Discussion

In this study, the strain LPPC159 (*Bacillus* sp.) promoted the greatest growth, followed by RAB9 (*Bacillus pumilus*), fertilizer, and control. These results highlight the potential of *Bacillus* strains, particularly

LPPC159, in promoting plant growth. This finding corroborates recent studies demonstrating the effectiveness of plant growth-promoting rhizobacteria (PGPR) in stimulating the development of agricultural and fruit crops, often surpassing conventional fertilizers (Rodrigues et al. 2022; Azeem et al. 2023; Khoso et al. 2024). Importantly, the superior performance observed for LPPC159 and RAB9 was not interpreted solely based on the presence of *in vitro* traits, but rather on the consistency between these biochemical capacities and the physiological responses observed *in vivo*. The higher efficacy of the strains may be associated with their ability to produce phytohormones, solubilize nutrients, and induce systemic resistance, thereby enhancing seedling performance (Singh et al. 2023; Wahab et al. 2024; Ehis-Eriakha et al. 2025). In this context, the growth responses detected in banana seedlings suggest that these microbial traits were effectively expressed under rhizosphere conditions, contributing to improved nutrient acquisition and root system establishment during acclimatization.

In the present study, the bacterial strain RAB9 produced indoleacetic acid, whereas LPPC159 produced siderophores and solubilized phosphate. These mechanisms are closely related to plant growth promotion and provide a mechanistic basis for the effects observed on banana seedlings in this study. The production of indoleacetic acid by RAB9 is likely associated with stimulation of root elongation and lateral root formation, increasing the absorptive surface area and favoring nutrient uptake, particularly during the early stages of seedling establishment. Conversely, the phosphate-solubilizing and siderophore-producing capacity of LPPC159 may have enhanced phosphorus and micronutrient availability in the substrate, which is consistent with the greater accumulation of P, Ca, N, and K observed in inoculated plants. The seedlings showed gains in height, pseudostem diameter, root development, and biomass, in addition to the accumulation of nutrients such as N, P, K, and Ca. These results are similar to other studies with growth-promoting microorganisms. For example, Shah et al. (2022) found that a phosphate-solubilizing bacterium that also produces siderophores and indoleacetic acid provided increases in variables related to banana growth, such as height, number of leaves, and pseudostem diameter.

These effects were potentiated when the bacteria were associated with mycorrhizae. In another study, Tatung and Deb (2023) showed that bacterial strains originating from the rhizosphere of *Musa itinerans*, which solubilize phosphate and produce siderophores and indoleacetic acid, had positive impacts on the growth of *Cicer arietinum* plants, such as root and shoot length, and fresh and dry mass of shoots and roots. All these results reinforce the interaction of the different mechanisms produced by microorganisms and their effects on promoting plant growth. Therefore, the present findings strengthen the hypothesis that the simultaneous expression of multiple PGPR traits may generate complementary effects in the rhizosphere, resulting in improved nutrient mobilization, root architecture modulation, and biomass accumulation.

Our findings indicate the high potential of the bacterial strains to promote radial growth of banana seedlings, possibly through mechanisms such as auxin production, nutrient solubilization, and synthesis of bioactive compounds (Wahab et al. 2024; Yavuz et al. 2024). The *Bacillus* sp. strain LPPC159 was able to solubilize phosphate in the soil/substrate, making phosphorus available to micropropagated banana

seedlings and improving their growth. This mechanism is of fundamental importance for the establishment of the microorganism in the soil, because with the increased growth of the root system and the presence of root exudates, there is a favoring of microbial colonization in the rhizosphere region of the plants (Pantigoso et al., 2023). This interaction likely created a positive feedback process in which enhanced root growth promoted greater rhizosphere competence and microbial persistence, while the continued microbial activity increased nutrient availability and plant vigor. Such rhizosphere-mediated interactions are particularly relevant during acclimatization of micropropagated seedlings, a stage characterized by physiological instability and reduced microbial association capacity.

These results of the present study indicate that the timing of application influences foliar development of the seedlings, and this response is modulated by the type of treatment. This underscores the importance of application timing and suggests that the efficacy of biological treatments may depend on temporal factors, such as the plant's phenological stage or the dynamics of microbial colonization in the rhizosphere (Bardin et al. 2015; Coen & Prusinkiewicz 2024). Regarding the colonization of banana rhizospheres, in a study by our research group (Rodrigues et al., 2022), strains of *Bacillus* sp. (1.2×10^9 CFU/ml) were inoculated during the acclimatization phase of the seedlings. In this process, only one bacterial application was performed on the rhizosphere of the seedlings, and at the end of 60 days, quantifications were carried out, showing an average bacterial population of $3.80\text{--}4.93 \times 10^4$ CFU/g. This study reinforces the need for more applications during banana cultivation during acclimatization, as well as in other phases under greenhouse conditions. Therefore, in the present study, in addition to the application at transplanting, we reapplied the bacteria at 30 days to the seedlings. Although only two applications were made during the 60-day acclimatization phase, these two inoculations provided considerable increases in variables related to growth promotion in micropropagated banana seedlings. The positive response obtained after reapplication suggests that maintenance of active bacterial populations in the rhizosphere may be essential for sustaining PGPR effects over time, particularly in sterile substrates where microbial community succession is initially limited. In this sense, repeated inoculations may have favored recolonization processes and prolonged metabolite production, increasing the interaction time between *Bacillus* strains and the developing root system. In future studies, our team intends to test more application times during the acclimatization phase. Furthermore, we used sterilized soil/substrate and pathogen-free micropropagated seedlings. Although we did not perform quantification at the end of the experiment, it is expected that the bacterial population did not suffer such a drastic drop. However, to ensure that the effects are indeed from the microorganisms applied to the seedlings, this quantification or visualization by other methods, such as fluorescence microscopy, is essential (Kumar et al., 2021; Pal et al., 2022). In future studies, our team will again include these validations of the effects of bacterial strains on banana plants. A limitation of the present study is the absence of direct assessment of bacterial colonization and persistence in the rhizosphere throughout the experimental period. Consequently, it was not possible to determine whether the differences observed among treatments were associated with variations in microbial establishment, persistence, or recolonization capacity over time. Additionally, future studies integrating microbial

population monitoring with plant physiological analyses will be important to better elucidate the temporal dynamics of *Bacillus* establishment and persistence in the banana rhizosphere.

It is worth highlighting that the timing of microorganism application has a significant influence on their rhizosphere colonization dynamics; the earlier the microorganism establishes itself in these ecological niches, the greater the positive impacts on the biological control of soil pathogens and increases in nutrient cycling (Choudhary & Johri, 2009). Early applications and the use of *Bacillus* or mineral fertilizers favor root development through mechanisms such as phytohormone production, phosphate solubilization, and siderophore synthesis (Silva et al. 2018; Timofeeva et al. 2022; Wahab et al. 2024; Yavuz et al. 2024). From a mechanistic perspective, successful early colonization may increase the efficiency of root-microbe signaling and improve the competitive establishment of beneficial microorganisms before occupation of ecological niches by other microbes. This may partially explain the superior responses observed in inoculated treatments compared with the non-inoculated control. As observed in this study, both RAB9 and LPPC159 exhibited distinct plant growth-promoting traits.

Biomass production is one of the main indicators of plant physiological and nutritional performance and is strongly influenced by the action of plant growth-promoting microorganisms, such as *Bacillus* (Fonseca et al. 2022; Terra et al. 2024). This finding supports the hypothesis that the activity of *Bacillus* is more directly associated with the rhizosphere, promoting root development through phytohormone production, nutrient solubilization, and systemic resistance induction (Glick 2012; Timofeeva et al. 2022; Wahab et al. 2024). This response may be associated with the strains' capacity to solubilize nutrients and modulate root metabolism (Matos et al. 2017). Moreover, the increase in biomass observed in inoculated seedlings likely reflects an integrated physiological response involving improved nutrient use efficiency, enhanced root functionality, and greater metabolic allocation to plant growth, rather than isolated effects of a single microbial trait.

The second application timing favored Ca and P accumulation, possibly due to higher microbial activity at more advanced stages of plant development (Hossain et al. 2022), whereas the first timing was more effective for N and K, nutrients essential during early growth stages. A significant interaction between application timing and treatments was observed for phosphorus, with higher levels in treatments combining fertilizer, LPPC159 and RAB9 at the second timing, suggesting a synergy between the phenological stage and P solubilization, in agreement with previous reports (Jensen et al. 2024). This temporal variation in nutrient accumulation reinforces the idea that PGPR-mediated effects are dynamic and dependent on the developmental stage of the host plant and the stability of microbial establishment in the rhizosphere. In particular, the enhanced phosphorus accumulation at later stages may indicate that the phosphate-solubilizing activity became more effective after establishment of a more structured root system and greater microbial adaptation to the substrate environment. Recent studies have reinforced the effectiveness of *Bacillus* strains in inducing physiological responses that favor growth in various crops, including tropical fruit species, emphasizing their role as bioinputs in sustainable agricultural systems (Akinrinlola et al. 2018; Patani et al. 2024).

Conclusions

The temporal application of *Bacillus* strains significantly influenced the growth and nutrition of micropropagated banana plantlets, with strain LPPC159 standing out by promoting the greatest gains in height, stem diameter, root development, biomass, and accumulation of N, P, K, and Ca. Early applications favored N and K accumulation, whereas late applications were more effective for Ca and P, highlighting the importance of the inoculation timing.

These findings suggest that temporal application strategies of plant growth-promoting rhizobacteria (PGPR) can optimize seedling production, providing a sustainable and cost-effective alternative to conventional nutrient management in agricultural systems.

Declarations

Conflict of interest statement

The authors declare no conflicts of interest.

Author Contribution

J.L.M: methodology and investigation. J.M.R.S: methodology and investigation. B.A.B: methodology and investigation. A.L.S.J: methodology and investigation. A.G.A.: formal analysis, validation, visualization and supervision. C.A.K.T: formal analysis, validation, visualization and supervision. C.F.B.S: formal analysis, validation, visualization, writing (review and editing), funding acquisition and resources, supervision.

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Figures

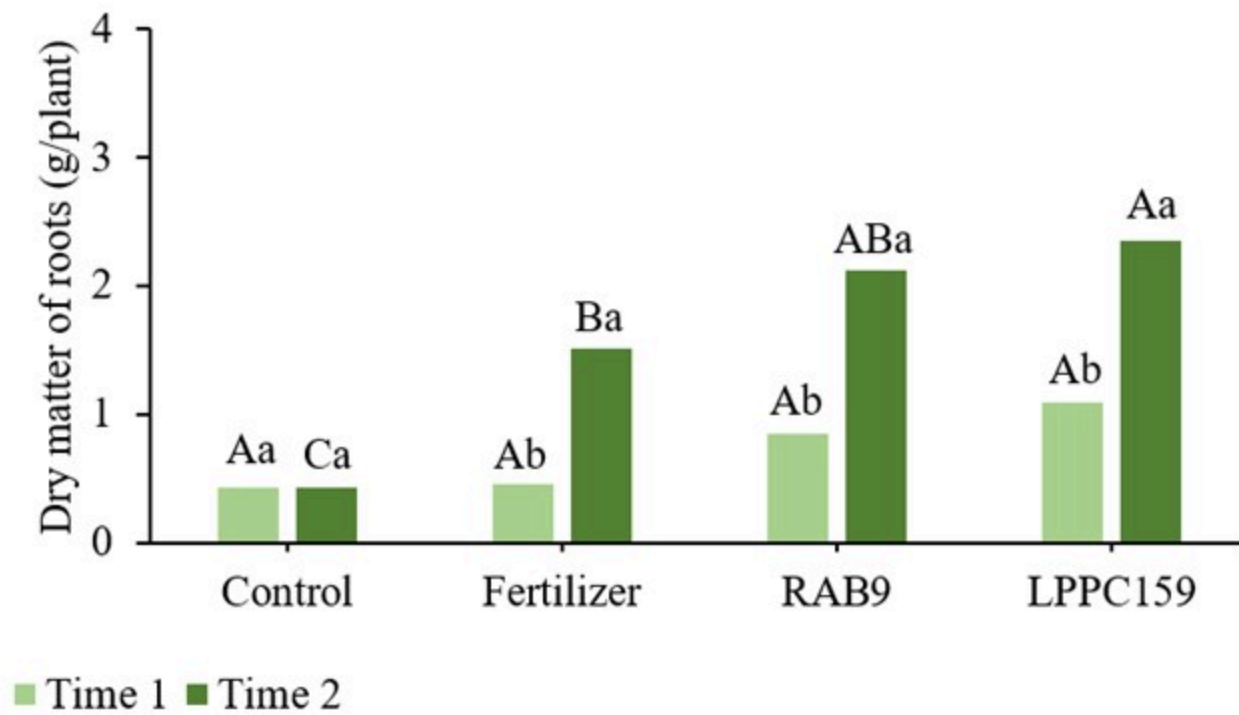


Figure 2

Breakdown of the interaction between application timings and treatments on root dry matter production of banana seedlings.