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Analysis of the Abilities of Endophytic Bacteria Associated with Banana Tree Roots to Promote Plant Growth

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A total of 40 endophytic bacterial isolates obtained from banana tree roots were characterized for their biotechnological potential for promoting banana tree growth. All isolates had at least one positive feature. Twenty isolates were likely diazotrophs and formed pellicles in nitrogen-free culture medium, and 67% of these isolates belonged to the genus *Bacillus* sp. The isolates EB-04, EB-169, EB-64, and EB-144 had N fixation abilities as measured by the Kjeldahl method and by an acetylene reduction activity assay. Among the 40 isolates, 37.5% were capable of solubilizing inorganic phosphate and the isolates EB-47 and EB-64 showed the highest solubilization capacity. The isolate EB-53 (*Lysinibacillus* sp.) had a high solubilization index, whereas 73% of the isolates had low solubilization indices. The synthesis of indole-3-acetic acid (IAA) in the presence of L-tryptophan was detected in 40% of the isolates. The isolate EB-40 (*Bacillus* sp.) produced the highest amount of IAA (47.88 μg/ml) in medium supplemented with L-tryptophan and was able to synthesize IAA in the absence of L-tryptophan. The isolates EB-126 (*Bacillus subtilis*) and EB-47 (*Bacillus* sp.) were able to simultaneously fix nitrogen, solubilize phosphate and produce IAA *in vitro*. The results of this study demonstrated that the isolates analyzed here had diverse abilities and all have the potential to be used as growth-promoting microbial inoculants for banana trees.

Keywords: *Musa* spp. nitrogen fixation, indole-3-acetic acid, phosphate solubilization, *Bacillus* sp.

Introduction

Several studies have shown that bacteria and fungi engage in a close relationship with their host plants and are efficient in acquiring nutrients, whether by biological nitrogen fixation (Baldani and Baldani, 2005), the solubilization of insoluble phosphates (Baldotto *et al*., 2010) or the production of plant growth regulators (Kuss *et al*., 2007; Cassán *et al*., 2009).

The use of endophytic bacteria that are able to perform nitrogen fixation, mainly in non-leguminous plants, has been the object of several studies (Araujo *et al*., 2000; Mia *et al*., 2007; Teixeira *et al*., 2007). These bacteria are an attractive alternative to the use of nitrogen fertilizers. Species of the genera *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Azotobacter*, *Beijeinckia*, *Methylobacterium*, and *Azospirillum* have been found to have nitrogen fixation capacity in diverse cultures (Perin *et al*., 2003; Cavalcante *et al*., 2007).

Although many previous studies have focused on clarifying the mechanisms of biological nitrogen fixation by diazotrophic bacteria, the mechanisms underlying the phosphorus solubilization process and the synthesis of auxins have received more attention in recent years (Chagas Junior *et al*., 2010). Genera with the ability to solublize phosphorus and synthesize auxins include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Flavobacterium*, *Erwinia* (Rodrigues and Fraga, 1999), *Ewingella*, *Enterobacter* (Whitelaw, 2000; Ribeiro and Cardoso, 2012), and *Paenibacillus* (Mota *et al*., 2008). It is estimated that 80% of the bacteria isolated from rhizospheres have the capacity to produce auxins that regulate plant growth. Specifically, these auxins promote increased root length and numbers of lateral roots and hair. These morphological changes are due to a rapid response that leads to an increase in cell elongation, division and differentiation in the plants (Dobbelaere *et al*., 2003).

Banana trees grow rapidly and require great quantities of nutrients in the soil for their development and fruit production. Banana trees have adventitious roots that develop horizontally in the topsoil region, mainly in tropical soil conditions. This growth pattern imposes a limitation on the root system that may impair the development of the species and fruit production, as it significantly affects the absorption of nutrients and water and reduces the capacity of rhizobacteria and endophytic bacteria to produce phytohormones (Stover and Simmonds, 1987; Price, 1995). Despite this restraint, bananas are the most consumed fruit worldwide and their production is concentrated mainly in tropical countries. In Brazil, bananas grow in several ecosystems. This fruit has great nutritional, economic and social importance, given that growing banana trees is a highly cost-effective business that generates jobs in the country and in the city and aids in the

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development of the regions involved in its production (Fioravanço, 2003).

Given the importance of the banana tree crop and the absence of studies of the bacterial community associated with this species, this study aimed to analyze the capacity of endophytic bacteria isolated from banana tree roots for biological nitrogen fixation, calcium phosphate solubilization, phosphatase activity and the *in vitro* production of indole-3-acetic acid.

Materials and Methods

Bacterial strains and growth conditions

The endophytic bacteria used in the present study were previously isolated from the roots of the banana tree cultivar 'Prata Anã'. The banana roots were collected from different counties in the Minas Gerais state, and root fragments were immersed in 70% ethanol for 1 min, followed by immersion in 4% sodium hypochlorite (NaClO) for 3 min. The frag-

Table 1. Evaluation of the mechanisms of promotion of banana tree growth: pellicle formation in nitrogen-free medium, phosphate solubilization and IAA production by isolates of endophytic bacteria associated with banana tree roots grown in the cultivar Prata Anã. Whether these bacteria have *nifH* is also indicated.

Isolate	Most closely related genus/species	Pellicle formation	Phosphate solubilization	IAA production		*nifH* Gene[a]	GenBank Accession no.
				Trp	No Trp		
EB-04	*Bacillus subtilis**	+	-	+	+	+	AY741264.1
EB-15	*Bacillus pumilus**	-	-	-	-	-	GQ917222.1
EB-23	*Klebsiella pneumoniae**	+	-	+	-	+	JN201948.1
EB-24	*Bacillus thuringiensis*		-	-	-	+	JF947357.1
EB-25	*Bacillus cereus**	+	-	-	-	+	GU451184.1
EB-26	*Bacillus* sp.	-	-	+	+	-	HM209756.1
EB-28	*Paenibacillus* sp.		+	+	-	+	EF178460.1
EB-30	*Bacillus axarquienses**	-	-	-	-	-	JF414764.1
EB-34	*Bacillus pumilus**	-	+	+	-	-	JN215511.1
EB-37	*Bacillus* sp.*	-	-	-	-	-	JN215502.1
EB-38	*Bacillus* sp.	-	+	+	+	+	EU931559.1
EB-40	*Bacillus* sp.*	+	-	+	-	+	GQ340516.1
EB-44	*Bacillus* sp.*	-	+	-	-	-	GU122948.1
EB-45	*Lysinibacillus* sp. *	+	-	+	+	+	JN215512.1
EB-46	*Bacillus pumilus**	-	-	-	-	-	FJ236809.1
EB-47	*Bacillus* sp.*	+	+	+	-	+	FJ611939.1
EB-49	*Bacillus licheniformis**	+	-	-	-	+	EU366371.1
EB-50	*Bacillus* sp.*	+	-	-	-	+	HM769816.1
EB-51	*Bacillus pumilus**	+	+	-	-	+	HQ218993.1
EB-53	*Lysinibacillus* sp. *	-	+	+	-	-	JN215512.1
EB-55	*Bacillus subtilis**	-	-	+	+	-	HQ334981.1
EB-56	*Bacillus* sp.*	+	-	+	-	+	GU269573.1
EB-57	*Bacillus safensis**	-	+	-	-	-	JN092810.1
EB-58	*Bacillus pumilus**	-	-	-	-	-	JN082265.1
EB-60	*Lysinibacillus* sp. *	-	+	-	-	-	JF906500.1
EB-63	*Bacillus pumilus**	-	+	-	-	-	GQ917222.1
EB-64	*Bacillus pumilus**	+	+	-	-	+	JF271873.1
EB-68	*Bacillus safensis**	-	+	-	-	-	JN092818.1
EB-71	*Bacillus* sp.		-	-	-	+	HM461161.1
EB-87	*Bacillus tequilensis**	+	-	+	+	+	HM770882.1
EB-88	*Bacillus flexus**	+	+	-	-	+	DQ870687.1
EB-126	*Bacillus subtilis**	+	+	+	-	+	HM769817.1
EB-127	*Sporolactobacillus* sp. *	+	-	+	-	+	D16282.1
EB-133	*Bacillus* sp.*	+	-	+	-	+	AB301022.1
EB-136	*Bacillus subtilis**	+	-	-	-	+	AB301012.1
EB-144	*Paenibacillus* sp. *	+	-	-	-	+	EF178460.1
EB-147	*Bacillus subtilis**	-	+	-	-	-	EU977724.1
EB-169	*Bacillus pumilus* *	+	-	-	-	+	FJ189791.1
EB-194	*Bacillus* sp. *	+	-	-	-	+	FJ405377.1
EB-196	*Enterobacter* sp. *	-	-	-	-	-	GQ260081.1

* Isolates used in the nitrogen-free culture medium assay. (-) not detected; (+) detected; Trp: 10% TSA with tryptophan; No Trp: 10% TSA without tryptophan: (-) not detected; (+) detected.
[a] Data published by Souza *et al.* (2013).

ments were subsequently were washed three times in sterile distilled water. These fragments were macerated, and the suspensions were diluted 10-fold. Aliquots (0.1 ml) were plated in various media, including nutrient yeast dextrose agar (NYDA), potato dextrose agar (PDA) and tryptic soy agar (TSA). The plates were incubated for 48 h at 25±1°C and in a 12 h photoperiod (Souza *et al.*, 2013). A partial sequence of the 16S rRNA from the bacteria was determined with two primers: 27 [5′-AGAGTTTGATC(AC)TGGCTC AG-3′] and 1492R (5′-ACGG(CT)TACCTTGTTACGAC TT-3′). These sequences were filed in the EMBL/GenBank databank (Table 1). The isolates were incubated in TSA in test tubes for 24 h at 28°C. To obtain bacterial suspensions, TSB (Tryptic Soy Broth) was inoculated with bacteria, and the cultures were incubated at 28°C for 48 h with 100 rpm shaking. After this stage, the suspension was centrifuged for 10 min at 10,000 rpm to pellet the bacterial cells. The cells were then resuspended in a 0.85% saline solution in an aseptic laminar flow cabinet. The concentration of bacterial cells in the suspension was determined by reading the absorbance at a wavelength (λ) of 540 nm on a spectrophotometer (approximately 10^8 CFU/ml), and each culture was adjusted to have an optical density (OD) equal to 1.0 ABS.

Ability of the isolates to grow in nitrogen-free culture medium

Bacterial isolates were screened by PCR analysis for the presence of partial nif*H* gene sequences. Isolated DNA was amplified with the universal primers 19fF (5′-GGAATTCT GTGACCTAAAGCTGA-3′) and 407R (5′-AGCATACATT GCCATCATTTCACC-3′), and an amplification product was detected in 24 of the 40 isolates evaluated (Souza *et al.*, 2013) (Table 1). Only these 24 isolates containing *nif*H were tested for their ability to grow in nitrogen-free culture medium. The selected isolates were inoculated into culture tubes containing 15 ml of NFb semi-solid nitrogen-free culture medium. One liter of NFb medium contained the following: 5.0 g of malic acid, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of NaCl, 0.02 g of $CaCl_2{\cdot}2H_2O$, 2 ml of a micronutrient solution (0.04 g of $CuSO_4{\cdot}5H_2O$, 1.20 g of $ZnSO_4{\cdot}7H_2O$, 1.40 g of H_3BO_3, 1.00 g of $Na_2MoO_4{\cdot}2H_2O$, and 1.175 g of $MnSO_4{\cdot}H_2O$, in one liter of solution), 2 ml of a 0.5% bromothymol blue solution in 0.2 N KOH, 4 ml of a 1.64% Fe-EDTA solution, 1 ml of a vitamin solution (10 mg of biotin and 20 mg of pyridoxal-HCl in 100 ml of solution of dH_2O) and 4.5 g of KOH. The pH of this medium was adjusted to 6.8, and 1.8 g of agar was added to create a semisolid media. Five microliters of adjusted bacterial suspension was transferred (DO=1.0 ABS, λ=540 nm) into test tubes containing 15 ml of semisolid NFb medium (Döbereiner *et al.*, 1995). The test tubes were incubated at 28°C for seven days. After this period, the presence of a pellicle, which is characteristic of free-living diazotrophs, was visually determined, and the number of isolates that could grow and form pellicles in the nitrogen-free media was determined.

Nitrogen fixation capacity

The acetylene reduction activity assay (ARA) was performed on all twenty isolates that were able to grow and form pellicles in the semi-solid free-nitrogen culture medium. Approximately 1 ml of the NFb cultures was transferred to flasks containing 10 ml of semisolid media without nitrogen. The flasks were fitted with rubber plugs and incubated at 30°C for seven days. Acetylene was added to a final concentration of 12% (v/v), and ethylene production was determined after 24 h with a gas chromatograph (Shimadzu GC-14A). Pure ethylene was used as a standard. Studies were performed in triplicate.

The nitrogen content in the twenty isolates was measured by the Kjeldahl method (Bremner, 1965). Briefly, 600 µl of

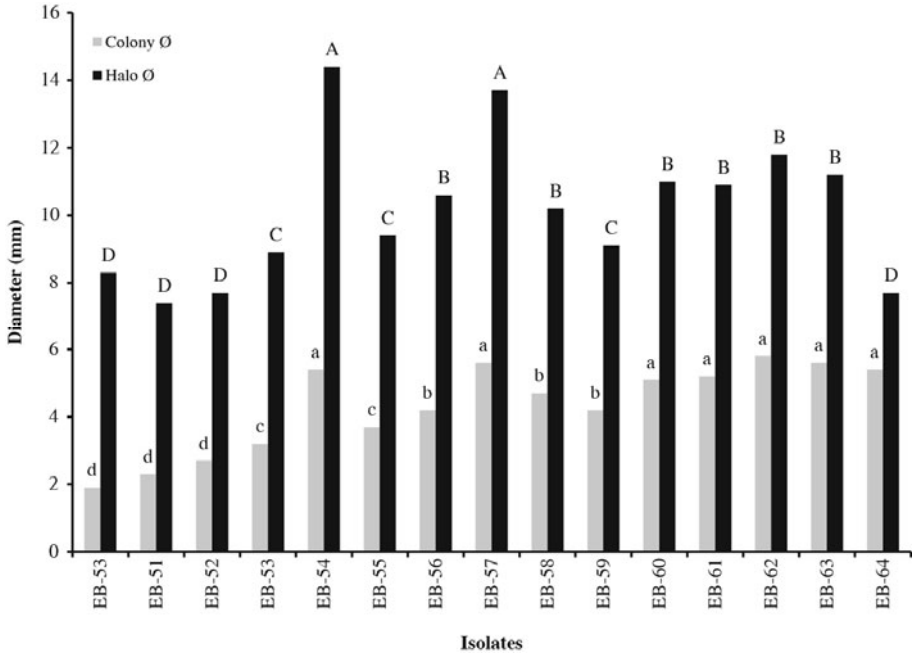

Fig. 1. Average colony and halo diameters (colony Ø and halo Ø) formed by endophytic bacteria, isolate from banana tree roots of the cultivar Prata Anã, in the phosphate solubilization assay in LGI_m medium supplemented with tribasic calcium phosphate [$Ca_3(PO_4)_2$]. Bars marked by different upper and lower case letters significantly differ between one another in halo and colony diameter, respectively, as determined by the Scott-Knott test (P<0.05).

Table 2. Solubilization indices (SI) for endophytic bacteria grown in LGI_m medium supplemented with tribasic calcium phosphate [$Ca_3(PO_4)_2$] and their classification according to the efficiency scale proposed by Silva Filho and Vidor (2000).

Isolate	Most closely related genus/species	Solubilization index	
		SI	Classification[a]
EB-53	*Lysinibacillus* sp.	3.56a	HS
EB-51	*Bacillus pumilus*	2.28b	MS
EB-44	*Bacillus amyloliquefaciens*	2.08b	MS
EB-63	*Bacillus pumilus*	1.94b	LS
EB-28	*Paenibacillus* sp.	1.76c	LS
EB-88	*Bacillus flexus*	1.70c	LS
EB-47	*Bacillus* sp.	1.49c	LS
EB-34	*Bacillus pumilus*	1.48c	LS
EB-60	*Lysinibacillus* sp.	1.21d	LS
EB-64	*Bacillus pumilus*	1.21d	LS
EB-57	*Bacillus safensis*	1.16d	LS
EB-38	*Bacillus* sp.	1.09d	LS
EB-68	*Bacillus safensis*	1.06d	LS
EB-147	*Bacillus subtilis*	1.03d	LS
EB-126	*Bacillus subtilis*		VL

Means followed by different letters differ from one another, as determined by the Scott-Knott test ($P<0.05$).
[a] HS, high solubilization; MS, medium solubilization; LS, low solubilization; VL, very low solubilization.

the adjusted bacterial suspension (DO=1.0 ABS, λ=540 nm) was transferred into test tubes containing 10 ml of semisolid NFb medium, and the samples were incubated at 30°C for seven days. Subsequently, each semisolid culture was digested, and the nitrogen concentration was determined by the Kjeldahl method. The quantities of nitrogen reported in the present study are the average of triplicate cultures. The results obtained were submitted to variance analysis with the statistical software Sisvar (Ferreira, 2008).

Screening of endophytes for inorganic phosphate solubilization

In order to select the isolates with capacity to solubilize inorganic phosphate, the methodology proposed by Katznelson and Bose (1959) was used. A modified LGI culture medium (Cavalcante and Döbereiner, 1988; Saravanan *et al.*, 2007) supplemented with 5.0 g/L of $Ca_3(PO_4)_2$ was used. Approximately 10 µl of each bacterial suspension was inoculated on the medium surface in three equidistant spots. The Petri dishes were incubated at 28°C for 15 days, and the halo of solubilization was analyzed. The diameter of the halo of solubilization and the bacterial colony were measured by using a caliper. The solubilization index (SI) was estimated by the formula proposed by Berraqueiro *et al.* (1976). The efficiency of solubilization was established according to the scale proposed by Silva Filho and Vidor (2000). The phosphate solubilization assays were performed in a randomized way with 40 treatments and three replicates. The solubilization indices were analyzed, and the results obtained were submitted to variance analysis with the statistical software Sisvar (Ferreira, 2008).

Quantification of inorganic phosphate solubilization

To quantify the solubilization of calcium phosphate the liquid medium NBRIP (Nautiyal, 1999) was used. Bacterial isolates were cultivated in liquid TSB medium for 48 h and approximately 1 ml of the adjusted bacterial suspension (DO=1.0 ABS, λ=540 nm) was transferred into Erlenmeyer flasks containing 50 ml of liquid NBRIP medium, supplemented with 0. 25 g/L of $Ca_3(PO_4)_2$. The control consisted of flasks without inoculum. The samples were incubated at 24°C in constant agitation for three days. After incubating the liquid medium was filtrate. The resulting filtrate was used to determine P concentration by the ascorbic acid method in a spectrophotometer at wavelength of 725 nm (Braga and Defelipo, 1974). The pH of the filtrate was also measured. Solubilized P was estimated by the difference between soluble P in the inoculated samples and in the uninoculated flasks. The quantification of inorganic phosphate solubilization assays were carried out in a randomized design with 16 treatments and three replicates.

Indole-3-acetic acid quantification

The production of indole-3-acetic acid (IAA) was performed following the methodology described by Kuss *et al.* (2007).

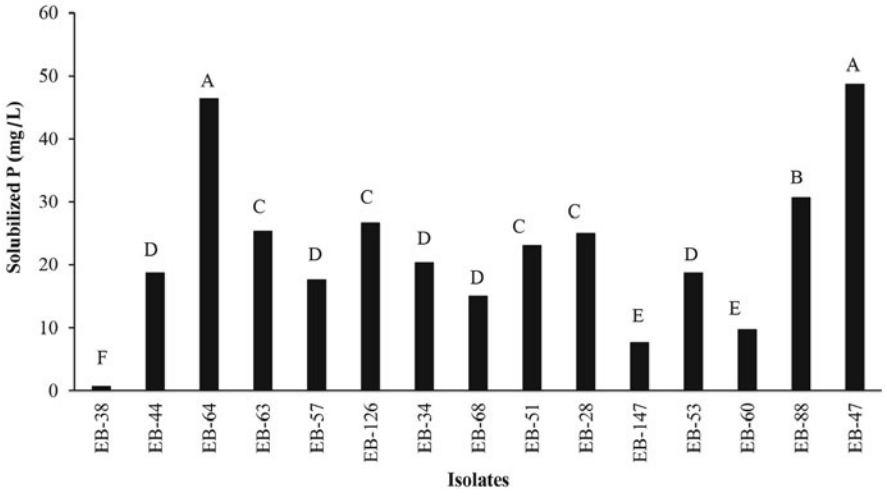

Fig. 2. Calcium phosphate solubilization by endophytic bacteria isolate from banana tree roots of the cultivar Prata Anã in NBRIP liquid culture medium. Means followed by different letters differ by the Scott-Knott test ($P<0.05$).

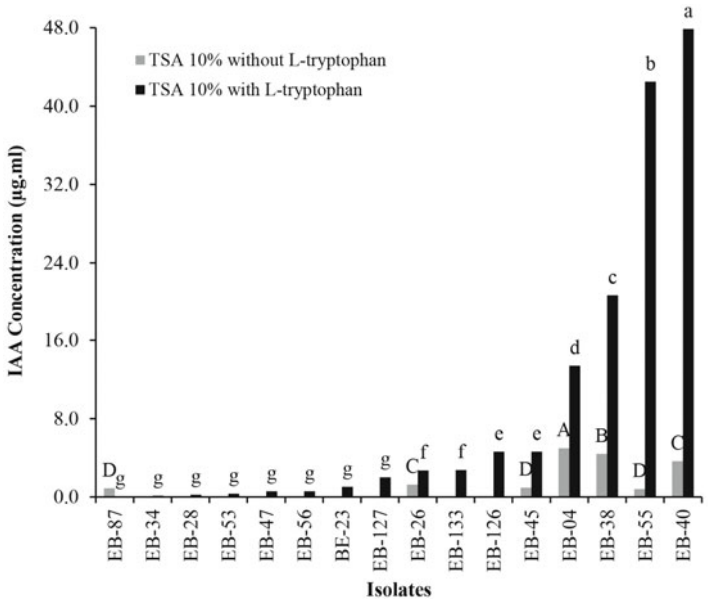

Fig. 3. Indole-3-acetic acid concentration (µg/ml) in TSA 10% medium, with and without L-tryptophan, by endophytic bacteria. The bars marked by different upper and lower case letters differ from one another, as determined by the Scott-Knott test ($P<0.05$).

The bacterial suspensions were inoculated into culture medium supplemented with 100 µg/ml of tryptophan and incubated for 48 h at 28°C under constant shaking (120 rpm) in the dark. Subsequently, 2.0 ml of Salkowski reagent was added (Sarwar and Kremer, 1995). The test tubes were incubated for 30 min in the dark. A reading was performed at 530 nm on a spectrophotometer. To obtain a standard curve, the absorbances of known concentrations of commercial IAA were determined. The production of a reddish-pink color in the samples indicated the production of IAA. For the isolates that were able to synthesize IAA in TSA medium supplemented with 10% L-tryptophan, they were next inoculated in TSA medium without L-tryptophan to test whether they could use an alternative pathway without tryptophan. The tests were performed in a randomized design, with 40 treatments and three repetitions. The IAA production levels were analyzed, and the results were submitted to variance analysis with the statistical software Sisvar (Ferreira, 2008).

Results

Analysis of the ability of the isolates to grow in nitrogen-free culture medium and fix nitrogen

Among the 24 isolates used in the nitrogen fixation assay, 83% were able to grow and form a pellicle in the NFb culture medium (Table 1). One isolate from the species *Bacillus thuringiensis* (EB-24), two isolates that belong to the genus *Bacillus* sp. (EB-38 and EB-71) and one isolate from the genus *Paenibacillus* sp. (EB-28) did not grow in the nitrogen-free medium. Among the twenty isolates that did grow in the semi-solid nitrogen-free culture medium, only EB-04, EB-64, EB-144, and EB-169 were able to fix nitrogen. EB-04 (*Bacillus subtilis*), EB-64 (*Bacillus pumilus*) and EB-169 (*Bacillus pumilus*) had low rates of N fixation as measured by the Kjeldahl method, producing 15, 9 and 15 µg/N/ml, respectively. Low levels of N fixation were measured with the ARA assay for EB-04, EB-144 (*Paenibacillus* sp.) and EB-169, which produced 12.2, 0.2, and 6.0 nmol C_2H_4/h/vial, respectively.

Screening of endophytes and quantification of inorganic phosphate solubilization

Of the 40 isolates analyzed, 37.5% were capable of solubilizing inorganic phosphate (Table 1). Different species that belong to the genus *Bacillus* were able to solubilize insoluble calcium phosphate, including *Bacillus axarquienses*, *B. pumilus*, *B. amyloliquefaciens*, *B. safensis*, *B. flexus*, and *B. subtillis*. Two isolates that belong to genus *Lysinibacillus* sp. and one isolate of genus *Paenibacillus* sp. were also able to solubilize phosphate.

There was variability in the colony and halo diameters in the phosphate solubilization assay. The largest colony was formed by isolate EB-68 (*B. safensis*), with an average diameter of 5.8 mm, while the smallest colony was observed for isolate EB-53 (*Lysinibacillus* sp.), with an average diameter of 1.9 mm. Isolate EB-51 (*B. pumilus*) had an average halo diameter of 7.4 mm, whereas isolate EB-28 (*Paenibacillus* sp.) had an average halo diameter of 14.4 mm (Fig. 1).

The isolates analyzed presented solubilization indexes ranging from very low solubilization [0.42 by isolate EB-126 (*B. subtilis*)] to high solubilization (3.56 by isolate EB-53 (*Lysinibacillus* sp.) (Table 2).

The 15 bacterial isolates evaluated showed distinct solubilization potentials. At the end of the incubation time in the liquid NBRIP culture medium the pH ranged from 4.6 (isolate EB-64) to 5.7 (isolates EB-38 and EB-63) (data not shown). The production of soluble phosphate ranged from 0.67 mg/L (EB-38) to 48.66 mg/L (Fig. 2).

Indole-3-acetic acid (IAA) production

The production of IAA in TSA medium supplemented with 10% L-tryptophan was observed in 40% (16) of the bacte-

rial isolates, and seven of these isolates were capable of producing IAA by an alternative pathway in TSA 10% medium without L-tryptophan (Table 1). Of the 16 isolates, 11 belong to the genus *Bacillus* sp., two belong to the genus *Lysinibacillus* sp., and one each belong to the genera *Paenibacillus* sp., *Sporolactobacillus* sp., and *Klebsiella* sp. (Table 1).

Isolates of the genera *Bacillus* sp. (EB-38 and EB-40) and *Lysinibacillus* sp. (EB-45) and the species *B. methylotrophicus* (EB-26), *B. subtilis* (EB-04 and EB-55) and *B. tequilensis* (EB-87) were able to synthesize IAA in media with and without L-tryptophan.

The isolates synthesized a wide range of IAA in TSA medium 10% supplemented with L-tryptophan. The isolate from the genus *Bacillus* sp. (EB-40) produced the highest IAA concentration, 47.88 µg/ml, whereas the isolate from the specie *B. tequilensis* (EB-87) produced the lowest IAA concentration, 0.05 µg/ml (Fig. 3). Among the isolates that synthesized IAA in TSA medium 10% supplemented with L-tryptophan, approximately 44% synthesized amounts less than 2.0 µg/ml, including isolates from the genera *Bacillus* sp. (EB-47 and EB-56), *Lysinibacillus* sp. (EB-53), *Paenibacillus* sp. (EB-28) and the species *K. pneumoniae* (EB-23), *B. pumilus* (EB-34), and *B. tequilensis* (EB-87).

Variation in the synthesis of IAA among the isolates cultivated in TSA 10% medium without L-tryptophan was also observed. The synthesis of IAA ranged from 0.80 µg/ml (isolate EB-55 [*B. subtilis*]) to 4.39 µg/ml (isolate EB-04 [*Bacillus* sp.]) (Fig. 3).

Discussion

The isolates of endophytic bacteria analyzed in the present study had different features and were capable of several biochemical reactions that indicate their potential use for growth promotion in banana trees.

The existence of different genera of nitrogen-fixing diazotrophic bacteria in banana trees have been reported by several groups (Weber *et al*., 1999; Cruz *et al*., 2001; Martinez *et al*., 2003). In this study, five different genera, *Bacillus* sp., *Klebsiella* sp., *Lysinibacillus* sp., *Paenibacillus* sp., and *Sporolactobacillus* sp., were able to form a pellicle in semi-solid N-free culture medium, and among these, three species, *B. subtilis*, *B. pumilus*, and *Paenibacillus* sp., were capable of fixing N. Despite the fact that all the isolates analyzed in this work contained *nifH* (Souza *et al*., 2013), we observed only a low level of N fixation.

nifH is considered to be an important marker in diazotrophic bacteria, and is a highly conserved region in the genome of these bacteria (Rosado *et al*., 1999). However, biological nitrogen fixation is a complex process that requires the expression of the *nif* regulon, which encodes several proteins involved in the nitrogen fixation process and regulates their expression (Teixeira, 1997). The high number of the genes in the *nif* regulon may explain the fact that four isolates did not form a pellicle in NFb medium and had low levels of N fixation, as detected by the ARA assay and the Kjeldahl method.

Seven different species in the genus *Bacillus* sp. were capable of growing in NFb medium. Teixeira *et al*. (2007) identified 47 different species of endophytic bacteria associated with *Manihot esculenta* L. and representatives of the species *B. cereus*, *B. pumilus*, and *B. megaterium* were capable of growing in NFb culture medium. Studies performed with *Zea mays* also showed that bacteria from the genera *Bacillus* sp., *Klebsiella* sp., and *Paenibacillus* sp. were capbale of growing in NFb medium, which indicated that they were diazotrophic bacteria (Cerigioli, 2005; Ikeda, 2010). Studies performed by Martinez *et al.* (2003) demonstrated more diversity, compared to previous studies and higher growth-producing potential of diazotrophs associated with banana trees. Four different genera (*Enterobacter* sp., *Citrobacter* sp., *Klebsiella* sp., and *Rhizobium* sp.) were identified, and some were able to fix high amounts of N.

Our results indicate that it is possible to have several diazotrophic bacteria present in banana tree roots, though the isolates that were analyzed had low rates of N fixation. Tests that are more specific must be conducted to confirm the effectiveness of the isolates at providing a nitrogen supply for banana trees.

The occurrence of endophytic bacteria with the ability to solubilize inorganic phosphate has been known since the beginning of the 20th century (Kucey, 1988). Since then, several studies have been performed to isolate and identify these micro-organisms and to clarify the mechanisms underlying the solubilization of P (Barroso *et al*., 2001; Inui, 2009; Silva *et al*., 2011). To our knowledge, this is the first report detailing an association between banana tree roots and endophytic bacteria with the ability to solubilize phosphate.

In the present work, six different species that belong to the genus *Bacillus* sp., two isolates from the genus *Lysinibacillus* sp. and one isolate from the genus *Paenibacillus* sp. were capable of solubilizing calcium phosphate.

Similar variations in the phosphate solubilization index were observed in earlier reports. Fifteen isolates of endophytic bacteria and 15 isolates of rhizobacteria isolated from sugar cane had varying scores in the phosphate solubilization index, ranging from 1.00 to 2.33 in endophytic bacteria and from 1.21 to 3.48 in rhizobacteria (Santos *et al*., 2012). All isolates used in the present study were collected from banana tree roots. Barea *et al*. (2005) reported that phosphate-solubilizing micro-organisms that live in the rhizosphere are, in most cases, metabolically more active than isolates from other niches. This difference occurs because the rhizosphere is exposed to an abundance of carbon sources in the soil due to root exudation (Taurian *et al*., 2010).

The analytical method to determine the solubilization of calcium phosphate used in the present study showed the relation between pH and amount of solubilized P. The decreased in pH of the culture medium is probably caused by production of organic acids by the bacteria. Despite the isolate EB-64 (*Bacillus* sp.) had been classified as low efficiency of solubilization according to the scale proposed by Silva Filho and Vidor (2000) it was characterized by one of the most promising isolate. Previous studies report high variations in efficiency of P solubilization by endophytic bacteria (Baig *et al*., 2011; Ribeiro and Cardoso, 2012). According to Gyaneshwar *et al.* (2002) most phosphate-solubilizing microorganisms solubilize Ca-P complexes, and only a small

number can solubilize Fe-P and Al-P complexes. Cunningham and Kuiack (1992) suggested that other components of the medium may affect the ability of the microorganisms to solubilize phosphate, such as the source of sugar and the nature of the source of N that are added to the culture medium.

The synthesis of phytohormones by endophytic bacteria is known to directly act on plants to promote growth (Lodewyckx *et al*., 2002). Indole-3-acetic acid is one of the most studied phytohormones and, according to several authors, is capable of promoting increases in root size and distribution, thus resulting in a higher absorption rate of nutrients and water from the soil (Lee *et al*., 2004; Sobral *et al*., 2004).

Among the isolates analyzed, we found that isolate EB-40, of the genus *Bacillus* sp., produced the most IAA (47.88 µg/ml). This isolate was also capable of growth in N-free culture medium. Only two out of the 40 isolates studied, EB-126 (*B. subtilis*) and EB-47 (*Bacillus* sp.), were able to grow in N-free culture medium, solubilize phosphate and produce auxin in TSA 10% medium supplemented with L-tryptophan. These isolates should be studied further and, if their potential to aid in banana tree growth is confirmed, they can be utilized as inoculants in nurseries growing banana suckers. Approximately 25% of the isolates were able to form a pellicle in the semi-solid nitrogen-free medium and synthesize IAA, and 17.5% were able to both produce IAA and solubilize phosphate.

Several species of the genus *Bacillus* known to support plant growth by synthesizing auxins, mainly indole-3-acetic acid, solubilizing insoluble phosphates, producing siderophores or fixing atmospheric nitrogen (Tsavkelova *et al*., 2006; Araujo *et al*., 2012; Luo *et al*., 2012; Phetcharat and Duangpaeng, 2012).

The results of this study demonstrate that the isolates analyzed had varying abilities to perform nitrogen fixation, solubilize phosphate, and produce IAA. In light of these results, it is clear that additional studies should be performed to further examine the potential of these micro-organisms to be used to promote plant growth in banana trees by introducing these isolates in greenhouses.

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