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Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (*Musa acuminata* Colla)

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ABSTRACT

Banana is the second largest export crop in Colombia. To meet the demand of international markets, high amounts of chemical fertilizers are required, which represent high costs and can be hazardous to the environment. Plant growth promoting rhizobacteria (PGPR) can, at least partially, replace chemical fertilizers. In this paper, we evaluated the effect of nine PGPR of the genera *Bacillus* and *Pseudomonas* on banana growth. Banana seedlings were produced through tissue culture and acclimatized in the greenhouse core. Plants were inoculated with the rhizobacteria and growth parameters (plant height, leaf number, leaf area, pseudostem thickness, root and shoot fresh weight, root and shoot dry weight) were assessed after 55 days. The two best performing PGPR, Bs006 and Ps006 previously identified as *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, respectively, promoted banana growth similarly or even slightly superior to 100% chemical fertilization, and were selected for further characterization of root colonization by both eletron microscopy and confocal microscopy of fluorescence *in situ* hybridization (FISH)–stained root tissues. Both *P. fluorescens* Ps006 and *B. amyloquifaciens* Bs006 showed ability to colonize banana roots, but Bs006 appeared faster than Ps006 in the colonization dynamics. This work demonstrated that inoculation of rhizobacteria *Bacillus amyloliquefaciens* Bs006 and *Pseudomonas fluorescens* Ps006 could partially replace the chemical fertilization of tissue cultured banana plants, and therefore could be used for the formulation of a new biofertilizer.

1. Introduction

The area for banana crop in Colombia is about 477,000 ha, which results in a total production of 5.4 millions metric tons, making the country as the fourth exporter of banana Cavendish worldwide (ASBAMA, 2016). Commercial cultivation of bananas requires large amounts of nitrogen and potassium fertilizers, followed by phosphorus, calcium and magnesium, to maintain high yields (Mia et al., 2010a).

Biofertilizers are an environmental–friendly alternative to enhance absorption of both water and mineral nutrients by the plant. Recently, it was shown that biofertilizers based on rhizobacteria played an important role in maintaining soil fertility (Mia et al., 2010a; Akhtar et al., 2012; Posada et al., 2016). Beneficial bacteria isolated from the rhizosphere habitat can be inoculated on autochtonous or allochtonous crops, in order to induce a positive effect on plant growth. These bacteria are usually designated as PGPR (plant growth-promoting rhizobacteria) and include a wide range of species having the ability to increase seed germination, plant biomass and crop yield (Kloepper and Bay-Peterson, 1991; Raupach and Kloepper, 1998; Kloeppe et al., 1999; Esitken et al., 2010; Rowe et al., 2010; Ahemad and Kibret, 2014; Gupta et al., 2015). Seed treatment with PGPR has been used to reduce germination time and to increase the growth of various crops such as vegetables (Cleyet-Marel et al., 2001; Qin et al., 2015; Kisiel and Kępczyńska, 2016; Zhang et al., 2016) and fruits like strawberry, apple, cherry, citrus, raspberry, blueberry and apricot (Esitken et al., 2002, 2003; 2006; Orhan et al., 2006; Aslantas et al., 2007; Karlidag et al., 2007).

Plant growth promotion can be achieved through direct interaction of beneficial microorganisms and the host plant or indirectly through their antagonistic activity against plant pathogens. The mechanisms of plant growth promotion by PGPR include, among others, the ability to produce plant hormones such as auxins (Spaepen and Vanderleyden,

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2011; Glick, 2012), cytokinins (Shilev, 2013), gibberellins (Kang et al., 2010) and ethylene (Shilev, 2013), which make the PGPR contributers to further proliferation of root hairs thus increasing the absorption of nutrients such as nitrogen, iron and phosphorus (Sharma et al., 2013; Ahemad and Kibret, 2014). These bacteria colonize the plant root surface, transforming root exudates into plant hormones, (Raupach and Kloepper, 1998; Kloeppe et al., 1999; Rowe et al., 2010; Miransari and Smith, 2014). Other modes of action are asymbiotic N_2 fixation (Gaby and Buckley, 2012), inorganic phosphate solubilisation and mineralisation of organic phosphates and / or other nutrients (Khan et al., 2010; Bhattacharyya and Jha, 2012). PGPR have also showed antagonistic activity against phytopathogenic microorganisms by siderophore production, synthesis of antibiotics, enzymes, anti-fungal compounds, nutrients, competition for binding sites and the recently discovered RHESt mechanism (Cornelis, 2010; Lanteigne et al., 2012; Arora et al., 2013; Mousa et al., 2016).

Beneficial activities have been reported for PGPR strains belonging to the genera *Azoarcus, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Clostridium, Enterobacter, Gluconacetobacter, Pseudomonas* and *Serratia*, among others (Kloepper and Bay-Peterson, 1991; Somers et al., 2004; Bhattacharyya and Jha, 2012). The genera *Bacillus* and *Pseudomonas* have been widely studied as PGPR in various crops, and some biofertilizers on the market are based on species belonging to both genera. Both microorganisms have also been tested on banana cultivars Great Dwarf and Calcutta (Rivera-Cruz et al., 2008), but so far no reports of their effect is known on banana cv. Williams, which is of great economic interest for several banana–producing countries (FAO, 2018).

To understand the ecology of PGPR, microscopy studies are well suitable, since they allow a better understanding of the ecology of the inoculated bacteria and their interaction with the plant, including rhizocompetence and dynamics of root tissue colonization (Cardinale, 2014). Root colonization by rhizobacteria can be analyzed with Scanning Electron Microscopy (SEM). This tool is widely used in cell biology because, despite the lower capacity of magnification compared to transmission electron microscopy, it allows appreciating the three-dimensional textures of objects that have been fixed with a metal before observation. The root colonization pattern of microbes like *Bacillus* and *Pseudomonas* has been well characterized in several plants including cucumber (Kimani et al., 2016), *Arabidopsis thaliana* (Palmqvist et al., 2015), tomato (Subramanian et al., 2015) and grape (Ma et al., 2017); however, there is no information about bacterial primary root colonization pattern in banana cv. Williams. Plant root microbial colonization has been be revealed by Fluorescent in situ Hybridization (FISH) staining using fluorescently labelled DNA probes targeting the 16S or the 23S ribosomal RNA contained in ribosomes (Moter and Göbel, 2000). Fluorescence quenched by labelled microbes can be detected by confocal laser scanning microscopy (CLSM). FISH coupled with confocal microscopy (FISH-CLSM) is a powerful tool that allows an accurate localization of bacteria in the root habitat, and a reliable characterization of the colonization patterns (Cardinale, 2014).

The aim of this study was to evaluate the effect of nine rhizobacteria in promoting the growth of banana cv. Williams, and to characterize the two most efficient strains with respect to their root colonization pattern by both SEM and FISH-CLSM.

2. Materials and methods

2.1. Location of the study

The study was conducted at the Research Center “Caribia” of the Colombian Agricultural Research Corporation (AGROSAVIA), located in the Municipality of the Banana Zone, Magdalena Department (between parallels 10° 39′ and 10° 55′ north latitude and between the meridians 74° 06′ and 74° 17′ west of Greenwich, at an altitude of 30 m a.s.l.). This area has an average temperature of 30 °C, average relative humidity of 83%, average annual rainfall of 1300 mm and an average evaporation of 1412 mm annually. The climate of this region is dry with marked seasonal summer and winter.

2.2. Plant material

The banana (*Musa acuminata* Colla) cultivar Williams (Cavendish subgroup, genome AAA) was used in this study. The mother plants of the banana cv. Williams were obtained from the *ex vitro* Musa Bank of the Caribia Research Center of AGROSAVIA. Murashige and Skoog (MS) culture medium (Murashige and Skoog, 1962), supplemented with 0.1 g l^{-1} myo-inositol, 3 ppm Benzyl Amino Purine (BAP), 0.5 ppm indole acetic acid (IAA), 1 ppm vitamins (Thiamine hydrochloride), 30 g l^{-1} sucrose and pH 5.7, was used for the micropropagation of the seedlings (meristem extraction) in the laboratory. The seedlings were placed in the growth room under controlled conditions, with a temperature of 23 °C/20 °C ± 1 °C day/night, 16/8-h light/dark photoperiod, and relative humidity of 65% ± 10% for another 4 weeks. The Murashige and Skoog (MS) culture medium was also used for the in vitro propagation and rooting of the seedlings.

2.3. Bacterial inoculants

In this study, nine candidate PGPR strains were tested. The strains of *Bacillus* (*B. pumilus* Ap18, *B. subtilis* Ap279 and *B. pumilus* Ap280) were obtained from University of Auburn (Alabama, USA). One strain of *Pseudomonas fluorescens* was provided by the Universidad de Los Andes (Bogotá, Colombia). Five native strains, two *Bacillus* (*B. amyloliquefaciens* Bs006 and *Bacillus sp.* Bs003) and three *Pseudomonas* (*P. fluorescens* Ps006, *Pseudomonas sp.* Ps13 and *Pseudomonas sp.* Pf14), identified by biochemistry and 16S rRNA gene sequencing, were obtained from the microbial collection of AGROSAVIA. Strains Bs006 and Ps006 were previously identified at genome level as *Bacillus amyloliquefaciens* (isolated from *Physalis peruviana,* in Combita, Boyacá) (Gamez et al., 2015) and *Pseudomonas fluorescens* (isolated from *Furcraea andina*, in Totoro, Cauca) (Gamez et al., 2016), respectively.

2.4. Greenhouse experiments

2.4.1. Planting and microbial inoculation

Seedlings of around 3 cm height with three fully expanded leaves were transplanted into trays of 24 cones, and each cone was filled with 300 ml (354 g) of a sand:alluvion substrate mixture (3:1). The substrate had been previously sterilized with Agrodine® (fungicidal and bactericidal) in ratio of 3 ml L^{-1} of water for each 18 kg of substrate. All bacterial strains were grown in Luria Bertani agar (LBA, Sigma) and stored in liquid LB medium with 15% glycerol at −80 °C. The bacterial inocula were obtained from a single colony-inoculated LB medium grown at constant shaking (120 rpm) at 28 °C for 48 h and then adjusted to a concentration of 10^8 CFU ml^{-1} in a water suspension. Ten days before and eight days after planting the banana seedlings, the bacterial inoculation was carried out by applying 15 ml of each of the rhizobacterial suspensions. The final concentration of bacteria in the substrate was 5×10^6 CFU ml^{-1}.

The hardening phase lasted 55 days. Seedlings were grown under room temperature (28–41 °C) and relative humidity of 85%, and were irrigated via Dan Sprinkler irrigation spray (flow rate 28 LPH and flow pressure 30 Psi), five seconds every 5 min during the first 10 days. From the 11th day until the harvest, the irrigation occurred for 5 s every 10 min.

2.4.2. Experimental design and statistical analysis

A completely randomized design was applied with a factorial arrangement of two factors: “bacterial inoculation”, with ten levels (nine rhizobacteria + one uninoculated) and “fertilization”, with three levels (5%, 50% and 100%). The fertilization was performed by applying diammonium phosphate (DAP), urea and potassium chloride (KCl),

according to the typical requirements of banana (100% fertilization corresponded to 1.38 g kg^{-1} of P_2O_5, 0.54 g kg^{-1} of N, and 1.44 g kg^{-1} of K). For each fertilization + inoculation combined treatment (30 in total), four biological replicates were used; each replicate consisted in a pot with six plants. Therefore, a total of 720 banana cv. Williams plants were analyzed.

The following variables were evaluated after 55 days of growth: plant height (cm), number of leaves, leaf area (cm^2), pseudostem thickness (mm), fresh and dry weight (total, root and aerial part) (g $plant^{-1}$). For each replicate (pot), the mean value of the six plants was used. To measure fresh and dry weight, plants were removed from the tray and the remaining substrate was removed from the roots. Plant material was first weighed and then dried in an oven at 60 °C for 48 h.

Data were analyzed by ANOVA using a SAS GLM procedure (Systat version 9.2, Cary NC, USA 2001). Means were separated by the Tukey multiple range ($P \leq 0.05$) post-hoc test. For orthogonal contrasts, multiple comparison tests were applied. In cases where significant differences occurred, separation of means for the main effects was performed by Tukey post-hoc test ($P \leq 0.05$). To directly compare the effect of the two best-performing strains with the effect of the fertilization, a one way ANOVA was performed including the following treatments (fertilization/inoculation): 5%/non-inoculated (NI), 50%/NI, 100%/NI, 5%/*Pseudomonas fluorescens* Ps006, and 5%/*B. amyloliquefaciens* Bs006.

2.4.3. Microbial root colonization analysis by Scanning Electron Microscopy

Banana cv. Williams roots, collected from the proliferation area, were carefully washed in water to remove excess of substrate without wiping attached microorganisms. The roots were dried at 37 °C for 3 days and then cooled at 4 °C until processing. Roots were dehydrated with 20% ethanol and subsequently washed with acetone. Gold covering was done with a sputtering Hummer II (Technics, Springfield, VA) and SEM imaging and / or EDAX analysis was performed in the laboratory of Scanning Electron Microscopy Center Interfaculty Equipment - CEIF of the National University of Colombia, using a Quanta 200 (Thermo Fisher Scientific, Columbus, Ohio, USA).

2.4.4. Fluorescence in situ hybridization and confocal laser scanning microscopy (FISH-CLSM)

Root samples used for FISH analysis were taken from banana plants 24 h after inoculation with the strain *Bacillus amyloliquefaciens* Bs006 or *Pseudomonas fluorescens* Ps006, the two best performing PGPR. The root samples inoculated with *Pseudomonas* and the non-inoculated roots were fixed with 3:1 4% formaldehyde:ice-cold 1X PBS for 6 h, washed three times with ice–cold 1X PBS and stored in 1:1 96% ice–cold ethanol:ice–cold 1X PBS at −20 °C. Root samples inoculated with *Bacillus* were fixed directly with 1:1 96% ice–cold ethanol: ice–cold 1X PBS at −20 °C.

Root segments of 0.5–1 cm length were stained using the FISH technique according to (Cardinale et al., 2008) ("in tube-FISH"). Hybridization was performed at 42 °C for two hours with a mixture of two probes: the bacterial EUB338MIX universal probe (Amann et al., 1990) labelled with Cy3 and a probe specific for either Firmicutes (LGC354MIX, Meier et al., 1999) or Gammaproteobacteria (Gam42a, Manz et al., 1992) labelled with Cy5 and coupled with its competitor probe. Uninoculated roots were hybridized with all three probes together. As a FISH negative control, subsamples of inoculated roots were hybridized with a mixture of Cy3 and Cy5-labelled non-sense probes NONEUB (Wallner et al., 1993).

Samples stained by FISH were viewed with the confocal laser microscope Leica SP5 (Leica Microsystems, Mannheim, Germany). For the Cy3-labelled probe EUB338MIX, the 561 nm laser light was used for excitation and the emitted light in the range 565–618 nm was detected. For Cy5 labelled probes, the 633 nm laser light was used for excitation and the emitted light in the range 652–693 nm was detected; autofluorescence of the roots was detected in the range 428–510 using UV laser light of 405 nm for excitation. The three signals were combined in multicolored images, where the inoculated bacteria appeared yellow by overlapping the signals of the EUB338MIX probe (to which the red color code was assigned) and the specific probe (to which the green color code was assigned). Blue color code was assigned to the root tissue. Confocal series were taken using a Leica 63X objective, with a step of 0.45–0.80 µm between the confocal planes. Confocal series were visualized and three-dimensional models were created with the software Imaris 8.1 (Bitplane, Zurich, Switzerland). Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA) was used to assemble and label the final images.

3. Results

3.1. Greenhouse experiments

All measured parameters (plant height, leaf area, pseudostem thickness, root fresh weight, aerial fresh weight, total dry weight, root dry weight and aerial dry weight) were significantly affected by both factors, “bacterial inoculation” and “fertilization” (ANOVA Main Effect, $P \leq 0.001$; Tables 1 and 2). The most significant increase in plant height was obtained with *Pseudomonas fluorescens* Ps006 (12.65 cm), followed by *Bacillus amyloliquefaciens* Bs006 (12.46 cm), in comparison

Table 1
Effect of *Bacillus* and *Pseudomonas* rhizobacteria inoculation and chemical fertilization (factorial ANOVA) on plant height, number of leaves, leaf area and pseudostem thickness of banana cv. Williams plants. Different letters indicate significantly different means (Tukey test, $P < 0.05$).

Treatment	Height (cm)	Number of leaves	Leaf area (cm^2)	Pseudostem thickness (mm)
B. pumilus Ap18	11.72 abc	6.51 a	98.49 ab	8.75 ab
B. subtilis Ap279	11.89 abc	6.26 abc	99.25 ab	8.85 ab
B. pumilus Ap280	11.82 abc	6.13 abc	99.75 ab	8.96 ab
Bacillus sp. Bs003	11.80 abc	5.91 bc	97.24 ab	8.92 ab
B. amyloliquefaciens Bs006	12.46 ab	6.35 abc	105.96 a	9.38 a
P. fluorescens (Univ. Andes)	11.65 bc	6.43 ab	93.25 ab	8.75 ab
P. fluorescens Ps006	12.65 a	5.95 abc	104.02 a	9.27 ab
Pseudomonas sp. Ps013	11.41 c	5.84 c	87.46 b	8.64 b
Pseudomonas sp. Pf 14	12.10 abc	6.26 abc	99.50 ab	8.90 ab
Uninoculated control	8.78 d	5.95 abc	58.86 c	7.74 c
Rhizobacteria P value	*< 0.0001*	*0.0004*	*< 0.0001*	*< 0.0001*
Fertilization 5%	10.94 c	5.87 c	85.01 c	8.52 b
Fertilization 50%	11.58 b	6.17 b	94.57 b	8.86 a
Fertilization 100%	12.36 a	6.45 a	103.60 a	9.07 a
Fertilization P value	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*
Interaction Rizobacteria – Fertilization P value	*< 0.0001*	*0.0202*	*< 0.0001*	*< 0.0001*

Table 2
Effect of *Bacillus* and *Pseudomonas* rhizobacteria inoculation and chemical fertilization (factorial ANOVA) on total fresh weight, root fresh weight, aerial fresh weight, total dry weight, root dry weight, shoot dry weight of banana cv. Williams plants. Different letters indicate significantly different means (Tukey test, $P < 0.05$).

Treatment	Total fresh weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Aerial fresh weight (g plant^{-1})	Total dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Aerial dry weight (g plant^{-1})
B. pumilus Ap18	11.37 e	3.89 bc	7.46 e	1.38 ab	0.27 ab	1.11 ab
B. subtilis Ap279	14.55 bcd	4.66 ab	9.87 cd	1.50 a	0.35 a	1.15 ab
B. pumilus Ap280	14.18 cd	4.60 ab	9.58 cd	1.34 ab	0.31 ab	1.02 ab
Bacillus sp. Bs003	11.60 e	4.30 ab	7.29 e	1.34 ab	0.34 a	1.00 ab
B. amyloliquefaciens Bs006	16.71 ab	4.90 a	11.74 ab	1.48 ab	0.32 ab	1.16 ab
P. fluorescens (Univ. Andes)	12.35 de	4.16 abc	8.19 de	1.21 bc	0.26 ab	0.94 bc
P. fluorescens Ps006	17.71 a	4.87 ab	12.86 a	1.47 ab	0.29 ab	1.17 a
Pseudomonas sp. Ps013	14.87 bc	4.10 abc	10.76 bc	1.22 abc	0.24 b	0.97 ab
Pseudomonas sp. Pf14	14.44 bcd	4.58 ab	9.86 cd	1.40 ab	0.32 ab	1.07 ab
Uninoculated control	8.53 f	3.20 c	5.17 f	1.02 c	0.27 ab	0.74 c
Rhizobacteria P value	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.0014*	*< 0.0001*
Fertilization 5%	10.92b	3.98b	6.93b	1.20b	0.30a	0.90b
Fertilization 50%	14.83a	4.52a	10.25a	1.43a	0.31a	1.12a
Fertilization 100%	15.15a	4.48a	10.66a	1.37a	0.28a	1.09a
Fertilization P value	*< 0.0001*	*0.0020*	*< 0.0001*	*< 0.0001*	*0.1219*	*< 0.0001*
Interaction Rizobacteria–Fertilization P value	*< 0.0001*	*< 0.0001*	*< 0.0001*	*< 0.0001*	*0.0116*	*< 0.0001*

Fig. 1. (A) Plant height of uninoculated banana cv. Williams (left) compared to plants treated with *Bacillus amyloliquefaciens* Bs006 (right). (B) Root quality and quantity of uninoculated plant (left) compared to plants treated with *Bacillus amyloliquefaciens* Bs006 (right).

Fig. 2. Comparison of the complex banana cv. Williams radicular system after inoculation with the 3 rhizobacteria: (A) *Bacillus amyloliquefaciens* Bs006, (B) *B. pumilus* Ap280 and (C) *Pseudomonas fluorescens* Ps006.

to the 8.78 cm reached by the non-inoculated control plants (Table 1, Fig. 1), with about 44 and 41% increase, respectively.

In relation to the numbers of leaves, significant differences were found when banana plants were treated with *B. pumilus* Ap18, *P. fluorescens* (strain Universidad de Los Andes) and *B. amyloliquefaciens* Bs006, with means of 6.51, 6.43 and 6.35, respectively, as compared to the control untreated plants with a mean of 5.95 (Table 1).

Leaf area showed statistically significant differences where banana plants were treated with bacterial strains *B. amyloliquefaciens* Bs006, *P. fluorescens* Ps006, *B. pumilus* Ap280 and *Pseudomonas* sp. Pf14 (105.96, 104.02, 99.75 and 99.50 cm^2, respectively), corresponding to 80%, 76%, 69.4% and 69% increase, respectively, as compared to the control uninoculated plants (58.86 cm^2) (Table 1).

Thickness of pseudo-stem was also influenced by the treatment with rhizobacteria, the highest values corresponding to plants treated with *B. amyloliquefaciens* Bs006, *B. pumilus* Ap280 and *Bacillus* sp. Bs003 (9.38, 9.13 and 8.92 mm, respectively (Table 1), as compared to the control (7.74 mm).

In plants treated with rhizobacteria, the average root length was 17.2, 16.3 and 14.5 cm after inoculation with *P. fluorescens* Ps006, *B. amyloliquefaciens* Bs006 and *B. pumilus* Ap280, respectively, the three best performing rhizobacteria (Fig. 2). This corresponded to an increase of 49.5%; 46% and 40% with respect to the uninoculated control plants. The roots of the plants inoculated with PGPR presented greater thickness, more length and volume, abundant secondary roots and in general good architecture. In particular the plants treated with *P. fluorescens* Ps006 showed a larger root complex than the uninoculated plants, with more branches and secondary roots, and an abundant growth of the peliferous area, also.

For fresh and dry aerial weight, the rhizobacterium with the most significant effect was *P. fluorescens* Ps006 with 12.86 and 1.17 g plant^{-1}, respectively. The weight of fresh and dry roots treated with *B. amyloliquefaciens* Bs006 were 4.90 and 0.32 g plant^{-1}, respectively, compared to 3.20 and 0.27 g plant^{-1} of the uninoculated control plants (Table 2). Complexively, the best performing rhizobacteria were *P. fluorescens* Ps006 and *B. amyloliquefaciens* Bs006.

When concentration of chemical fertilizer was 50% of the recommended dosage, plant growth parameters as height, number of leaves, leaf area and pseudostem thickness reached a mean of 11.58, 6.17, 94.57 and 8.86, respectively (Table 1). Recommended fertilization (100%), exhibited a superior ($p < 0.05$) improvement of 12.36, 6.45, 103.60, and 9.07 in the above plant growth features, respectively. However, for most of the weight parameters (Table 2), 50% fertilization reached similar values than 100%.

The direct comparison of *P. fluorescens* Ps006- and *B. amyloliquefaciens* Bs006-inoculated plants with the fertilized, non-inoculated plants showed that these rhizobacteria had an effect comparable or even slightly superior to that of 100% fertilization (Fig. 3).

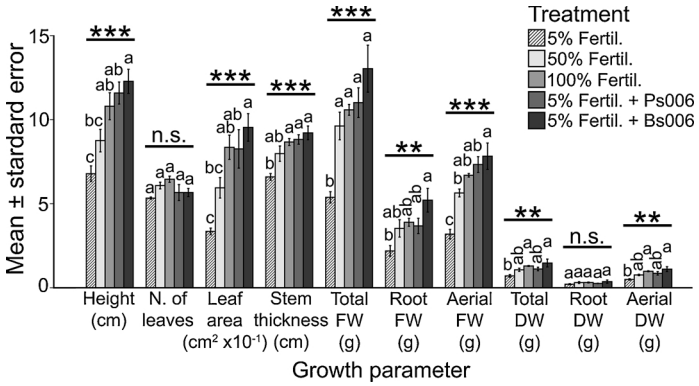

Fig. 3. Growth parameters of *Pseudomonas fluorescens* Ps006- and *Bacillus amyloliquefaciens* Bs006-inoculated banana plants compared to non-inoculated and fertilized plants. ANOVA *P* values: ** $P < 0.01$, *** $P < 0.001$, n.s. $P > 0.05$. Different letters above the bars indicate significantly different means (Tukey test, $P < 0.05$).

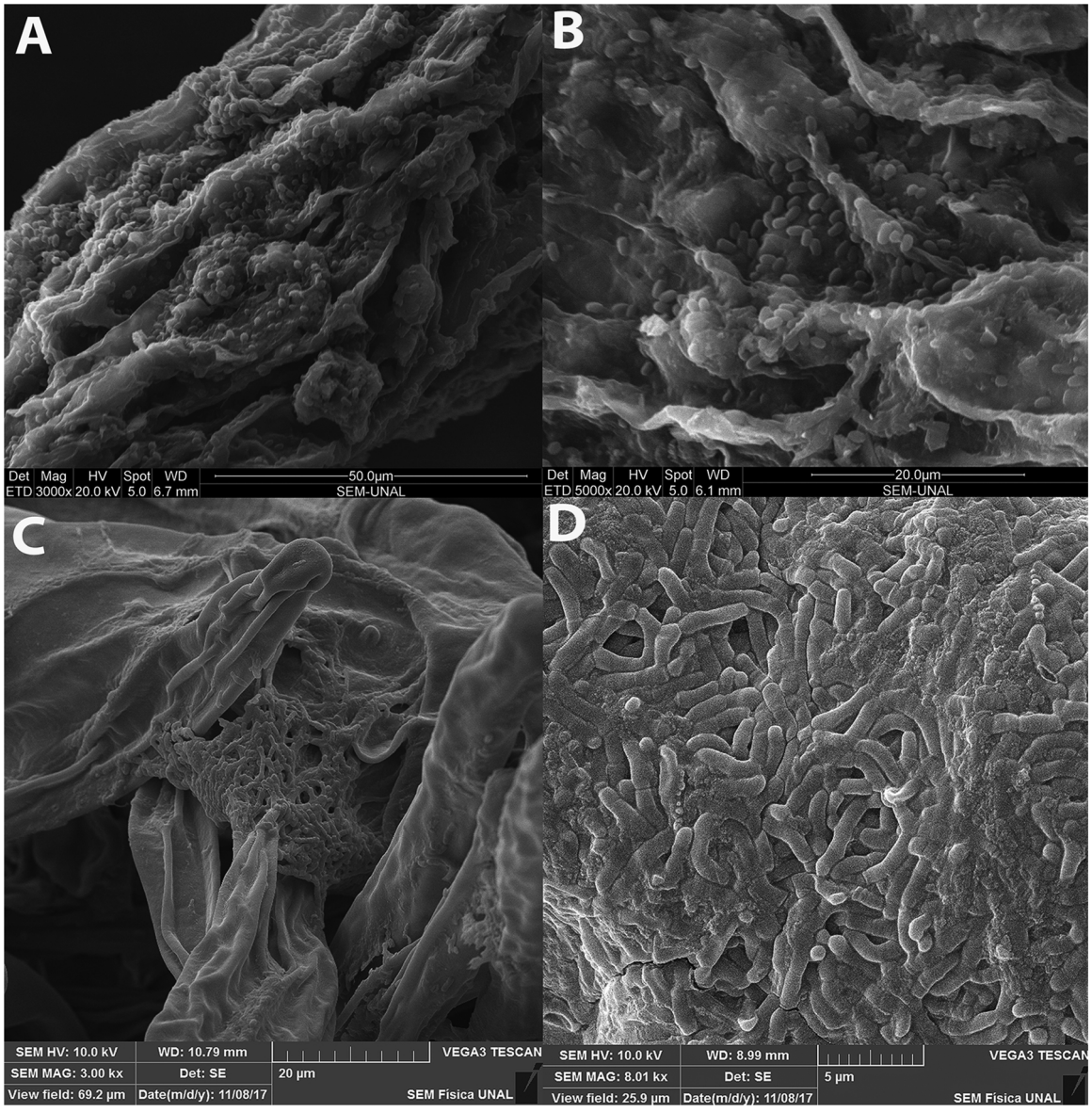

Fig. 4. Scanning Electron Microscope photomicrographs showing the colonization of banana cv. Williams roots by rizobacteria *Pseudomonas fluorescens* Ps006 (A–B) and *Bacillus amyloliquefaciens* Bs006 (C–D).

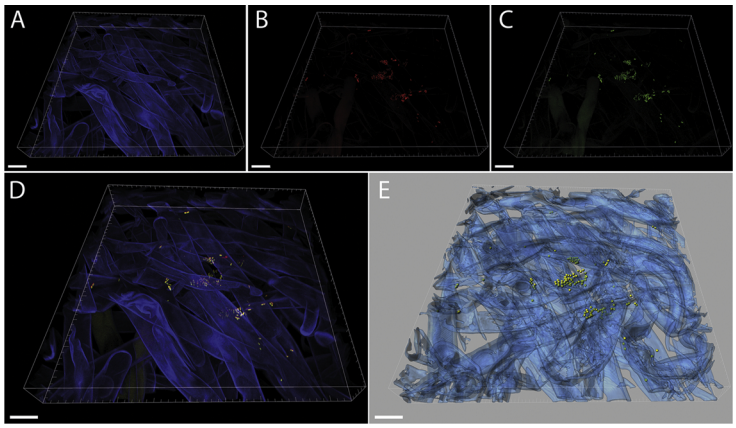

Fig. 5. FISH-CLSM image showing the colonization of banana cv. Williams roots by *Bacillus amyloliquefaciens* Bs006, 24 h after inoculum. A) root tissue autofluorescence; B) Signal of the Cy3-labelled EUB338MIX universal probe for bacteria; C) Signal of the Cy5-labelled LGC354MIX probe specific for Firmicutes; D) overlay of A–C; E) Three-dimensional model of image in D. Scale bars: 20 μm.

3.2. Plant root colonization by rhizobacteria

3.2.1. Scanning electron microscopy analysis

Strains *Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006 were selected for analysis of the colonization pattern, as they were the best performing PGPR. Both of them successfully colonized the root surface of banana seedlings obtained from tissue culture (Fig. 4). Scaning Electron Microscopy revealed that the bacterial cells of both *P. fluorescens* Ps006 and *B. amyloliquefaciens* Bs006 were present abundantly on root surfaces. The colonization pattern clearly showed that the bacterial cells of both strains were located in the root proliferation zone. *B. amyloliquefaciens* Bs006, showed a pattern of colonization characterized mainly by generating clusters or bacterial agglomerates through a matrix or biofilms, which looked very compact (Fig. 4C–D). In the case of *P. fluorescens* Ps006, colonization is also evident, however these bacteria appeared more dispersed in the roots and did not generate biofilms (Fig. 4A–B).

3.2.2. FISH-Confocal microscopy analysis

Both inoculated rhizobacteria were detected on both root hairs and root surface. *Bacillus amyloliquefaciens* Bs006 was often detected as small-to-thick colonies (Fig. 5 and Fig. S1). *Pseudomonas fluorescens* Ps006 was detected instead mostly as single cells, markedly less abundant than *Bacillus* (Fig. 6, arrows) and localized on the root hairs only. No signal was detected in the non-inoculated roots (Fig. 7A), nor in the inoculated roots hybridized with negative FISH–probe NONEUB (Fig. 7B–C).

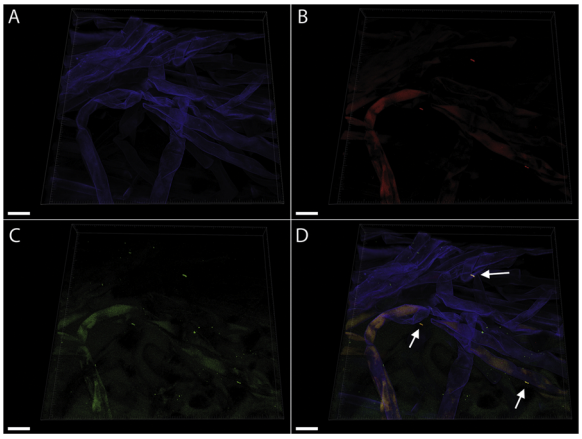

Fig. 6. FISH-CLSM image showing the colonization of banana cv. Williams roots by *Pseudomonas fluorescens* Ps006 24 h after inoculum. A) root tissue autofluorescence; B) Signal of the Cy3-labelled EUB338MIX universal probe for bacteria; C) Signal of the Cy5-labelled Gam42a probe specific for Gammaproteobacteria; D) overlay of A–C; arrows indicate *P. fluorescens* Ps006 cells. Scale bars: 20 μm.

4. Discussion

This study demonstrated that PGPR inoculation increased significantly plant growth and seedling development of tissue-cultured banana cv. Williams, under greenhouse conditions. Similar results about positive effects of inoculating PGPR in plant growth have been found in other species. For example, when tomato plants were treated with *Bacillus pumilus* WP8 and *Pseudomonas putida* RBP1 as PGPR, they showed increases in height in about 68% and 56%, respectively (Shen et al., 2012). Eight strains of both *Bacillus* and *Pseudomonas* genera (HJR1, HJR2, HJR3, HJR4, HJR5, MR6, HJR7, HJR8) promoted the growth of maize (*Zea mays* L.) grown in pots in greenhouse, with an increase between 98% and 54% in plant height, 30 and 60 days after germination (Zahid, 2015). More recently, (Batista et al., 2018) found that *Bacillus* sp. RZ2MS9 promoted an increase in corn root dry weight by 247.8%. Other species of *Bacillus*, like *Bacillus megaterium* in combination with a PGPR strain of *Paenibacillus polymyxa* increased dry weight in common bean in comparison to plants inoculated with rhizobia only (Korir et al., 2017). In other studies carried on in tomato, several types of PGPR such as *Pseudomonas*, *Azotobacter* and *Azospirillum*, have been evaluated, finding significant differences in both fresh and dry weight of the plant shoot and root, compared to tomato plants that did not received rhizobacteria (Sharafzadeh, 2012). Seeds of cucumber plants treated with the PGPR strains *Pseudomonas stutzeri*, *Bacillus subtilis*, *Stenotrophomonas maltophilia* and *Bacillus amyloliquefaciens* showed significant higher levels of germination, seedling vigor, and plant growth (Islam et al., 2016). Root length of mint plants has been improved after treatment with *Pseudomonas fluorescens* WCS417r (Zhang et al., 2007). Treated plants presented a greater lateral root formation and an increase in the root surface favoring probably a greater potential for plant nutrient uptake.

In general, our microbial treatment with *Bacillus amyloliquefaciens* Bs006 or *Pseudomonas fluorescens* Ps006 (the two best performing PGPR) led to comparable or superior values of plant growth parameters compared to 100% fertilization, suggesting that bacterial inoculation has a high potential to at least partially replace chemical fertilization. There are numerous experimental findings indicating that benefical soil bacteria would be able to improve nutrient use efficiency compared to or combined with the fertilizer application. Recent reports suggest that inoculation of *Azotobacter chroococcum* allows to reduce nitrogen fertilization doses up to 50% on cotton growth, thus representing a viable alternative to alleviate the environmental deterioration related to N pollution (Romero-Perdomo et al., 2017). Application of 75% recommended dose (RDNP) along with *Azospirillum* (HAU) + Phosphorus Solubilizing Bacteria (PSB) + Pink Pigmented Facultative Methylotrophs (PPFM), yielded to 2.91 t/ha seed cotton with higher net return (Rs 40.553) and net B:C ratio value of 1.94, while application of 100%

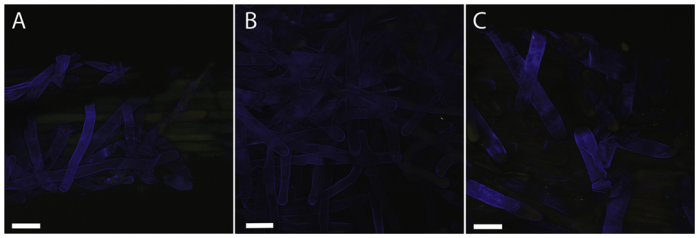

Fig. 7. CLSM images of FISH negative controls. A) not inoculated banana cv. Williams roots after staining with the Cy3-labelled EUB338MIX universal probes for bacteria, the Cy5-labelled LGC354MIX specific probe for Firmicutes and the FITC-labelled Gam42a specific probe for Gammaproteobacteria: only the root tissue autofluorescence was detected; no detectable signal from the probes EUB338MIX, LGC354MIX and Gam42a was observed. B) FISH negative control (Cy3- and Cy5-labelled NONEUB probes) of roots inoculated with *Bacillus amyloliquefaciens* Bs006 C) FISH negative control (Cy3- and Cy5-labelled NONEUB probe) of roots inoculated with *Pseudomonas fluorescens* Ps006. Scale bars: 30 μm.

RDNP without bio-inoculants yielded 2.54 t/ha (Nalayini et al., 2010). Yasmin et al. (2013) showed that the co-inoculation of *Pseudomonas aeruginosa* Z5 and *Bacillus fusiformis* S10 with half and 1/4th of the recommended N and P fertilizers improved both the cotton boll mass and lint, as well as seed yield compared to un-inoculated controls. Other studies provided evidence that *B. pumilus* S1r1 can biologically fix atmospheric N_2 and provide an alternative technique, besides plant breeding, to delay N remobilisation in maize plant for higher ear yield (up to 30.9%) with reduced N-fertiliser input (Kuan et al., 2016).

The growth promoting activity seen in our study after *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* inoculation in banana is thus coherent with several previous reports in other plant species. The presence of *Bacillus* spp. in raspberry roots has been related to plant performance, and specifically to plant growth and nutrient content increase (Orhan et al., 2006). *Bacillus* OSU 142 and M3 stimulated performance and quality parameters of sugar beet and barley, as well (Cakmakcı et al., 2001). When micropropagated banana plants variety 'Gran Enano' were inoculated with mycorrhiza and rhizobacteria (monoculture and combined), plant growth parameters as total fresh weight, shoot dry weight, stem length and leaf area were significantly higher than in non-inoculated banana plantlets (Rodríguez-Romero et al., 2005).

In relation to root growth, Mia et al. (1998) showed that inoculation of rhizobacteria could stimulate root growth and development, involving both primary and secondary roots. An important feature is that these roots are usually longer and with larger volume, even increasing 43% over the control, similar to the results obtained here in banana cv. Williams.

The Integrated System of Plant Nutrition (IPNS) improves soil productivity after the implementation of several activities, such as the balanced use of soil nutrients, the use of chemical fertilizers combined with organic sources (including bio-inoculants) and nutrient transfer through agroforestry systems. These alternatives are adapted to the agricultural systems of both irrigated and rainfed agriculture. The extensive use of chemical fertilizers in horticultural crops deteriorates soil health, affecting in turn the productivity (Sangeeth and Suseela Bhai, 2015). Only the application of new and sustainable alternatives of soil fertilization will allow facing the food security and sustainability challenges of the coming decades, however it will require considerable changes in the management of nutrients and water (Mueller et al., 2012; Zhang et al., 2015).

Colonization of host plant roots by PGPR is essential for long-term association, for three reasons: (i) if the bacteria do not bind to the root of the plant, the substances excreted by them will diffuse into the rhizosphere and will be consumed by the versatile nutrition of other microorganisms before arriving at the plant, (ii) without firm attachment, water can wash the bacteria away from the rhizosphere, (iii) root areas that do not have PGPR association are more vulnerable to pathogen colonization (Mia et al., 2010a). According to Mia et al. (2010b), the pattern of colonization is the formation of a fibrillar material that favors the massive colonization of the rhizobacteria in the root of the banana cultivar Berangan. It has also been shown that rhizobacteria have a predilection for areas where mucilage is present (Benizri et al., 2001; Compant et al., 2005, 2010; Liu et al., 2014; Mhlongo et al., 2018). In response, rhizobacteria increase secretions of plant growth promoting substances such as auxins, gibberellins and cytokinins (Gutiérrez-Mañero et al., 2001; Ortíz-Castro et al., 2008; Miransari and Smith, 2014). Some studies reported that *Bacillus* and *Pseudomonas* PGPR strains induced a significant effect on plant germination and seedling growth even under abiotic stress (Miransari and Smith, 2014; Kumar et al., 2016; Sorty et al., 2016). On the other hand the effect of bacterial colonization is more concentrated in the root hairs (Fournier et al., 2008).

Scanning electron microscopy (SEM) analysis demonstrated the rhizobacteria establishment and colonization in *Lycopodium cernuum* L. (Ghosh et al., 2016). Cell clusters of *Bacillus amyloliquefaciens* UCMB5113, have been reported colonizing the roots of cucumber. Later, a greater amount of adhered bacteria revealed by SEM was further confirmed by quantitative fluorescence measurements (Palmqvist et al., 2015).

Molecular techniques like qPCR not only have added evidences about colonization capabilities of PGPR, but have also detected and quantified precisely the abundance of root-colonizing microbes like *B. amyloliquefaciens* and *B. firmus* in commercial maize and soybean hybrids (Mendis et al., 2018), or *P. fluorescens* in maize roots and rhizoplane (Mosimann et al., 2017). Recently, a coordinated action of colonization and surfactin-mediated antibacterial activity has been proposed as a mechanism of bacterial interaction of *B. subtilis* with melon roots (Fan et al., 2017). Besides pathogen antibacterial activity, *Bacillus* spp. root colonization has also been connected to *Fusarium oxysporum* f. sp. *cubense* suppression, suggesting that this microorganism might be associated to banana diseases suppression. *Bacillus* spp. relative abundance is highly linked to a wealthy soil microbiome associated to plant growth promotion (Yuan et al., 2013; Xue et al., 2015).

In our study, it was possible to visualize *B. amyloliquefaciens* Bs006 and *P. fluorescens* Ps006 colonizing and dividing on banana plant root surface. Both SEM and FISH-CLSM microscopical approaches revealed bacterial cell clusters formation of *B. amyloliquefaciens* Bs006 on roots. The higher extent of biofilm-like structures would be related to its effects as a PGPR on banana plants. In banana, plant organic compounds as fumaric acid were significantly involved in *B. amyloliquefaciens* plant growth promoting activity as well as in biofilm formation and linked to gene expression (Yuan et al., 2015). In contrast, *P. fluorescens* Ps006 colonization was scanty, without visible biofilm formation especially when observed by confocal microscopy after FISH staining. This could indicate that this strain requires longer time to firmly adhere to the banana roots compared to *B. amyloliquefaciens* Bs006. It is also possible that *P. fluorescens* Ps006 is better adapted to the endophytic environment; therefore, the bacterial cells must first entry the plant tissues, to proliferate and then provide the host with their beneficial effects (Subramanian et al., 2015; Fox et al., 2016; Ma et al., 2017). Similar results of microbial adhesion has been reported by Palmqvist et al. (2015) on *Brassica napus* and *Arabidopsis thaliana* roots. The presence of potential endophytic *Bacillus* spp. and *Pseudomonas* spp. from banana roots has been recently reported by (Su et al., 2017), association that has been related to a bacterial effect against the nematode *Meloidogyne javanica*.

5. Conclusions

The results of the present study suggest that inoculation with growth promoting rhizobacteria are a suitable alternative for chemical fertilization on banana cv. Williams plants. Inoculation with rhizobacteria increased significantly the growth features such as height, pseudostem thickness, leaf area, fresh weight and dry weight in micropropagated banana plants. In particular, the strains *Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006, able to colonize the banana roots at different extent, are the best candidates to be used as bio-fertilizers to improve the growth and development of seedlings of banana cv. Williams, to at least partially replace mineral fertilization. Further studies are necessary to evaluate the long-term dynamics of colonization and the mechanisms of action of these two promising strains.

Conflict of interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.11.006.

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