UNIVERSIDADE ESTADUAL DO SUDOESTE DA BAHIA
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ASSOCIATION OF GROWTH-PROMOTING MICROORGANISMS AND FERTILIZERS IMPROVES THE QUALITY OF BANANA SEEDLINGS

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Thesis presented to the State University of Southwest Bahia, as part of the requirements of the Graduate Program in Agronomy, area of concentration in Crop Science, to obtain the title of Doctor.

Advisor: Prof. Dr. Paulo Araquém Ramos Cairo
Coadvisor: Dr. Fernando Haddad

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GENERAL ABSTRACT

MOREIRA, F.M. **Association of growth-promoting microorganisms and fertilizers improves the quality of banana seedlings.** Vitória da Conquista – BA, UESB, 2021. 73 p. (Thesis: Doctor in Agronomy; Area of Concentration: Crop Science)*.

The inoculation of plant growth promoting microorganisms (PGPM) has been a promising and ecologically sustainable alternative to improve the quality of fruit seedlings. Based on the knowledge of the physiological and biochemical characteristics of these microorganisms, they can be managed in order to eliminate, totally or partially, the use of mineral fertilizers, or to enhance the effect of organic fertilizers on agricultural crops, without harming productivity. The two goals of this study were: (1) to investigate the ideal mixture of soil and organic compound with *Bacillus* sp. (CNPMF 1009) and *Trichoderma asperellum* (CNPMF 1007) to optimize the growth and nutrient content of banana seedlings, cultivar BRS Princesa; and (2) to evaluate the agronomic efficiency of some strains of nitrogen-fixing bacteria (NFB), associated with different doses of nitrogen (N) fertilization, on morphophysiological, biochemical and nutritional characteristics related to the growth of banana seedlings, cultivar Prata Anã. Two experiments were conducted in a greenhouse. In the first one, a factorial scheme (3×2×2)+3 was used, with the following factors: (a) substrate with different ratios of organic compost and soil (20:80, 40:60, and 60 :40); (b) inoculations with and without *Bacillus* sp. (B+ and B-); (c) inoculations with and without *Trichoderma asperellum* (T+ and T-), plus control treatments consisting of a 0:100 ratio, with the combinations [B+T-], [B-T+], and [B-T-]. In the second experiment, a factorial scheme 6×5 was used, with the following factors: (a) no inoculation (SI) and inoculations with BFN BR11005, BR11674, BR12137, BR12157, and BR12158; and (b) doses of N (0, 50, 100, 150, 200 mg kg-1 substrate). Plant height, pseudostem diameter, number of leaves, total leaf area, root volume, dry mass of aerial parts and root, photosynthesis, contents of chlorophyll, carotenoids, and proline, and concentrations of nitrogen, phosphorus, and potassium in the plant were evaluated. The results showed that the substrate with a 40:60 ratio and inoculation with *Bacillus* sp. and/or *Trichoderma asperellum* provided the highest morphophysiological quality for BRS Princesa banana seedlings. Regarding the cultivar Prata Anã, NFB BR11005 and BR12157 were the ones that promoted more growth, and the last strain had greater agronomic efficiency, as it was able to totally replace N fertilization. The benefits of inoculation with PGPM, alone or together, were better than those of organic fertilization, in relation to promoting seedling growth, due to its multiple abilities, such as biological N fixation, nutrient solubilization, and synthesis of auxin and hydrolytic enzymes.

Keywords: Organic fertilization, *Azospirillum baldaniorum*, *Bacillus* sp., BRS Princesa, *Burkholderia* sp., endophytics, mineral nutrition, *Trichoderma asperellum*, *Musa* spp., Prata Anã.

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RESUMO GERAL

MOREIRA, F.M. **Associação de microrganismos promotores de crescimento e fertilizantes melhora a qualidade de mudas de bananeira**. Vitória da Conquista – BA, UESB, 2021. 73 p. (Tese: Doutorado em Agronomia; Área de Concentração: Fitotecnia)*.

A inoculação de microrganismos promotores de crescimento em plantas (MPCP) tem sido uma alternativa promissora e ecologicamente sustentável para melhorar a qualidade de mudas de fruteiras. Com base no conhecimento das características fisiológicas e bioquímicas desses microrganismos, pode-se manejá-los de forma a eliminar, total ou parcialmente, o uso de fertilizantes minerais, ou potencializar o efeito de fertilizantes orgânicos em cultivos agrícolas, sem prejuízos à produtividade. Os dois objetivos deste estudo foram: (1) investigar a mistura ideal de solo e composto orgânico com inoculações de *Bacillus* sp. (CNPMF 1009) e *Trichoderma asperellum* (CNPMF 1007) para otimizar o crescimento e o teor de nutrientes em mudas de bananeira, cultivar BRS Princesa; e (2) avaliar a eficiência agronômica de algumas cepas de bactérias fixadoras de nitrogênio (BFN), associadas a diferentes doses de adubação com nitrogênio (N), sobre características morfofisiológicas, bioquímicas e nutricionais relacionadas ao crescimento de mudas de bananeira, cultivar Prata-Anã. Em casa de vegetação, conduziram-se dois experimentos. No primeiro, adotou-se um esquema fatorial (3×2×2)+3, com os seguintes fatores: (a) substrato com diferentes proporções de composto orgânico e solo (20:80, 40:60 e 60:40); (b) inoculações com e sem *Bacillus* sp. (B+ e B-); (c) inoculações com e sem *Trichoderma asperellum* (T+ e T-), acrescidos dos tratamentos controle, constituídos da proporção 0:100, com as combinações [B+T-], [B-T+] e [B-T-]. No segundo experimento, adotou-se o esquema fatorial 6×5, com os seguintes fatores: (a) sem inoculação (SI) e inoculações com as BFN BR11005, BR11674, BR12137, BR12157 e BR12158; e (b) doses de N (0, 50, 100, 150, 200 mg kg^{-1} de substrato). Foram avaliados altura de plantas, diâmetro do pseudocaule, número de folhas, área foliar total, volume radicular, massa seca de parte aérea e radicular, fotossíntese, teores de clorofilas, carotenoides e prolina, e concentrações de nitrogênio, fósforo e potássio na planta. Os resultados mostraram que o substrato com proporção 40:60 e inoculação com *Bacillus* sp. e/ou *Trichoderma asperellum* proporcionou a melhor qualidade morfofisiológica para as mudas de bananeira BRS Princesa. Com relação à cultivar Prata-Anã, as BFN BR11005 e BR12157 se destacaram como promotoras de crescimento, sendo que esta última apresentou maior eficiência agronômica, devido ao seu potencial para substituir totalmente a adubação nitrogenada. Os benefícios da inoculação com MPCP, de forma isolada ou em conjunto, foram melhores que os da adubação orgânica, em relação à promoção do crescimento das mudas, devido às suas múltiplas habilidades, como fixação biológica de N, solubilização de nutrientes e síntese de auxina e enzimas hidrolíticas.

Palavras-chave: Adubação orgânica, *Azospirillum baldaniorum*, *Bacillus* sp., BRS Princesa, *Burkholderia* sp., endofíticos, nutrição mineral, *Trichoderma asperellum, Musa* spp., Prata Anã

***Orientador:** Prof. Dr. Paulo Araquém Ramos Cairo, UESB, e **Co-orientador:** Dr. Fernando Haddad, Embrapa Mandioca e Fruticultura.

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A	Net photosynthesis
Chla	Chlamydospores
CFU	Colony-forming unity
CHLO	Total chlorophyll
GIP	Growth increase percentage
H	Plant height
Ha	Hectare
H/D	Plant height and pseudostem diameter ratio
Hyp	Hyphae
IRGA	Infrared Gas Analyzer
NA	Nutrient Agar
NFB	Nitrogen-fixing bactéria
NL	Number of leaves
PGPM	Plant growth-promoting microorganisms
PSA	Potato Sucrose Agar
PSD	Pseudostem diameter
RCP	Root colonization percentage
RDM	Root dry mass
ROS	Reactive oxigen species
RV	Root volume
SDM	Shoot dry mass
TLA	Total leaf área

SUMMARY

1. GENERAL INTRODUCTION

The banana plant (*Musa* spp.) has great cultural and commercial importance and is one of the most widely consumed fruits in the world. Brazil is one of the largest banana producers in the world, but their production is limited by nutritional and phytosanitary factors. To mitigate these limitations, new cultivars that are more adaptable to the edaphoclimatic conditions in the production regions and having different levels of resistance to major banana diseases have been developed (Ploetz, 2015; Ribeiro et al., 2018).

Prata Anã (AAB) (from the subgroup Prata), is one of the most traditionally grown banana cultivars in Brazil. BRS Princesa (YB42-07) is a newly generated tetraploid hybrid (AAAB) from the Silk subgroup, which is gradually being accepted in the Brazilian market. Agronomic evaluation of genotypes provides information to the Banana Genetic Breeding Program, which is currently seeking to develop the two main groups, Prata and Maçã (Gonçalves et al., 2018; Rebouças et al., 2018).

Mineral nutrition is one of the primary factors that limits banana productivity. The proper cultural management of orchards depends on the intensive use of chemical fertilizers during the different stages of banana cultivation, which significantly alters the microbial composition and soil quality, and thus affects plant health and productivity. To reduce the use of mineral fertilizers, organic compounds from composting agricultural waste as well as bioinoculants have been recommended (Chen et al., 2011; Huang et al., 2015; Cai et al., 2017; Cedeño et al., 2021).

Organic compounds can aid in improving the supply of water and nutrients that help to sustain plants. Depending on their chemical and physical characteristics, these compounds can be used as fertilizers and/or soil conditioners, which supply nutrition and/or improve the morphophysiological quality of seedlings (Amaral et al., 2016; Santos et al., 2017; Moreira et al., 2018; Moreira et al., 2021). However, it is necessary to evaluate the ideal proportion of organic compounds in the composition in the substrate, because excessive amounts of organic compounds can led to nutritional imbalances and the appearance of visible anomalies (Moreira et al., 2018).

On the other hand, plant growth-promoting microorganisms (PGPMs) can partially or completelly replace chemical fertilizers (Souza et al., 2016), and this may result in the enhancement of plant growth and productiviy (Moreira et al., 2021). PGPM acts on several biological processes, such as N-fixation, solubilization of phosphorus and iron (siderophores), synthesis of growth regulators (auxins, gibberellins, and cytokinins), and synthesis of hydrolytic enzymes (cellulase, protease, phosphatase, and chitinase) that decompose organic waste. Thus, PGPMs aid in plant nutrition and improve the biocontrol of phytopathogens, thereby providing greater protection from adverse environmental conditions (Chen et al., 2011; Fox et al., 2016; Kuan et al., 2016; Lopez-Coria et al., 2016; Gopalakrishnan et al., 2017; Kumar et al., 2017; Su et al., 2017; Silva et al., 2019; Wang et al., 2019).

PGPMs, also referred to as endophytes, are microorganisms which usually form symbiotic relationship with plants such that they provide plants with metabolites that improve their morphophysiological, biochemical, and nutritional characteristics (Doni et al., 2014; Shen et al., 2015; Lopez-Coria et al., 2016; Moreira et al., 2021). In turn, the host plant provides photoassimilates and physical protection against possible adverse conditions within the rhizosphere. The presence of inter-and/or intracellular endophytes, in different parts of the plant, such as the root, stem, and leaf, does not cause damage or disease symptoms. The compounds or nutrients provided by PGPMs are completely utilized by the plants, with little or no loss to the external environment (Mia et al., 2010).

Plant responses to inoculation with endophytes may vary depending on the plant species and biochemical characteristics of the strains. In general, inoculation leads to increases in plant height, dry mass, number of leaves, leaf area, photosynthetic pigment content, photosynthesis rate, root length and volume, and concentration of nutrients such as nitrogen, phosphorus, and potassium (Rozpadek et al., 2015; Souza et al., 2016; Wang et al., 2019; Zhai et al., 2019; Moreira et al., 2021). Therefore, the use of PGPM strains with multiple abilities is relevant in improving the agronomic efficiency of banana plants and obtaining healthy plants, that are adaptable to adverse field conditions.

Endophytes, in addition to enhancing plant growth, also promote morphophysiological and biochemical changes that can mitigate the negative effects of abiotic stresses on plants (Su et al., 2017; El-Esawi et al., 2018; Silva et al., 2019). It has been reported that an increase in carotenoid and proline content, resulting from inoculation with endophytes, prevents the synthesis of reactive oxygen species and lipid peroxidation. This, in turn, provides greater membrane stability, cell homeostasis, and enhanced protection of the photosynthetic apparatus (Amira et al., 2017; El-Esawi et al., 2018; Alexander et al., 2019).

Inoculation with PGPM strains, while reducing the use of chemical fertilizers, may not be successful if the substrate is not suitable (Timmusk et al., 2017). In this study, we hypothesized that inoculation with PGPM strains, isolated or mixed with organic or nitrogen fertilizer, would improve the performance of growth-related morphophysiological and nutritional traits of banana seedlings. This would help to restore the beneficial microbiota and produce seedlings without microorganisms. Therefore, two experiments were carried out using banana seedlings, focusing on: (1) investigating an ideal mixture of soil and organic compost, associated with *Bacillus* sp. and *Trichoderma asperellum*, to optimize nutrient contente and growth of banana seedlings; and (2) evaluating the agronomic efficiency of using certain nitrogen-fixing bacteria (NFB) strains associated with different levels of nitrogen fertilization on growth-related morphophysiological, biochemical and nutritional traits of banana seedlings, cultivar Prata Anã.

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ARTICLE I

Investigating the ideal mixture of soil and organic compound with *Bacillus* sp. and *Trichoderma asperellum* inoculations for optimal growth and nutrient content of banana seedlings*

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Investigating the ideal mixture of soil and organic compound with *Bacillus* sp. and *Trichoderma asperellum* inoculations for optimal growth and nutrient content of banana seedlings

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ABSTRACT

Organic compounds from the compost of agricultural waste have been increasingly used in the formulation of substrates for fruit seedlings production. Microorganisms can enhance the beneficial effects of fertilizers on the plant but reports on the use of endophytic strains to promote banana plant growth are rare. The goal of this study was to investigate the ideal mixture of soil and organic compound with *Bacillus* sp. and *Trichoderma asperellum* inoculations for optimal growth and nutrient content of banana seedlings. A Greenhouse experiment was arranged in a factorial scheme (3×2×2)+3, five times replicated, with the following factors: (a) substrates consisting of three organic compound and soil ratios – 20:80, 40:60, 60:40 (v/v); (b) *Bacillus* sp. inoculation – with [B+] and without [B-]; and (c) *Trichoderma asperellum* inoculation – with [T+] and without [T-]. Additionally, the following controls for substrates consisting of soil free of organic compound (0:100) were also considered: [B+T-], [B-T+] and [B-T-]. After 100 days from planting, plant height (H), pseudostem diameter (D), H/D ratio, number of leaves, total leaf area, root volume, shoot and root dry mass, net photosynthesis and contents of chlorophyll, carotenoid, nitrogen (N), phosphorus (P) and potassium (K) were evaluated. Results showed that the substrate 40:60 with *Bacillus* sp. or *Trichoderma* *asperellum* inoculation provides the best morphophysiological quality to banana seedlings. The strains also showed potential to promote plant growth in different ways, either by helping phosphate solubilization or by favoring auxins and hydrolytic synthesis.

Keywords: *Musa* spp., bioinoculant, co-inoculation, compost, mineral nutrition.

3.1. Introduction

Nutritional and phytosanitary factors are among the main limitations to banana production in Brazil. Some genotypes have been highlighted due to their agronomic and phytosanitary features that help to mitigate these limitations, such as the cultivar BRS Princesa (YB42-07), a tetraploid hybrid (AAAB) of the "Maçã" type. Organic compounds from the compost of agricultural waste have been increasingly used in the formulation of substrates for fruit seedlings production (Martins et al., 2011; Medeiros et al., 2015; Santos et al., 2017), to reduce or replace the use of mineral fertilizers in agriculture (Scheer et al., 2010). Organic compounds are sources of macro and micronutrients and organic matter and have a high water retention capacity (Amaral et al., 2016; Moreira et al., 2018). When applied to substrates, they promote improvements in chemical, physical and biological characteristics (Huang et al., 2017; Moreira et al., 2018), and can supply the nutritional demand for banana seedlings (Santos et al., 2017). However, over-application of organic compounds can result in nutritional imbalance, which negatively affects morphological characteristics, reduces chlorophyll content and causes the appearance of leaf anomalies (Moreira et al., 2018).

Conversely, microorganisms with complementary ecological functions can enhance the beneficial effects of fertilizers on the plant, reducing the amount of its application and providing the accumulation of organic carbon and microbial biomass in the soil (Baig et al., 2012; Xiao et al., 2013; Shen et al., 2015a; Larsen et al., 2017; Suleman et al., 2018; Zhai et al., 2019). Microorganisms participate in several biochemical processes, such as fixing atmospheric nitrogen and solubilizing phosphate, iron and other nutrients (Baig et al., 2012; Xiao et al., 2013), decomposition of organic matter and chelation of compounds (Jiang et al., 2011). When associated with the plant, they can become plant growth promoting microorganisms (PGPM) (Doni et al., 2014; Lopez-Coria et al., 2016). Besides, host plants can benefit from intermediate and / or final exudates of secondary metabolism, such as phytohormones (auxin, gibberellin and cytokinin), chelators (iron-siderophore complex), enzymes (nitrogenase) and organic acids that solubilize nutrients (Baig et al., 2012; Jimtha et al., 2014; Lopez-Coria et al., 2016; Su et al., 2017).

Bacteria of the genus *Bacillus* and fungi of the genus *Trichoderma* are among the most widely studied PGPM. Both contain endophytic strains and bring benefits to the plant, both for growth and nutrition, minimizing the effects of adverse conditions (Ahmad et al., 2018; Silva et al., 2019a), alone or together (Karuppiah et al., 2019). When they are endophytic species, these microorganisms reside inter or intracellularly in the tissues of the plant and do not cause visible damage or morphological changes. However, reports on the use of endophytic strains to promote banana plant growth are rare. A better understanding of plant growth promotion mixed with the potential of banana endophytic microorganisms will be useful for the establishment of a sustainable environment in the agricultural production system. Besides, these two genera have been referenced for biological control diseases caused by Fusarium (Shen et al., 2015b; Amira et al., 2017), in several species, including banana (Wuang et al., 2016; Hadadd et al., 2018). Therefore, the inoculation of beneficial microorganisms that play on multiple fronts, such as on nutrient solubilization, as well as on promoting the synthesis of plant hormones and cell wall degradation enzymes (cellulase, protease, lipase), a be considered a useful technological resource for farmers, either by promoting plant growth or by its disease-preventing action.

According to Timmusk et al. (2017), the integration of beneficial plant-microbe and microbiome interactions can be seen as an ecologically sustainable and promising solution to improve agricultural production. However, only the use of beneficial microorganism inoculation into the soil may not be successful if it is not mixed with an appropriate organic compost. Isolated or synergistic effects of these microorganisms mixed with organic compost on plant growth depend on the species, the biochemical and physiological characteristics of the microorganisms, and the quality and quantity of the fertilizer (Moreira et al., 2018; Silva et al., 2019b). Therefore, the goal of this study was to investigate the ideal mixture of soil and organic compound with *Bacillus* sp. and *Trichoderma asperellum* inoculations for optimal growth and nutrient content of banana seedlings.

3.2. Materials and methods

3.2.1. Site description and experimental design

A Greenhouse experiment was carried out under temperature control, at EMBRAPA Cassava and Fruits, in Cruz das Almas, Bahia, Brazil (12º40’39 ”S, 39º06’23”, 226 m asl). The local climate is Af type (rainy tropical forest), according to

Köppen and Geiger classification, with average annual temperature at 23 ºC. During the experimental period, the temperature in the greenhouse was kept at 26 ± 2 ºC.

The experimental design was completely randomized, arranged in a factorial scheme (3×2×2)+3 (Banzatto and Kronka, 2013), five times replicated, with the following factors: (a) substrates consisting of three organic compound and soil ratios – 20:80, 40:60, 60:40 (v/v); (b) *Bacillus* sp. inoculation – with [B+] and without [B-]; and (c) *Trichoderma asperellum* inoculation – with [T+] and without [T-]. Additionally, the following controls for substrates consisting of soil free of organic compound (0:100) were also considered: [B+T-], [B-T+] and [B-T-].

3.2.2. Substrates composition

The substrates preparation was based on different organic compound and soil ratios, with or without *Bacillus* sp. and *Trichoderma asperellum* inoculation. The choice of the microorganisms based on their potential for biological control of *Fusarium oxysporum* f. sp. *cubense*. The soil was a Dystrophic Yellow Latosol collected from the sub-superficial layer (> 40 cm deep), where there is a poor incidence of pathogens and spontaneous plant seeds, and the nutrient level is low, thus constituting an inert material.

The organic compounds were prepared from a compost heap of pruned garden grass, chopped banana stalks, cattle manure and poultry litter, in a 1:3:1:3 ratio, irrigated and turned every 15 days. Four compost heaps were prepared, two of which were inoculated monthly with a suspension of *Bacillus* sp. (Optical Density = 1.0 Absorbance, λ = 540 nm). The physical and chemical analysis of the substrates with and without

Bacillus sp. inoculation was performed at the EMBRAPA's Laboratory of Soil and Plant Nutrition (Table 1).

On the other two heaps, after 90 days from compost, suspensions of *Trichoderma asperellum* spores ($1x10^8$ spores mL^{-1} of sterile distilled water) were added to a heap non inoculated and another to a heap with *Bacillus* sp. inoculation. *Thichoderma asperellum* inoculation took place during the maturation of the organic compound when the temperature of the organic compound was still similar to that of the environment since the fungus is poorly tolerant of high temperatures.

Table 1. Chemical and physical attributes of the substrates prepared from different organic compound and soil ratios, with [B+] or without [B-] *Bacillus* sp. inoculation.

Attributes	Substrates							
	Soil		20:40		40:60		60:40	
	[B-]	[B+]	[B-]	[B+]	[B-]	[B+]	[B-]	[B+]
pH H_2O	4.2	4.3	6.8	6.6	7.6	7.9	8	7.9
P (mg dm^{-3})	2	3	106	144	349	477	722	734
K^+ (cmolc dm^{-3})	0.28	0.26	1.18	1.58	4.96	3.17	10.57	4.59
Ca^{2+} (cmolc dm^{-3})	0.48	0.5	2.8	3.22	3.16	3.78	4.59	5.23
Mg^{2+} (cmolc dm^{-3})	0.35	0.34	1.2	1.33	1.7	1.92	3.51	4.23
Al^{3+} (cmolc dm^{-3})	1.1	1.1	0	0	0	0	0	0
Na^+ (cmolc dm^{-3})	0.03	0.03	0.09	0.1	0.16	0.17	0.16	0.19
H+Al (cmolc dm^{-3})	2.7	2.25	0.22	0.33	0	0	0	0
SB (cmolc dm^{-3})	1.14	1.13	5.27	6.23	9.98	9.04	18.83	14.24
CEC (cmolc dm^{-3})	3.84	3.38	5.49	6.56	9.98	9.04	18.83	14.24
V (%)	29.7	34.5	96	95	100	100	100	100
OM (g kg^{-1})	1	3	8	15	37	31	47	51
Umidity at 10kPa (m^3 m^{-3})	23.3	-	25.4	-	28.4	-	46.1	-
Umidity at 30kPa (m^3 m^{-3})	23.2	-	23.6	-	24.1	-	34.7	-

Phosphorus (P); Potassium (K^+); Calcium (Ca^{2+}); Magnesium (Mg^{2+}); Aluminum (Al^{3+}); Sodium (Na^+); Hydrogen (H); Sum of Bases (SB); Cation Exchange Capacity (CEC); Base Saturation (V%); Organic Matter (OM).

Strains of *Bacillus* sp. (CNPMF 1009) and *Trichoderma asperellum* (CNPMF 1007), obtained from the collection of microorganisms from the EMBRAPA's Laboratory of Phytopathology, were reactivated in a nutrient medium containing agar and potato sucrose agar (PSA), and then incubated at 28 and 25 ± 1 ºC, at photoperiod of 12 h, respectively. The *Bacillus* sp. inoculum was prepared in potato sucrose, agitated at 100 rpm, at 28 ºC, for 24 h and then adjusted to the optical density of 1 Abs, at a wavelength of 540 nm. The suspension of *Trichoderma asperellum* was prepared by scraping the mycelium (grown for seven days) in distilled and sterile water, and subsequently counting the spores in the Neubauer chamber.

Microorganisms were evaluated for their ability to synthesize extracellular hydrolytic enzymes, siderophores, hormones, solubilize inorganic phosphate, and inhibit mycelial growth (Kuss et al., 2007; Kapri and Tewari, 2010; Cuzzi et al., 2011; Lamichhane and Varvaro, 2013; Isaias et al., 2014) (Table 2).

Table 2. Biochemical and physiological characteristics of *Bacillus* sp. and *Trichoderma asperellum*.

Characteristics	Microorganism	
	Bacillus sp.	*Trichoderma asperellum*
Inorganic phosphate solubilization		
$Ca(H_2PO_4)$	103.5 mg mL^{-1}	75 mg mL^{-1}
Synthesis		
Indol-3-acetic acid (IAA)	-	+
With L- tryptophan		9.9 µg mL^{-1}
Without L- tryptophan		8.6 µg mL^{-1}
Siderophores	-	-
Enzymes		
Urease	-	-
Pectinase	-	-
Cellulase	+	-
Amylase	+	+
In vitro antifungal activity		
Foc[1]	+	+
Bacillus sp.		+
Trichoderma asperellum	+	

[1]*Fusarium oxysporum* f. sp *cubense*. Signs (+) and (-) represent positive and negative reaction, respectively.

The chemical and physical characteristics of organic compounds are described in Table 3, according to BRASIL/MAPA (2017). Regarding the potential risks to the environment, according to the Brazilian Standard NBR 10.004 for Solid Waste, waste such as grass pruning, banana stems, cattle manure and poultry litter are classified as Waste

Class II A – not inert. Both the organic compounds and the soil were air dried, sieved in a 5 mm mesh and homogenized according to the treatments, and then placed in polyethylene pots (0.12 x 0.17 m, 2 dm^3).

Table 3. Chemical and physical characteristics of the organic compounds, with [B+] or without [B-] *Bacillus* sp. inoculation.

Characteristics	Organic compounds	
	[B-]	[B+]
pH (H_2O)	8.8	9.9
Humidity at 110 ºC (%)	1.43	1.31
Organic Matter (g kg^{-1})	77	75
Total Organic Carbon (g kg^{-1})	35.1	34.72
Nitrogen (g kg^{-1})	14.35	15.1
C/N ratio	2.45	2.3
Electrical Conductivity (µS cm^{-1})	13.56	13.56
Phosphorus (P_2O_5) (g kg^{-1})	18.08	6.23
Potassium (K_2O) (g kg^{-1})	26.90	24.33
Calcium (g kg^{-1})	25.61	15.13
Magnesium (g kg^{-1})	5.07	4.55
Sulfur (S) (g kg^{-1})	1.86	1.03
Copper (Cu) (g kg^{-1})	0.17	0.05
Manganese (Mn) (g kg^{-1})	0.32	0.23
Zinc (Zn) (g kg^{-1})	0.24	0.12
Iron (Fe) (g kg^{-1})	10.07	7.10
Sodium (Na) (g kg^{-1})	1.29	0.95

3.2.3 Plant material and growing conditions

Banana seedlings BRS Princesa about 3 cm high, 0.2 cm in diameter and a pair of extended leaves were washed in distilled water to remove substrate residues added to the roots, and then transplanted into the pots, one seedling per pot. Irrigation was performed daily to maintain the substrate moisture at 80% from the pot capacity. Every two weeks, suspensions of *Bacillus* sp. or *Trichoderma asperellum* were added to the substrates, according to the respective treatments. For the substrate containing both [B+T+] strains, alternate inoculations were performed.

3.2.4. Growth-related traits

After 100 days from planting, the following growth-related traits were measured: plant height (H), based on the length from soil to shoot apex; pseudostem diameter (D), at 1 cm above the soil level; H/D ratio; number of leaves (NL); total leaf area (TLA), using a leaf area integrator (LI 3100, LI-COR, USA); and root volume (RV). Then, the plants were dried in an oven at 65 ± 5 °C up to reach constant weight, to obtain shoot (SDM) and root dry mass (RDM). Clarification and coloring of fungal structures and the cultivation of Koch's Postulate were also performed to verify endophytic colonization, following Phillips and Hayman (1970) and Brundett et al. (1996).

3.2.5. Photosynthetic pigments and net photosyntesis

Photosynthetic pigments were extracted from leaf tissues using 80% acetone, in dark conditions. Absorbances in extracts were verified in a spectrophotometer, and chlorophyll and carotenoid contents were determined and expressed in mg g^{-1} of fresh leaf mass, according to Arnon (1949).

Net photosynthesis (*A*) (μmol CO_2 m^{-2} s^{-1}) was measured from 8 to 10 am in random plants, taking the third or fourth leaf from apex to base, using an Infrared Gas

Analyzer (IRGA) Portable Photosynthesis System model (ADC BioScientific Limited, UK) with an air flow of 2000 mL min^{-1} and a coupled light source of 1200 µmol m^{-2} s^{-1}.

3.2.6. N-P-K content

The main macronutrients were determined in leaf and root dry mass. Nitrogen (N) was quantified by sulfuric digestion (Kjeldahl), according to Bremner (1996). Phosphorus (P) and Potassium (K), in turn, after nitro-perchloric digestion, were analyzed in a spectrophotometer (model V-100D) at 660 nm; an ascorbic acid solution was used for P, while for K, a flame photometer (Mehlich, 1953). Values were shown in g kg^{-1}.

3.2.7. Data analysis

Data were previously submitted to homoscedasticity (Cochran) and normality (Lilliefors) tests, followed by analysis of variance and Tukey test ($p < 0.05$), using the SISVAR statistical program (Ferreira, 2014). Nutrient contents were transformed using $\sqrt{0,5x}$.

3.3. Results

Results showed that growth-related traits, photosynthesis and nutritional status were affected by changes in organic compost and soil ratios, as well as by *Bacillus* sp. and *Trichoderma asperellum* inoculation. The interaction of organic compound (OC) x *Bacillus* sp. (B) x *Trichoderma asperellum* (T) factors was significant for plant height (H), pseudostem diameter (D), chlorophyll content (Chl), net photosynthesis (*A*) and root dry mass (RDM). There was also significant OCxB, OCxT and BxT interactions, depending on the variable studied.

3.3.1. Growth-related traits

Plants grown in 40:60 substrate, either with *Bacillus* sp. [B+T-] or *Trichoderma asperellum* [B-T+] inoculation, alone or with both inoculations [B+T+], showed increases in height, pseudostem diameter and root dry matter (Table 4).

Plant height (H) in substrates 40:60 and 60:40 non-inoculated (B-T-) (17.3 and 9.8 cm, respectively) was much less than those of the other treatments. Perhaps could be speculated as a tendency that these plants to be higher than those of the control non-inoculated (substrate 0:100, B-T-); nevertheless, they did not differ statistically. The largest pseudostem diameters (D) were found in substrate 40:60, with inoculations [B+T+] (32.8 mm), [B-T+] (31.7 mm) and [B+T-] (30.0 mm). The highest root dry mass (RDM) (34.5 g) was found in substrate 40:60 [B-T+]. Conversely, the RDM was lower in substrates 0:100, 40:60 and 60:40 not inoculated with *Trichoderma asperellum*.

Results suggest that the number of leaves (NL) may have been positively influenced by *Trichoderma asperellum* inoculation [T+]. In substrate 60:40, for example, NL in [T+] (6.5) was higher than in [T-] (5.2). Similarly, the root volume (RV) may also have been favored by *Trichoderma asperellum* inoculation: in substrate 40:60, RV in [T+] was 67% higher than in [T-] (Table 5).

Table 4. Effects of the organic compound (OC) x *Bacillus* sp. (B) x *Trichoderma asperellum* (T) interaction on plant height, pseudostem diameter and root dry mass in banana seedlings BRS Princesa, after 100 days from planting.

	OC (0:100)		OC (20:80)			OC (40:60)			OC (60:40)			Total
Plant height (cm)												
	[B-]	[B+]	[B-]	[B+]	Mean	[B-]	[B+]	Mean	[B-]	[B+]	Mean	Mean
[T-]	6.5	28.0	27.6Bbα	40.1Aaα	33.8b	17.3Bbβ	46.2Aaα	31.7b	9.8Bbβ	39.0Aaα	24.4b	30.0b
[T+]	10.0		38.8Aaα	41.2Aaα	40.0a	46.0Aaα	47.0Aaα	46.5a	40.5Aaα	44.0Aaα	42.2a	42.9a
Mean			33.2B	40.7A		31.6B	46.6A		25.2B	41.5A		
	Total mean		36.9αβ			39.1α			33.3β			
	Total mean		30.0B	42.9A								
Pseudostem diameter (mm)												
	OC (0:100)		OC (20:80)			OC (40:60)			OC (60:40)			Total
	[B-]	[B+]	[B-]	[B+]	Mean	[B-]	[B+]	Mean	[B-]	[B+]	Mean	Mean
[T-]	5.6	19.1	18.7Bbα	28.7Aaα	23.4b	9.0Bbβ	30.0Aaα	19.5b	4.7Bbβ	27.3Aaα	16.0b	19.6b
[T+]	7.1		30.6Aaα	31.0Aaα	30.8a	31.7Aaα	32.8Aaα	32.2a	25.5Aaβ	27.0Aaβ	26.2a	29.7a
Mean			24.6B	29.6A		20.3B	31.4A		15.1B	27.1A		
	Total mean		27.1α			25.8α			21.1β			
	Total mean		20.0B	29.4A								
Root dry mass (g)												
	OC (0:100)		OC (20:80)			OC (40:60)			OC (60:40)			Total
	[B-]	[B+]	[B-]	[B+]	Mean	[B-]	[B+]	Mean	[B-]	[B+]	Mean	mean
[T-]	6.5	14.8	16.2Abα	19.3Abα	17.7b	6.5Bbα	23.1Baα	14.8b	6.0Bbα	27.8Aaα	16.9a	16.5b
[T+]	5.5		23.2Baαβ	29.8Aaα	26.5a	34.5Aaα	30.6Aaα	32.5a	13.3Aaβ	21.5Aaα	17.4a	25.5a
Mean			19.7A	24.6A		20.53A	26.8A		9.7B	24.6A		
	Total mean		22.1αβ			23.70 α			17.18 β			
	Total mean		16.6B	25.38 A								

Same capital letters in the column, lowercase letters in the line and Greek letters among organic compounds (OC) do not differ by Tukey's test ($p < 0.05$).

Shoot dry mass (SDM) was positively influenced by *Trichoderma asperellum* inoculation in all substrates, except in the control (0:100). In addition, the lowest SDM (8.7 g) was found in substrate 60:40 [B-] (Table 5).

Referring particularly to the effects of *Bacillus* sp. and *Trichoderma asperellum* inoculation, irrespectively of the substrates with different organic compound and soil ratio

(Table 6), the lowest plant height and pseudostem diameter ratio (H/D) (1.44, 1.47 and 1.49) were found in substrates [B-T+], [B+T-] and [B+T+], respectively. The highest SDM (29.6 and 26.6 g) were found in substrates with [T+] and [B+] inoculation, respectively. The lowest total leaf area (TLA) (599.2 cm^2) occurred in non-inoculated substrate, while the highest values were found in [B+T-], [B-T+] and [B+T+] – that is, 1557.0, 1705.9 and 1823.9 cm^2, respectively.

Table 5. Effects of the organic compound (OC) x *Trichoderma asperellum* (T) interaction, in substrates with [B+] or no [B-] *Bacillus* sp. inoculation, on number of leaves (NL), root volume (RV) and shoot dry matter (SDM), in banana seedlings BRS Princesa, after 100 days from planting.

Number of leaves (units $plant^{-1}$)					
Trichoderma asperellum	OC (0:100)	OC (20:80)	OC (40:60)	OC (60:40)	Mean
[T-]	3.4	5.6Aa	5.9Aa	5.2Ba	5.5b
[T+]	3.6	5.3Ab	6.6Aa	6.5Aa	6.1a
Mean		5.4B	6.2A	5.8AB	
Root volume (cm^3 $plant^{-1}$)					
Trichoderma asperellum	OC (0:100)	OC (20:80)	OC (40:60)	OC (60:40)	Mean
[T-]	66.0	97.0Ba	108.5Ba	116.0Aa	107.1b
[T+]	36.0	138.0Aa	161.5Aa	87.5Bb	129.0a
Mean		117.5AB	135.0A	101.7B	
Shoot dry mass (g $plant^{-1}$)					
Trochoderma asperellum	OC (0:100)	OC (20:80)	OC (40:60)	OC (60:40)	Mean
[T-]	6.5	15.6Ba	12.1Ba	14.4Ba	14.0b
[T+]	6.2	30.0Aa	32.6Aa	21.6Ab	28.1a
Mean		22.8a	22.3A	18.0A	
Shoot dry mass (g $plant^{-1}$)					
Bacillus sp.	OC (0:100)	OC (20:80)	OC (40:60)	OC (60:40)	Mean
[B-]	6.5	24.1Aa	19.6Aa	8.7Bb	17.5b
[B+]	10.1	21.5Aa	25.1Aa	27.3Aa	24.7a
Mean		22.8a	22.3A	18.1a	

Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test ($p < 0.05$).

Table 6. Effects of *Bacillus* sp. and *Trichoderma asperellum* inoculation on plant height and pseudostem diameter ratio (H/D), total leaf area (TLA) and shoot dry mass (SDM) in banana seedlings BRS Princesa grown in substrates with different organic compound and soil ratio, after 100 days from planting.

	H/D			TLA (cm^2)			SDM (g)		
	[B-]	[B+]	Mean	[B-]	[B+]	Mean	[B-]	[B+]	Mean
[T-]	1.85Ba	1.47Ab	1.66a	599.2Bb	1557.0Aa	1078.1b	8.3Bb	21.1Ba	14.7b
[T+]	1.44Aa	1.49Aa	1.47b	1705.9Aa	1823.9Aa	1764.9a	26.6Aa	29.6Aa	28.1a
Mean	1.65^{a}	1.48A		1152.63B	1690.50A		17.4B	25.3A	

Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test ($p < 0.05$).

Endophytic colonization in roots was observed in substrates [B-T+], either by fungal structures found in root epidermis, cortex and vascular cambium (Fig. 1), or by *in vitro* fungal isolation (Fig. 2). Colonization of *Bacillus* sp. in roots was observed both in substrate [B+T-] and in [B+T+] (Figure 2). In substrate [B+T+], the mycelia of the strains were 18% inhibited. Although the microorganisms have shown antagonism under in vitro condition (Table 2), both PGPM showed positive effects on roots under in vivo co-inoculation, which was performed alternating every two weeks (Table 4 and 6).

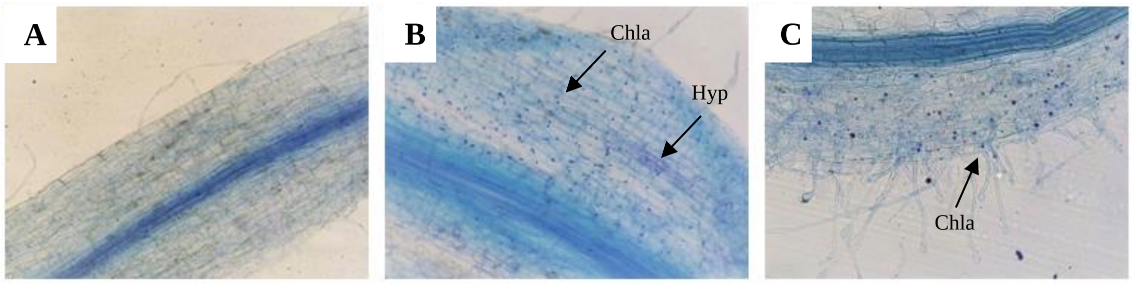

Fig. 1. Endophytic colonization in roots of banana seedlings BRS Princesa, 100 days after transplanting (photomicrograph). Cross section of root tissue from substrate [B-T-] (A) and substrate [B-T+] (B and C). Presence of hyphae (Hyp), in epidermis, cortex and vascular cambium (B), and chlamydospores (Chla), in secondary roots (B) and (C).

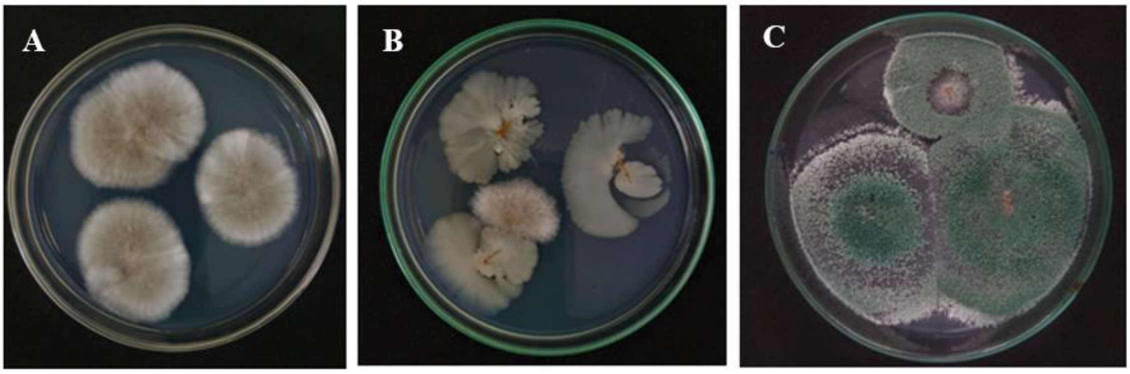

Fig. 2. Isolation of roots of banana seedlings BRS Princesa, 100 days after transplanting, in substrates non-inoculated [B-T-] (A); substrates [B+T-] and [B+T+] (B); and substrates [B-T+] (C).

3.3.2. Photosynthetic pigments and net photosyntesis

Results show an increase in chlorophyll content, in substrates with increasing organic compound and soil ratio, since 40:60, especially with *Trichoderma asperellum* inoculation. The highest chlorophyll content (30.3 mg g^{-1}) was found in substrate 60:40 [B-T+] (Table 7).

Net photosynthesis, in turn, was inhibited in substrate 60:40 [B-T-] non-inoculated, where the rate was the lowest (639 µmol CO_2 m^2 s^{-1}). Significant differences on net photosynthesis, however, were not found among the other substrates (Table 7).

Referring particularly to the effects of *Bacillus* sp. and *Trichoderma asperellum* inoculation on carotenoid content, irrespectively of the substrates with different organic compound and soil ratio, results showed higher carotenoid content in substrates with *Trichoderma asperellum* inoculation, regardless of *Bacillus* sp. inoculation. The lowest carotenoid content (1.9 mg g^{-1}) was found in non-inoculated substrates [B-T-] (Table 8).

Table 7. Effects of the organic compound (OC) x *Bacillus* sp. (B) x *Trichoderma asperellum* (T) interaction on chlorophyll content and net photosynthesis in banana seedlings BRS Princesa, after 100 days from planting.

Chlorophyll content (mg g^{-1})													
	OC (0:100)		OC (20:80)			OC (40:60)			OC (60:40)			Total
	[B-]	[B+]	[B-]	[B+]	Mean	[B-]	[B+]	Mean	[B-]	[B+]	Mean	Mean
[T-]	10.7	17.3	4.3Aaαβ	7.0Aaα	5.7a	11.7Aaα	11.1Aaα	12.4a	3.5Bbβ	13.2Aaα	7.3b	8.5b
[T+]	10.2		9.3Aaβ	6.7Aaα	8.0a	15.2Aaβ	10.9Aaα	13.1a	30.3Aaα	13.9Abα	22.1a	14.4[a]
Mean			6.8A	6.9A		13.4A	12.0A		16.9A	12.5A		
	Total mean		6.8β			12.77 α			14.72α			
	Total mean		12.4A	10.5A								

Net photosynthesis (µmol CO_2 $m^{-2}s^{-1}$)													
	OC (0:100)		OC (20:80)			OC (40:60)			OC (60:40)			Total
	[B-]	[B+]	[B-]	[B+]	Mean	[B-]	[B+]	Mean	[B-]	[B+]	Mean	Mean
[T-]	1042	1035	1023Aaα	1028Aaα	1025a	669 Abα	1112Aaα	891a	639Baα	1239Aaα	939a	951[a]
[T+]	1195		871Aaα	1055Aaα	963a	1170Aaα	792Aaα	981a	944Aaα	1119Aaα	1031a	991[a]
Mean			947A	1041A		920A	952A		792B	1179A		
	Total mean		994α			935α			985 α			
	Total mean		886B	1057A								

Same capital letters in the column, lowercase letters in the line and Greek letters among organic compounds (OC) do not differ by Tukey's test ($p <0.05$).

Table 8. Effects of *Bacillus* sp. (B) x *Trichoderma asperellum* (T) interaction on carotenoid content in banana seedlings BRS Princesa, after 100 days from planting.

Carotenoid content (mg g^{-1})			
	[B-]	[B+]	Mean
[T-]	1.9Bb	2.7Aa	2.3b
[T+]	4.8Aa	3.7Ba	4.2a
Mean	3.4A	3.2A	

Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test ($p <0.05$).

3.3.3. N-P-K content

The shoot nitrogen (N) content was higher in substrates non-inoculated [B-T-]. In the roots, however, N content was higher in substrates with *Bacillus* sp. and *Trichoderma asperellum*, alone or together (Table 9). The root N content did not show difference among the different organic compound and soil ratio, when inoculated with *Bacillus* sp.

However, the highest N content was found in substrate 60:40 non-inoculated with *Bacillus* sp. (Fig. 3A).

The increase in organic compound and soil ratio promoted an increase in root phosphorus (P) content, especially with *Bacillus* sp. inoculation (Fig. 3B). There was no significant difference for potassium (K) content among the substrates, when inoculated with *Bacillus* sp. and *Trichoderma asperellum* (Table 9).

Table 9. Effects of *Bacillus* sp. (B) x *Trichoderma asperellum* (T) interaction on shoot and root nitrogen (N) content, and root potassium (K) content in banana seedlings BRS Princesa, after 100 days from planting.

	Shoot N content (g kg^{-1})		
	[B-]	[B+]	Mean
[T-]	-	-	11.1a
[T+]	-	-	4.94b
Mean	14.16[a]	1.86B	
	Root N content (g kg^{-1})		
	[B-]	[B+]	Mean
[T-]	9.8Bb	18.1Aa	13.9a
[T+]	19.1Aa	15.0Aa	17.1a
Mean	14.4[a]	16.5A	
	Root K content (g kg^{-1})		
	[B-]	[B+]	Mean
[T-]	23.9Bb	31.9Aa	27.9b
[T+]	45.7Aa	31.7Aa	38.7a
Mean	34.8[a]	31.8A	

Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test ($p < 0.05$).

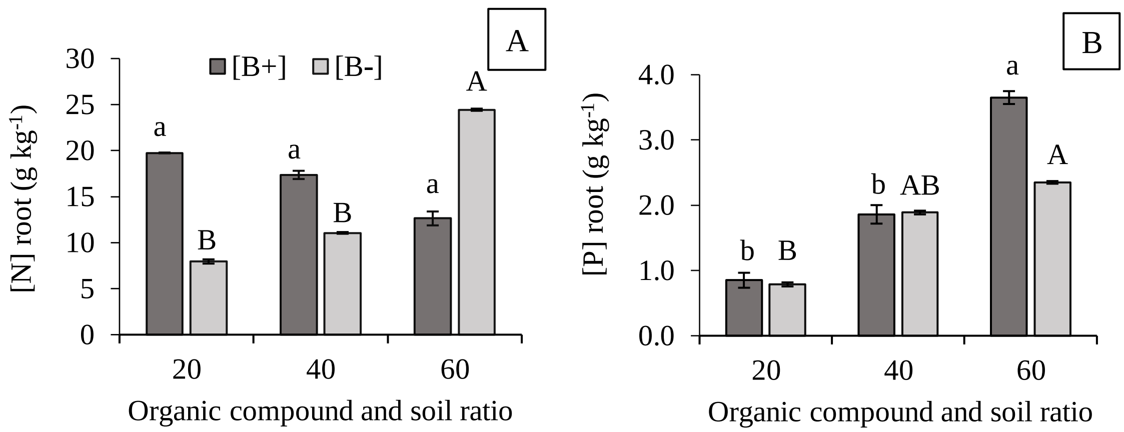

Fig. 3. Effects of substrates with different organic compound and soil ratio, [B-] and [B+], on nitrogen [N] (Figure 3A) and phosphorus [P] content (Figure 3B) in roots of banana

seedlings BRS Princesa, after 100 days from planting. Same capital letters indicate no significant difference among substrates [B-], whereas lowercase letters indicate no significant difference among [B+] treatments, by Tukey's test ($p < 0.05$).

3.4. Discussion

The changes in morphophysiological growth-related traits and nutrient status that were found in banana seedlings BRS Princesa can be attributed to changes in chemical and physical characteristics of the substrates, pH, cationic complex, organic matter, moisture and water retention capacity. According to Borges (2004), even in substrate with low organic compound and soil ratio (20:80, for example), regardless of microorganisms inoculation, fertility tends to increase, reaching high levels of P and K. In general, the best responses have been found on substrates with 10 to 30% organic compound (Lima et al., 2009; Martins et al., 2011; Santos et al., 2017), although in this study it was in substrate 40:60, depending on the type of inoculation.

Through the synthesis of cell wall hydrolytic enzymes, such as chitinase, β-1,3 glucanase (Radhakrishnan et al., 2017), cellulase, protease, ACC-deaminase, as well as by releasing organic acids during phosphate solubilization (Matos et al., 2017), *Bacillus* sp. can increase the rate of organic matter degradation and, consequently, increase the availability of chemical elements. This explains why the pseudototal levels in organic compound [B+] were lower than in [B-] (Tables 2 and 3).

Besides the effects of organic compounds on seedling quality, the growth promoters present in inoculated substrates potentiated these effects, improving growth-related traits, such as plant height, pseudostem diameter, H/D ratio, number of leaves, total leaf area, root volume and shoot and root dry mass (Tables 4, 5 and 6). Physiological traits, such as photosynthetic pigments, net photosynthesis (Tables 7 and 8) and N, P and K content, were also favored by inoculation (Table 9 and Fig. 1). In fact, even in control substrate (0:100), the effects of *Bacillus* sp. or *Trichoderma asperellum* on some of these traits were similar or greater than in the other substrates.

PGPM colonization in roots is essential for the plant-microorganism association for the medium and long term, because of two reasons: (i) if the bacteria do not reach the roots, the excreted metabolites will diffuse in the rhizosphere and will be consumed by the other microorganisms before being absorbed by the plant, and (ii) root tissues not associated with PGPM are more vulnerable to the colonization of pathogens (Mia et al ., 2010). The presence of *Bacillus* sp. e *Trichoderma asperellum* in roots improves access to growth-promoting substances, such as auxins, soluble phosphorus, and hydrolytic enzymes, and maintains the microorganism-plant association for a long time (Figs. 1 and 2), besides to enhance plant resistance to pathogens, because of the disease-preventing action of the strains (Table 2).

Data indicate that substrates with *Bacillus* sp. and *Trichoderma asperellum* inoculation, alone or together, promoted a decrease in H/D ratio and an increase in total leaf area (Table 6), in addition to an increase in carotenoid content (Table 8). A lower H/D ratio is usually interpreted as a positive indicator, in terms of quality of banana seedlings, because it reduces the risk of tipping (Sturion and Antunes, 2000). A larger total leaf area may increase the possibilities of light interception.

Trichoderma asperellum inoculation provided an increase in chlorophyll content, even in control plants, in substrate 0:100 (Table 7). A high chlorophyll content is usually related to high photosynthesis rate, which can favor seedlings adaptation after transplanting (Afonso et al., 2017). The increase in carotenoid content, in turn, plays a relevant role in give structural stability to the assembly of light harvesting complexes and to protective mechanisms against oxidative stress (Cazzonelli, 2011; Zhai et al., 2016; Lal, 2018). Preventive action of fungi on oxidative stress was also observed in *Trichoderma harzianum* inoculation in *Olea europaea* L., which helped restore the balance between synthesis enzymes and degradation of reactive oxygen species (ROS) in roots, thus preventing cell damage (Amira et al., 2017). The regulation of the antioxidant apparatus appears to be the mechanism used by strains of Trichoderma during abiotic stress (Amira et al., 2017; Su eta l., 2017; Ahmad et al., 2018; Silva et al., 2019b). due to Trichoderma's ability to properly modulate its own ROS elimination pathways to ensure rapid ROS restoration and maintain acceptable ROS homeostasis (Amira et al., 2017).

Substrates with *Trichoderma asperellum* inoculation provided an increase in net photosynthesis (Table 7). These data corroborate previous studies that report positive effects of *Trichoderma asperellum* inoculation on increased number of leaves, dry mass and photosynthesis, resulting in higher plant growth rates (Akladious and Abbas, 2014; Doni et al., 2014; Silva et al., 2019b; Zhai et al., 2019).

According to Zhai et al. (2019), soils with *Trichoderma asperellum* inoculation have more N and P availability. In the present study, however, substrates containing organic compound and inoculated with *Bacillus* sp. and *Trichoderma asperellum*, alone or together, resulted in a lower root N and P content (Fig. 1). On the other hand, data showed an increase in dry matter, suggesting that inoculation plays a positive role on improving nutrient use efficiency. These results are similar to those of Chagas et al. (2016), in which cowpea plants inoculated with some *Trichoderma* species producing indole acetic acid (IAA) and phosphate solubilizers showed better P use efficiency.

The positive effect of *Trichoderma asperellum* on plant growth can be attributed to the studied strain's ability to produce auxins (IAA). The CNPMF – 1007 strain is able to synthesize IAA in the absence or presence of its precursor, L-tryptophan (Table 2). Auxins promote root elongation, favoring the soil nutrients absorption (Contreras-Cornejo et al., 2009). The relationship between phytohormone and phytomass production was evidenced by the greater root volume in substrate 40:60 with *Trichoderma asperellum* inoculation (Table 5), where more root hairs were formed (Fig. 2). These results are similar to those of Contreras-Cornejo et al. (2009), who also observed root hair formation caused by *Trichoderma virens* inoculation.

Some strains also produce ACC deaminase, which inhibits ethylene synthesis in plants, promoting their growth (Glick, 2014; Pourbabaee et al., 2016). Szilagyi-Zecchin et al. (2015) reported an increase in chlorophyll content and subsequent shoot growth in tomato seedlings grown under soil with organic fertilization and *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 inoculation. According to these authors, growth effects resulted from the synthesis of indole compounds and siderophores.

In this study, co-inoculation with *Bacillus* sp. and *Trichoderma asperellum* proved to be beneficial for most growth-related morphophysiological traits, except for number of leaves. These results corroborate previous studies in which the co-inoculation of microorganisms with complementary ecological functions also promoted the plant growth (Karuppiah et al., 2019; Silva et al., 2019b). The knowledge on the peculiarities of each strain expands possibilities for exploiting its potential benefits, either using them alone or mixed with biofertilizers. Current research increasingly assess the use of biotechnological resources that mix biological control of phytopathogens and growth promotion in banana and other species in current research (Wang et al., 2016; Amira et al., 2017; Karuppiah et al., 2019; Zhai et al., 2019). Nevertheless, the effect of microorganism inoculation, in terms of promoting growth in banana, continues to demand studies in field conditions, due to the complex understanding that remains discrepant on the relationships among plant genotype and physiological status, biochemical features of the microorganism strains, and soil environmental conditions, such as pH, fertility, and humidity (Karuppiah et al., 2019; Naik et al., 2019).

3.5. Conclusion

The results showed that substrates 40:60 with *Bacillus* sp. and/or *Trichoderma asperellum* inoculation provided the best performance of morphophysiological growth-related traits in banana seedlings BRS Princesa, after 100 days from planting. Substrates with increasing organic compound and soil ratio, inoculated with *Bacillus* sp. and *Trichoderma asperellum*, alone or together, decreased nitrogen content and increased phosphorus and potassium content in the roots. The strains showed potential to promote plant growth in different ways, either by helping phosphate solubilization, or by favoring auxins and hydrolytic enzymes synthesis. Therefore, we conclude that *Bacillus* sp. and *Trichoderma asperellum* is promising for further research on microorganism inoculation to promote banana growth in field conditions.

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ARTICLE II

Growth-promoting endophytic bacteria replace nitrogen fertilization in banana seedlings, cultivar Prata Anã*

Situation: Submitted

Article

Growth-promoting endophytic bacteria replace nitrogen fertilization in banana seedlings, cultivar Prata Anã*

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Abstract

Using bacteria which can fixate atmospheric nitrogen (N) may constitute an avenue for reducing the requirement of mineral fertilizers by agricultural crops, which would consequently reduce adverse environmental impacts. The objective of this study was to evaluate the agronomic efficiency of several strains of N-fixing bacteria, in combination with different levels of N fertilization, on morphophysiological, biochemical, and nutritional characteristics related to the growth of banana seedlings of a Prata Anã cultivar. The experiment was conducted in greenhouse in a 6 × 5 factorial scheme with the following factors: (1) N-fixing bacteria: BR11005, BR11674, BR12137, BR12157, and BR12158, and a treatment with no inoculation; and (2) doses of N (0, 50, 100, 150, or 200 mg kg^{-1} of substrate). Bacterial strains were characterized with respect to biochemical and physiological aspects. Ninety days after transplanting, plant height, pseudostem diameter, number of leaves, total leaf area, root volume, dry mass of aerial parts and roots, total content of chlorophyll, carotenoids, and proline contents, and N concentrations in the plants were evaluated. Only strains BR11005 and BR12157 showed potential to improve the performance of growth-related morphophysiological traits. The strains are effective for biological N fixation, potassium and phosphate solubilization, auxin synthesis, and production of siderophores, exopolysaccharides, and hydrolytic enzymes. The performance of plants treated with these strains exceeded those of the standard nitrogen fertilization treatment, and strain BR12157 showed higher agronomic efficiency due to its potential to completely substitute nitrogen fertilization.

Keywords: Bioinoculant • *Azospirillum baldaniorum* • *Burkholderia* sp. • nitrogen fixation • mineral nutrition

4.1 Introduction

Banana (*Musa* spp.) is a fruit tree of high cultural and commercial importance in Brazil and throughout the world. Crop productivity is typically reduced by biotic and abiotic stressors, however, their effects can be mitigated through the use of genetically more suitable cultivars (Gonçalves et al. 2018; Rebouças et al. 2018) and through adequate plantation management, which mainly involves fertilization during the production phase.

Inoculation with plant growth-promoting microorganisms (PGPM) has become an increasingly used management strategy to reduce the requirement for chemical substances without losses in crop productivity. PGPMs can establish more favorable environmental conditions to improve plant morphophysiology and reduce stress caused by various biotic and abiotic factors (Gopalakrishnan et al. 2017; Kumar et al. 2017; Bahulikar et al. 2021; Moreira et al. 2021). In general, these beneficial effects of PGPMs result in increased plant height, pseudostem diameter, leaf number, aboveground and root system biomass, number, length, and volume of roots, and root nitrogen (N) concentrations (Souza et al. 2016; Wang et al. 2019a; Moreira et al. 2021).

PGPMs can establish symbiotic relationships with several plant species including leguminous plants and other groups, and they stimulate plant growth through direct or indirect mechanisms. Benefits of this associations include the following aspects: increased nutrient uptake by the host plant due to the ability of microorganisms to fix atmospheric N; mobilization of nutrients such as phosphorus (P), zinc (Zn) and iron (Fe) through production of organic acids (solubilization of organic-metal complexes) and siderophores; assistance in modulating the concentrations of plant regulators such as auxin (indole-acetic acid) (Yuan et al. 2013; Andrade et al. 2014; Souza et al. 2016); and release of hydrolytic enzymes (e.g. cellulases, pectinases, and proteases) that degrade pathogen cell walls, occupy contact and entry sites in root tissue, and inhibit growth of other pathogens, thereby supporting plant defense mechanisms (Ho et al. 2015; Cedeño et al. 2021). These characteristics determine the classification of microorganisms according to their potential as inoculants for agricultural crops.

Inoculation with endophytic N-fixing bacteria (NFB) has been studied in some, mainly non-leguminous, plant species (Andrade et al. 2014; Gopalakrishnan et al. 2017; Emami et al. 2019; Bahulikar et al. 2021); however, respective information on banana plants is still scarce. According to Weber et al. (2000), inoculation with *Herbaspirillum* sp. and *Burkholderia cepacia* improves nutrient uptake in Caipira banana seedlings after the roots are colonized, and it increases growth by 50%, compared to that of plants which receive only N fertilization. Mia et al. (2010) recommend inoculation with *Azospirillum brasiliense* SP7 in combination with N fertilization at 20 mg kg^{-1} of substrate to obtain banana seedlings with better morphophysiological quality. However, Souza et al. (2016) found that production of dry mass in Prata Anã banana inoculated with NFB did not differ from that of plants treated only with complete mineral fertilization.

Some studies on NFB in banana plants showed marked diversity of N-fixing genera in roots, including *Azospirillum*, *Burkholderia*, *Bacillus*, *Herbaspirillum*, *Rhizobium*, *Agrobacterium*, and *Paenibacillus* (Weber et al. 2000; Andrade et al. 2014; Salvador-Figueroa et al. 2016; Souza et al. 2016; Pereira et al. 2018). *In vivo* inoculation with NFB of these genera increases the probability of obtaining seedlings with higher morphophysiological quality and productivity. Furthermore, some of these genera include species which may be used for biocontrol of plant pathogens, including the use of *Burkholderia cenocepacia* which helps control *Fusarium oxysporum* f. sp. *cubense* in banana plants (Ho et al. 2015).

Despite the affinity of several genera of NFB and banana plants, several other factors must be considered to facilitate efficient symbiotic relationships between bacteria and plants, including the genetic characteristics of a given cultivar, the physiological characteristics of a particular strain, and N concentrations in the substrate. Biological N fixation is more efficient when inoculation occurs under soil N-limited conditions, which facilitates an increase in the rate of root colonization with a consequent increase in the concentration of atmospheric N in the plant, and higher biomass production (Puri et al. 2016; Bahulikar et al. 2021).

Some previous studies corroborate the hypothesis that more efficient NFB strains in growth promotion can substitute banana crop N fertilization, completely or in part. The objective of the present study was to evaluate the effects of five NFB strains and different levels of nitrogen fertilization on morphophysiological, biochemical, and nutritional characteristics related to the growth of seedlings of a Prata Anã banana cultivar.

4.2 Material and Methods

4.2.1 Growing conditions and experimental design

The experiment was conducted in a greenhouse at Embrapa Cassava and Fruit Growing (12°40'39" S, 39°06'23" W, altitude 226 m) in Cruz das Almas - BA, Brazil, from October 2019 to January 2020, and it was repeated in from September to December 2020. According to Köppen and Geiger, the climate of Cruz das Almas is an Af climate with an average annual temperature of 23 ºC. During the experiment, the temperature in the greenhouse was maintained at 26 ± 2 ºC.

The experimental design was completely randomized in a 6 × 5 factorial scheme with eight repetitions and one plant per plot. The factors were: (1) N-fixing bacteria - no inoculation (NI) or inoculation with strains BR11005, BR11674, BR12137, BR12157, or BR12158; and (2) doses of N (0, 50, 100, 150, or 200 mg kg^{-1} substrate).

Doses of N were calculated based on the dosage of 200 kg ha^{-1} $year^{-1}$ which is considered the standard fertilization for banana field crops (Borges et al. 2004). Urea was used as the N source. N fertilization was used on six plots and was applied alternately to inoculations with bacteria. In all treatments, the following basic fertilization was used: 120 mg dm^{-3} P_2O_5 and 300 mg dm^{-3} K_2O, using superphosphate and potassium chloride as sources, respectively; 100 mg dolomitic limestone and 5 mg FTE BR12 (a nutrient mix) were supplied, according to Borges et al. (2004).

Polyethylene pots of 12 × 17 cm with 2 dm^{-3} volume were filled with coconut fiber-based substrate with the following chemical composition: 7.61 g kg^{-1} N, 0.93 g kg^{-1} P, 10.0 g kg^{-1} K, 10.51 g kg^{-1} Ca, 2.04 g kg^{-1} Mg, 1.73 g kg^{-1} S, 54.73 mg kg^{-1} Fe, 36.48 mg kg^{-1} Zn, 33.42 g kg^{-1} Mn, 24.64 mg kg^{-1} Cu, and 1.28 g Na; humidity -10 kPa (291.40 m^3 m^{-3}), pH 6.5. Micropropagated Prata Anã banana seedlings (Prata subgroup) of approximately 10 cm height, 0.3 cm in diameter, and with two pairs of extended leaves produced in the Biofactory Field at Embrapa Cassava and Fruit Growing were used. Irrigation was performed daily using a micro-sprinkler system to maintain humidity at approximately 60% of the pot capacity.

4.2.2 Identification and characterization of strains

Lyophilized bacterial strains from the microorganisms collection of Embrapa Agrobiology in Seropédica, RJ, Brazil, were reactivated in nutrient agar (NA) and were incubated at 28 ± 1 ºC under a 12-h photoperiod. Table 1 shows their identification and collection history.

Table 1. Identification, host plant, place origin and year of isolation for obtaining some NFB strains from Embrapa Agrobiology (Seropédica, RJ).

Strain	Identification	Host plant	Place origin	Year of isolation	Reference
BR 11005^T	*Azospirillum baldaniorum*	*Triticum aestivum*	Passo Fundo, RS	1979	Baldani et al. (1983); Ferreira et al. (2020)
BR 11647	*Azospirillum* sp.	*Sorghum bicolor*	Seropédica, RJ	1978	Baldani and Döbereiner 1980
BR 12137	*Herbaspirillum* sp.	*Musa* spp.	Itaguaí, RJ	1995	Weber et al. (1999)
BR 12157	*Burkholderia* sp.	*Musa* spp.	Uruburetama, CE	1999	Weber et al (2007)
BR 12158	*Burkholderia* sp.	*Musa* spp.	Ubajara, CE	1999	Weber et al (2007)

BR 11005^T first identified as *Azospirillum* sp. SP245^T and later classified as *Azospirillum baldaniorum* sp. nov

After reactivation, the bacterial strains were evaluated regarding their capacity to fixate free N_2 using JNFb and NFb culture medium (Cavalcante and Döbereiner 1988). Bacterial growth capacity in Döbereiner N-free culture medium indicated that all of them were non-symbiotic fixers (Table 2).

Table 2. Morphocultural, biochemical and physiological characteristics of the NFB strains.

Characteristics	NFB				
	BR11005	BR11674	BR12137	BR12157	BR12158
Morphocultural					
Growth speed	fast	fast	very slow	fast	very slow
Consistency	fluid	fluid	mucous	fluid	fluid
Lucidity	translucent	opaque	translucent	opaque	translucent
Color	yellow	white	yellow	beige	white
Biochemical and physiological					
N fixation	+	+	+	+	+
K solubilization	-	-	+	+	-
P solubilization	+	+	+	+	+
Solubilization index	0.62 (VL)	1.17 (L)	1.84 (L)	1.14 (L)	4.41 (VH)
Synthesis					
IAA[1]	+	-	-	-	-
Siderophores	+	+	-	+	-
Exopolysaccharides	-	+	-	+	-
Enzymes					
Urease	-	-	-	-	+
Pectinase	-	-	-	-	-
Cellulase	-	-	-	-	-
Amylase	+	+	+	+	+
Protease	+	+	-	+	+
Antibiotic activity *in vitro*					
Foc[2]	-	-	-	+	-
Bacillus sp.	-	-	-	-	-
Trichoderma asperellum	-	-	-	-	-

[1]Indol-acetic acid; [2]*Fusarium oxysporum* f. sp. *cubense*. VL: very low solubilizer (VL < 1,0), L: little (1 > L < 2), M: medium (2 > M < 3), H: high (3 > H < 4), VH: very high (VH > 4). The signs (+) and (-) represent positive and negative reaction to biochemical tests, respectively.

Morphocultural characterization was performed using pure colonies grown in NA with respect to shape, color, and consistency of the colonies, and the following criteria were recorded: (1) growth time (very fast = less than one day; fast = between one and two days; average = between two and three days; slow = between three and four days; and very slow = more than four days); (2) colony consistency (slimy, fluid, or mycelial); (3) brightness (bright, translucent, and opaque); and (4) color.

The microorganisms were also evaluated with respect to their ability to synthesize extracellular hydrolytic enzymes, siderophores, exopolysaccharides and growth regulators, solubilize P and potassium (K), antibiotic effects on *Fusarium oxysporum* f. sp. *cubense*, *Trichoderma asperellum* and *Bacillus* sp. (Kuss et al. 2007; Kapri and Tewari 2010; Cuzzi et al. 2011; Lamichhane and Varvaro 2013; Isaias et al. 2014) (Table 2).

Bacterial inocula were prepared in liquid nutrient medium under agitation (100 rpm) at 28 ºC for 48 h, and optical density was adjusted to 0.7 Abs at a wavelength of 540 nm, equivalent to 10^6 CFU mL^{-1}. Inoculation was performed every fifteen days, alternating with mineral fertilization.

4.2.3 Morphophysiological characteristics

All evaluations were performed after 90 days from transplanting. Plant height (H), pseudostem diameter (PSD) and total number of leaves (NL) were measured. To measure total leaf area (TLA), a leaf area integrator (LI 3100, LI-COR, USA) was used, and root volume (RV) was measured based on the displacement of water equivalent units (1 mL = 1 cm^3). Dry mass of aerial parts (DMAP), dry mass of roots (DMR), and total dry mass were determined after drying in a forced ventilation oven (65 ± 5 °C). The growth increase percentage (GIP) of inoculated seedlings based on controls without N fertilization and with standard fertilization was calculated according to the following equation:

$$GIP = \frac{(DMTinoculated - DMTfertilized)}{DMTfertilized} * 100$$

where DMT is total dry mass.

Koch’s postulate culturing was performed according to Phillips and Hayman (1970) to evaluate endophytic colonization and root colonization percentage (RCP), using morphological characteristics of the bacterial colonies to differentiate them. Root architecture was analyzed according to two of the criteria described by Lynch (1995): (i) growth in individual root segments and (ii) occurrence of daughter roots on each root segment.

4.2.4 Photosynthetic pigments and proline

Photosynthetic pigments were extracted from fresh leaf discs macerated in 80% acetone in the dark. After analysis in a spectrophotometer, total chlorophyll (CHLO) and carotenoid (CAROT) concentrations were determined according to Arnon (1949) as a function of mass per sample and the volume of acetone used. Proline levels were obtained according to Bates et al. (1973). Concentrations of photosynthetic pigments and proline levels were expressed in mg g^{-1} of leaf fresh mass.

4.2.5 N concentrations

N concentrations were measured in dry mass of aerial parts (NAP) and roots (NR) at the end of the experiment, using sulfuric digestion according to the Kjeldahl method proposed by Bremner (1996). Respective values were expressed in g kg^{-1}.

4.2.6 Statistical analysis

Data were tested with respect to homoscedasticity and normal distribution of residuals using Cochran's and Lilliefors tests, respectively. Subsequently, analysis of variance and multiple comparisons of means were performed using Tukey's test (at $p<0.05$). Data on morphophysiological and nutritional parameters of the plants were subjected to principal component analysis (PCA) to explore the relationships between variables and treatments using the FactoMineR package in R software (R Development Core Team, 2017).

4.3 Results

4.3.1 Morphophysiological characteristics

The results showed changes in morphophysiological characteristics as a function of inoculation with NFB strains and doses of N. Interaction effects between these factors were significant for all tested variables. The effects of inoculation with strains BR11005, BR12137, and BR12157 were superior to those in the control treatments NI (with no inoculation or fertilization) and standard fertilization. Among NFB without N fertilization treatments, BR12157 produced the strongest increase in H (35.4% and 25.0%), PSD (21.4% and 15.8%), NL (21.4% and 25%), TLA (30.7% and 26%), DMAP (30% and 33.9%), and DMP (9.8% and 26.7%), compared to treatments NI and standard fertilization, respectively. Compared with the NI treatment, RV increased by 12.8% (Table 3).

Table 3. Effect of NFB x Doses of N interaction on morphophysiological characteristics of banana seedlings, cultivar Prata Anã, after 90 days from transplanting.

Plant height (cm plant^{-1})						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	30.00 cC	33.74 abBC	34.21 abCD	34.66 aCD	34.73 aB	33.47 C
BR11005	38.39 aB	37.40 aB	32.64 bD	27.65 cE	26.53 cC	32.52 C
BR11674	21.46 bD	37.40 aB	37.23 aBC	38.26 aBC	35.40 aB	33.95 C
BR12137	37.03 bB	42.26 aA	40.99 abAB	44.37 aA	44.50 aA	41.83 B
BR12157	46.33 abA	46.69 aA	44.80 abA	41.79 bAB	42.44 abA	44.41 A
BR12158	30.44 aC	29.62 aC	30.87 aD	31.76 aDE	27.40 aC	30.02 D
Mean	33.94 c	37.85 a	36.79 ab	36.42 ab	35.16 bc	
CV (%)	12.91					
Pseudostem diameter (mm plant^{-1})						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	22.75 aAB	25.44 aAB	26.28 aAB	24.63 aAB	24.38 aAB	24.70 B
BR11005	26.25 aAB	25.94 abAB	24.00 abB	20.50 abB	20.03 bB	23.34 BC
BR11674	16.00 bC	24.46 aAB	23.31 aB	27.25 aA	24.31 aAB	23.07 BC
BR12137	25.19 aAB	30.44 aA	27.06 aAB	27.88 aA	27.00 aA	27.51 A
BR12157	28.94 aA	28.81 aA	30.81 aA	26.25 aAB	29.19 aA	28.80 A
BR12158	22.38 aB	21.21 aB	22.19 aB	22.94 aAB	18.50 aB	21.44 C
Mean	23.58 b	26.05 a	25.61 ab	24.91 ab	23.90 ab	
CV (%)	12.91					
Number of leaves (quantity plant^{-1})						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	5.50 aC	5.75 aC	5.00 aC	5.13 aB	5.25 aB	5.33 B
BR11005	6.50 aAB	6.63 aAB	6.75 aAB	6.38 aA	5.38 bB	6.33 A
BR11674	5.38 bC	7.14 aA	6.50 aAB	7.13 aA	6.63 aA	6.55 A
BR12137	5.88 cBC	6.63 abcAB	7.25 aA	6.38 bcA	7.00 abA	6.63 A
BR12157	7.00 aA	6.63 aAB	6.38 aB	6.63 aA	6.50 aA	6.63 A
BR12158	6.13 abBC	5.88 bBC	6.63 abAB	6.75 aA	6.75 aA	6.43 A
Mean	6.06 b	6.44 a	6.42 a	6.40 a	6.25 ab	
CV (%)	12.88					
Total leaf area (cm^2 plant^{-1})						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	673.65 aB	807.44 aAB	978.67 aAB	698.6 aABC	718.92 aAB	775.46 B
BR11005	728.90 aAB	593.06 abB	436.10 abC	342.83 bC	422.93 abB	504.77 C

BR11674	271.89 bC	705.60 aAB	736.49 aBC	606.0 abBC	611.79 abB	586.36 C
BR12137	797.32 aAB	813.87 aAB	740.80 aBC	1014.90 aA	974.59 aA	868 AB
BR12157	971.75 abA	980.17 abA	1164.55 aA	808.27 bAB	979.19 abA	980.78 A
BR12158	603.31 aBC	595.10 aB	513.09 aC	641.65 aBC	368.42 aB	544.32 C
Mean	674.47 a	749.21 a	761.62 a	685.38 a	679.31 a	
CV (%)	34.85					
Root volume (cm^3 $plant^{-1}$)						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	113.33 aA	140.00 aA	183.33 aA	140.00 aA	140.00 aAB	143.33 A
BR11005	113.33 aA	186.67 aA	126.67 abA	133.33 abA	160.0 abAB	144.00 A
BR11674	70.00 aA	140.00 abA	146.67 aA	160.00 aA	160.00 aAB	140.00 A
BR12137	113.33 bA	180.00 abA	116.67 bA	166.67 abA	210.00 aA	157.33 A
BR12157	130.00 aA	140.00 aA	180.00 aA	130.00 Aa	106.67 aB	134.62 A
BR12158	105.00 bA	126.67 abA	186.67 aA	146.67 abA	180.0 abAB	152.14 A
Mean	108.67 b	152.22 a	155.29 a	146.11 a	159.44 a	
CV (%)	21.38					
Dry mass of aerial parts (g $plant^{-1}$)						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	29.56 aB	34.26 aAB	32.69 aBC	28.85 aB	27.92 aCD	30.65CD
BR11005	32.29 abB	35.81 aAB	30.72 abBC	26.42 Bb	27.58 bCD	30.56 CB
BR11674	19.98 bC	35.60 aAB	31.43 aBC	37.05 Aa	32.21 aBC	31.25 C
BR12137	29.83 aB	39.97 aA	34.31 abB	38.56 aA	35.7 abAB	35.67 B
BR12157	42.22 aA	39.73 abA	44.18 aA	32.98 Bab	39.37 abA	39.70 A
BR12158	28.09 aB	30.02 aB	26.68 aC	29.83 Ab	23.30 aD	27.58 D
Mean	30.33 c	35.89 a	33.34 ab	32.28 bc	31.01 bc	
CV (%)	21.63					
Dry mass of roots (g $plant^{-1}$)						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	25.16 abAB	32.16 aA	31.48 aAB	24.03 bA	20.44 bB	26.6ABC
BR11005	30.34 aA	30.25aA	24.49 aBC	23.72 Aa	25.40 aAB	26.84AB
BR11674	22.04 bB	29.52 aA	24.45 abBC	29.77 Aa	27.90 abA	26.7ABC
BR12137	21.91 aB	27.86 aA	23.37 aC	28.04 aA	23.57 aAB	24.95 BC
BR12157	27.88 abAB	27.25 bA	34.87 aA	24.58 Ba	28.27 abA	28.57 A
BR12158	25.97 aAB	25.28 aA	23.11 aC	23.55 Aa	19.60 aB	23.50 C
Mean	25.55 b	28.72 a	26.96 ab	25.62 b	24.20 b	
CV (%)	28.28					

NI (No inoculation and no N fertilization); BR11005 (*Azospirillum baldaniorum*); BR11674 (*Azospirillum* sp.); BR12137 (*Herbaspirillum* sp.); BR12157 (*Burkholderia* sp.); BR12158 (*Burkholderia* sp.); CV: Coeficient of Variation. Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test ($p < 0.05$).

Strains BR11005 and BR12137 also improved morphophysiological characteristics, compared to the NI and standard fertilization treatments, respectively (Table 3). Treatment with strain BR11005 led to increases in H (21.8% and 9.5%), PSD (13.3% and 7.1%), NL (15.4% and 19.2%), TLA (7.6% and 1.4%), DMAP (8.5% and 13.5%), and DMP (17.1% and 32.6%). Plants treated with strain BR12137 showed increased H (19% and 6.2%), PSD (9.7% and 3.2%), NL (6.5% and 10.7%), TLA (15.5% and 9.8%) and DMAP (0.9% and 6.4%). Regarding strains BR11674 and BR12158, improvements in morphophysiological characteristics were smaller than those obtained with standard fertilization (Table 3).

The effects of N on morphophysiological characteristics varied between bacterial strains. Inoculation with BR11005 in absence of N as well as with 50 mg dm^{-3} N led to an increase in H (30.9%), PSD (23.7%), and NL (17.2%), compared with standard fertilization. The effects of BR11674 were positive only under addition of N fertilization, and with standard fertilization, inoculation led to an increase in H (39.4%), PSD (34.2%), TLA (55.6%), NL (18.9%), DMAP (38%), and DMP (21%), compared to the NI treatment. Doses of N did not influence morphophysiological characteristics in inoculation treatments with BR12137, BR12157, and BR 12158 (Table 3).

Seedlings inoculated with BR11005 and BR12157 showed higher GIP than the control treatments. Strains BR12137 and BR12158 showed higher GIP than the standard fertilization treatment. Growth did not increase after inoculation with BR11674, compared with the control treatments (Fig. 1).

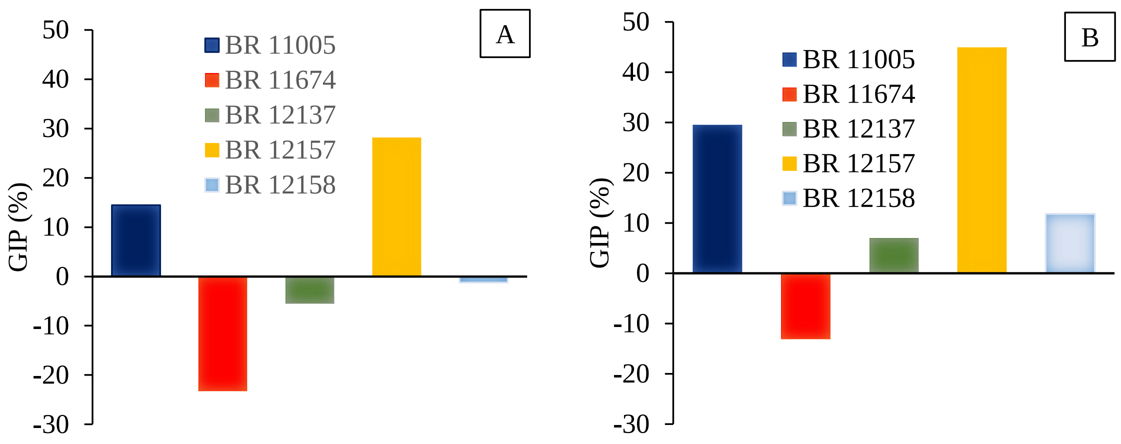

Fig. 1. Growth increase percentage (GIP) of banana seedlings, cultivar Prata Anã, inoculated with NFB, with standard fertilization (200 mg dm^{-3} of N) [A] and without N fertilization [B].

All strains endophytically colonized the roots of seedlings at different proportions as a function of doses of N, except for BR12158. Higher doses of N elicited an increase in RCP in the BR11005 and BR11674 treatments to a maximum of 150 mg dm^{-3}, and it decreased in the BR12137 and BR12157 treatments (Fig. 2).

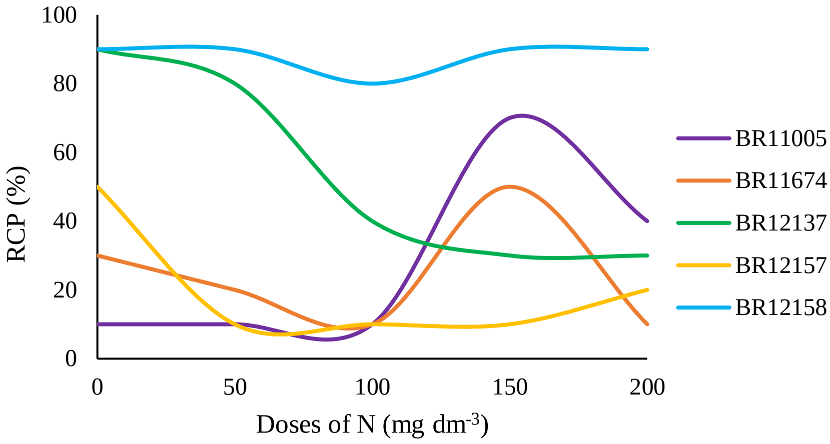

Fig. 2. Root colonization percentage (RCP) of NFB inoculations in banana seedlings, cultivar Prata Anã, after 90 days from transplanting, as a function of doses of N.

Strain BR12157 affected the morphology and stimulated the expansion of the seedlings' root system, thereby changing the root architecture and the number and arrangement of fine roots. In treatments with other strains, standard fertilization promoted growth of primary roots (Fig. 3).

Fig. 3. Effects of standard fertilization and inoculation with NFB strains on the growth of banana seedlings, cultivar Prata Anã, after 90 days from transplanting [A]. Change in the quantity and disposition of fine roots along the root system, in seedlings inoculated with BR12157 without N fertilization [B] and with standard fertilization [C].

4.3.2 Photosynthetic pigments and proline

Inoculation with BR12157 produced the highest total chlorophyll content in leaves, followed by treatments with strains BR11674, BR12158, and BR12137, which, however, did not differ from each other statistically. The other strains showed no significant effects. Strain BR12157 produced an increase in total chlorophyll content by 50% and 43.7%, compared to the NI and standard fertilization treatments, respectively.

Carotenoid content, however, was similar among inoculation treatments, regardless of N fertilization. For both photosynthetic pigments, N fertilization did not significantly influence the effect of inoculation (Table 4).

Table 4. Effects of the interaction between NFB and doses of N on total chlorophyll, carotenoids and proline content in leaves of banana seedlings, cultivar Prata Anã, after 90 days from transplanting.

Total chlorophyll (mg g^{-1} of fresh mass)						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	22.30 aD	27.71 aAB	26.04 aB	23.83 aC	25.51 aA	25.08 C
BR11005	21.25 abD	23.84 abB	26.47 aB	27.16 aBC	15.96 bB	22.94 C
BR11674	37.12 aAB	28.59 bAB	31.92 abAB	34.25 abB	29.65 abA	32.31 B
BR12137	27.35 aC	15.24 bC	29.65 aAB	31.02 aBC	28.01 aA	26.25 C
BR12157	44.56 aA	35.46 bA	38.12 abA	34.39 bB	30.60 bA	36.63 A
BR12158	34.29 bBC	26.13 cB	29.45 bcB	44.44 aA	30.11 bcA	32.88AB
Mean	31.15 a	26.15 b	30.28 a	32.52 a	26.64 b	
CV (%)	12.07					
Carotenoids (mg g^{-1} of fresh mass)						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	5.60 aA	5.33 aAB	5.32 aA	4.83 bA	5.01 abA	5.22 A
BR11005	4.15 abA	3.11 bBC	3.86 abA	5.82 aA	4.66 abA	4.32 AB
BR11674	5.43 aA	5.95 aA	4.69 aA	4.26 aAB	4.33 aA	4.93 A
BR12137	4.70 abA	2.86 bC	5.52 aA	4.77 abA	4.38 abA	4.44 AB
BR12157	3.44 bA	3.66 abABC	3.28 abA	3.89 abAB	4.30 aA	3.71 B
BR12158	3.26 abA	3.89 abABC	4.63 aA	2.01 bB	3.57 abA	3.47 B
Mean	4.05 a	4.18 a	4.55 a	4.26 a	4.37 a	
CV (%)	20.07					
Proline (mg g^{-1} of fresh mass)						
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	1.10 aA	0.53 dF	0.81 bA	0.36 eF	0.58 cD	0.68 E
BR11005	1.06 aB	0.55 eE	0.74 cC	0.89 bC	0.68 dC	0.78 A
BR11674	0.69 eC	0.71 dB	0.74 cC	0.99 aA	0.75 bB	0.77 B
BR12137	0.56 eD	0.75 cA	0.80 bB	0.90 aB	0.68 dC	0.74 D
BR12157	0.53 eE	0.65 dC	0.81 cA	0.85 bD	0.91 aA	0.75 C
BR12158	0.69 aC	0.63 bD	0.48 dD	0.50 cE	0.51 cE	0.56 F
Mean	0.77 a	0.64 e	0.73 c	0.75 b	0.68 d	
CV (%)	10.02					

NI (No inoculation and no N fertilization); BR11005 (*Azospirillum baldaniorum*); BR11674 (*Azospirillum* sp.); BR12137 (*Herbaspirillum* sp.); BR12157 (*Burkholderia* sp.); BR12158 (*Burkholderia* sp.); CV: Coeficient of Variation. Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test ($p < 0.05$).

An interaction effect of N fertilization and inoculation with NFB strains on proline content was observed. In the NI and inoculation treatments with BR11005 and BR12158, increasing doses of N led to decreased proline content in leaves. By contrast, in plants inoculated with BR11674, BR12137, or BR12157, proline content increased with increasing doses of N (Table 4).

4.3.3 N concentrations

Inoculation with NFB strains and doses of N showed an interaction effect on N concentrations in aerial parts of the seedlings. Standard fertilization yielded the highest N concentrations (Table 5). In roots, an isolated effect of the strains was observed. Treatment with strain BR12137 produced the highest N concentrations (8.9 g kg^{-1}) (Fig. 4).

Table 5. Effect of the interaction between NFB and doses of N on the N concentration in aerial part of banana seedlings, cultivar Prata Anã, after 90 days from transplanting.

	N concentration in aerial parts (g kg^{-1})					
NFB	Doses of N (mg kg^{-1})					Mean
	0	50	100	150	200	
NI	8.82 abAB	8.07 abAB	7.23 bC	8.24 abA	11.34 aA	8.74 B
BR11005	6.72 aB	8.15 aAB	8.57 aC	9.58 aA	9.41 aAB	8.48 B
BR11674	11.43 aA	9.24 aAB	10.84 aABC	9.66 aA	8.49 aAB	9.93 AB
BR12137	9.41 abAB	7.22 bB	12.43 aAB	10.92 abA	9.66 abAB	9.93 AB
BR12157	9.16 abAB	8.23 abAB	8.07 abC	11.51 aA	6.64 bB	8.72 B
BR12158	8.99 bcAB	11.85 abA	13.02 aA	7.90 cA	12.02 aA	10.82 A
Mean	9.085 a	8.79 a	10.02 a	9.63 a	9.65 a	
CV (%)	13.59					

NI (No inoculation and no N fertilization); BR11005 (*Azospirillum baldaniorum*); BR11674 (*Azospirillum* sp.); BR12137 (*Herbaspirillum* sp.); BR12157 (*Burkholderia* sp.); BR12158 (*Burkholderia* sp.); CV: Coeficient of Variation. Same capital letters in the column and lowercase letters in the line do not differ by Tukey's test (p <0.05).

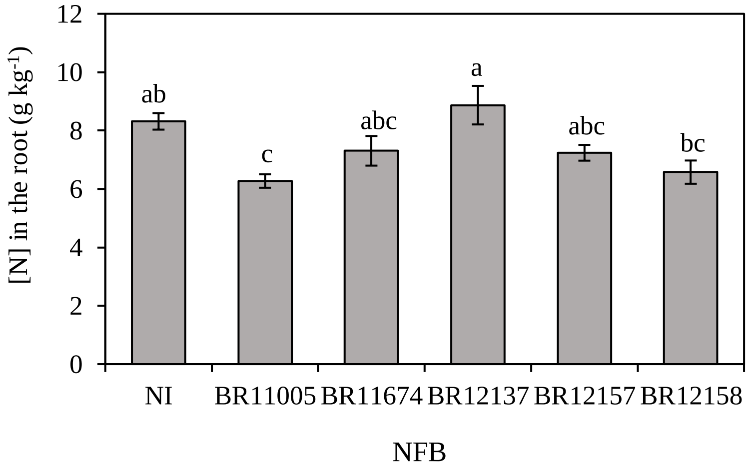

Fig. 4. N concentration in the root of banana seedlings, cultivar Prata Anã, after 90 days from transplanting, with no inoculation (NI), no N fertilization, and inoculation with BR11005, BR11674, BR12137, BR12157 and BR12158.

4.3.4 PCA

The PCA showed that the variables were positively correlated in two principal components which accounted for 38.31% and 13.16% of the overall variation, respectively. The characteristics leaf number, root volume, aerial and root dry mass, total chlorophyll and N content in the aboveground parts of the seedlings were strongly affected by inoculation with strain BR12157 (Figs. 5A and 5B).

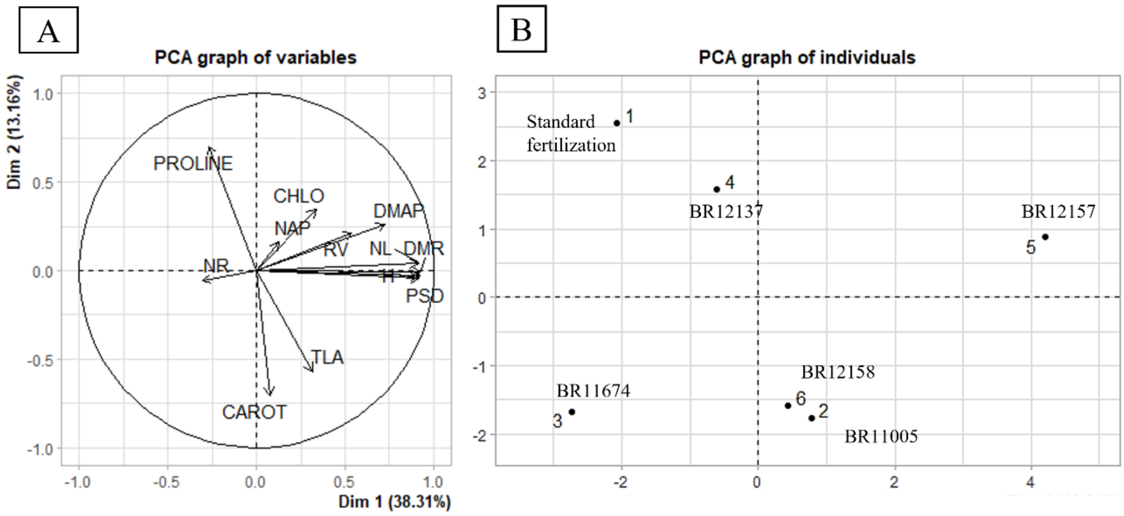

Fig. 5. Principal component analysis graph (PCA) [A] and principal component analysis score [B] for the morphophysiological and nutritional variables of banana seedlings, cultivar Prata Anã, after 90 days from transplanting, as a function of inoculation with NFB and doses of N.

4.4 Discussion

Inoculation with strains BR11005 and BR12157 resulted in improved performance of almost all morphophysiological characteristics, apart from root volume. These inoculation treatments provided even better results than those obtained with standard fertilization (Table 3). The positive effects of these inoculations on morphophysiological characteristics may be indirectly related to exudation of intermediate and/or final products of the secondary metabolism of NFB strains, such as growth regulators (auxin, gibberellin, and cytokinin), chelators (iron-siderophore complex), enzymes (nitrogenase, phosphatase), and organic acids that solubilize nutrients (Fox et al. 2016; Kuan et al. 2016; Gopalakrishnan et al. 2017; Kumar et al. 2017; Wang et al. 2019b), thereby making them available to plants. The results suggest that inoculation with these strains can substitute N fertilization during this stage of development and may help reduce the time seedlings remain in the nursery.

The evaluated strains showed the following functions: N fixation, K and inorganic P solubilization at different levels of intensity, and production of amylases and proteases. Some strains such as BR11005 showed outstanding performance with respect to auxin synthesis and production of siderophores and exopolysaccharides, and BR12158 which appeared to be efficient in terms of P solubilization (Table 2). Both siderophores and K and P solubilizers can increase the mobilization of K, Fe, P, and Zn through production of organic acids and acid phosphatases, making them soluble and available to plants (Yuan et al. 2013; Andrade et al. 2014; Souza et al. 2016).

The benefits derived from the multiple effects of NFBs depend on their localization with respect to the plant root system. Plant growth promotion is most effective using inoculation with endophytic bacteria or when the microorganisms can reproduce in plant tissues (Fox et al. 2016; Kandel et al. 2017; Liu et al. 2017). Strains BR12137, BR12157, and BR12158 were isolated from internal banana plant tissues, which explains the microbe/plant affinity observed in terms of the percentage of root colonization in the present study. Furthermore, all strains colonized the roots endophytically (Fig. 2), which may have favored an increase in the concentrations of N, P, K, Fe, Zn, and auxins in this tissue, resulting in stronger nutrient uptake by the plant. This beneficial effect of inoculation on nutritional state of the plants was corroborated by the similarity observed between inoculation treatments with NFB and standard fertilization, in terms of N concentration, both in aerial parts and roots (Table 5, Fig. 3).

In addition to promoting an increase in nutrient concentrations, the strains can colonize different root niches (Mia et al. 2010; Yegorenkova et al. 2016), increase the proportion of root colonization, and consequently limit the severity and frequency of infection with phytopathogens (Cedeño et al. 2021) due to their biochemical and physiological characteristics (Table 2).

The NFB strains were compatible with *Trichodema asperellum* and *Bacillus* sp. which are PGPM also used in banana cultivation (Moreira et al. 2021). By contrast, strain BR12157 was incompatible with *Fusarium oxysporum* f. sp. *cubense,* which is the causal agent of the 'fusarium blight' disease in banana plants (Hadadd et al. 2016), showing mycelial inhibition of 35.9% (Table 2). These compatibility responses between NFB and other PGPM are considered relevant, especially because the selection of microorganisms with multiple aptitudes expands the possibilities of applications of biotechnological products, not only for disease treatment purposes, but also in terms of preventive measures.

Although the volume of roots was more strongly affected by standard fertilization than by inoculation treatments (Table 3), the strains modified the architecture, number, and quantity of fine roots (Fig. 3), which resulted in a more robust root system and thereby contributed to improving water and nutrient uptake. Some previous studies reported such effects of inoculation with endophytes on root surface area and on the number and length of root hairs (Yegorenkova et al. 2016; Gamez et al. 2019; Wang et al. 2019a). In plants inoculated with BR12157 and which received no N fertilization, greater lateral root formation and occurrence of multiple branches (Fig. 3) were observed at the expense of the primary root, which contributes to improved N uptake and dry mass production (Table 3) per unit of absorbed N, in addition to promoting the ability to absorb nutrients such as calcium through root interception (Yegorenkova et al. 2016; Emami et al. 2019).

Regarding photosynthetic pigments, inoculation with BR12157 led to increased total chlorophyll content (Table 4, Fig. 5). Previous studies found some endophytic NFB strains to stimulate the production of compounds that favor carbon assimilation in the leaf (Rozpadek et al. 2015; Marcos et al. 2016) and root-to-leaf transport of CO_2 originating from endophyte respiration (Hibberd and Quick 2002). Thus, endophytic colonization affects photosynthetic processes of host plants, from light energy uptake to carbon assimilation (Rozpadek et al. 2015).

The increase in chlorophyll was accompanied by a decrease in proline content in plants inoculated with BR12157 (Table 4), indicating a metabolic change that is necessary for the acceleration of cell growth by increasing the photosynthetic rate, resulting in larger biomass (Table 3). Moreover, proline can be used as an alternative metabolic substrate to sucrose to maintain N-fixing activity in microorganisms during abiotic stress (Verdoy et al. 2006), which explains the concentration of N in aerial parts and roots of inoculated plants without N fertilization. The preventive effects of PGPM regarding oxidative stress caused by low substrate fertility was previously observed in *Arachis hypogea* and *Cicer arietinum* plants inoculated with rhizobacteria, which showed lower proline and hydrogen peroxide content in leaves, resulting in higher cell membrane stability and cellular homeostasis (Alexander et al. 2019; El-Esawi et al. 2019). Without N fertilization, however, proline content increased in plants inoculated with BR11005, BR11674, BR12137, and BR12158 (Table 4), suggesting that these strains do not help prevent oxidative stress. Proline plays an important role in the protection of the photosynthetic apparatus in plants subjected to abiotic stressors because it is a compatible solute for osmotic adjustment, a metal chelator, an activator of detoxification pathways, and it helps eliminate reactive oxygen species and maintain stability of subcellular structures and membranes, including photosystem II and other molecular structures, to alleviate oxidative damage (Hayat et al. 2012; Abd-Allah et al. 2016).

Doses of N affected neither N concentrations in aerial parts nor total chlorophyll content in seedlings inoculated with BR12157 (Tables 4 and 5), showing that N in plants inoculated with this strain originated from NFB activity. The main advantage of using N obtained through biological fixation is complete utilization of ammonium which can be fully assimilated inside the plant, with no risks to the environment (Mia et al. 2010; Kuan et al. 2016).

The results of the current study showed the potential of strain BR12157 as a bioinoculant for promoting banana plant growth during the seedling phase. However, further studies are needed to examine the effects of this and other strains during subsequent growth stages under field conditions to better understand potential interactions between the performance of endophytic NFB and factors such as fertility and soil moisture as well as their effects on the plants' morphophysiological, biochemical, and nutritional characteristics (Tshikantwa et al. 2018).

4.5 Conclusions

Banana seedlings of the Prata Anã cultivar can be produced at sufficient quality without N fertilization, and inoculation with BR11005 or BR12157 can improve the performance of morphophysiological characteristics related to growth. This is attributed mainly to the ability of the bacteria to positively influence some biochemical, metabolic, and nutritional mechanisms such as biological N fixation, K and P solubilization, auxin synthesis, and production of siderophores, exopolysaccharides, and hydrolytic enzymes. The performance of plants inoculated with these strains exceeded those of plants receiving only standard N fertilization. Strain BR12157 showed the most promising effects due to its potential to completely substitute N fertilization. Thus, inoculation with BR12157 can be considered a sustainable strategy for the production of banana seedlings with high morphophysiological quality.

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5. FINAL CONSIDERATIONS

This study broadens and deepens our knowledge of the use of bioinoculants as another technological resource to improve the performance of growth-related morphophysiological, biochemical, and nutritional characteristics of banana seedlings. The results demonstrated that the benefits of inoculations with *Bacillus* sp. and/or *Trichoderma asperellum* are more effective when their use is concomitant with a substrate consisting of a mixture of organic compounds and soil at 40:60. Due to their positive effect on the synthesis of auxins and hydrolytic enzymes, these bioinoculants are promising candidates for further studies investigating the microbial inoculation for growth promotion of banana plants under field conditions.

This study also revealed that the endophytic NFB strains BR11005 and BR12157 enhanced the performance of growth-related morphophysiological traits of banana seedlings. The strains were able to promote biological N fixation, potassium and phosphate solubilization, auxin synthesis, and production of siderophores, exopolysaccharides and hydrolytic enzymes. These strains, especially BR12157, proved to be more efficient than standard nitrogen fertilization in agronomic terms due to their potential to fully meet the N demand of the plant.