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Bioprospecting of aerobic endospore-forming bacteria with biotechnological potential for growth promotion of banana plants

Luisa F. Posada[a,b], María Ramírez[c], Natalia Ochoa-Gómez[a,b], Tatiana Z. Cuellar-Gaviria[a], Luz Edith Argel-Roldan[d], Camilo A. Ramírez[c], Valeska Villegas-Escobar[a,*]

[a] Research Group CIBIOP, Process Engineering Department, Universidad EAFIT, Colombia
[b] Universidad Nacional de Colombia, Sede Medellín, Colombia
[c] Institute of Biology, Universidad de Antioquia, Colombia
[d] Banana Research Center, AUGURA, Colombia

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ABSTRACT

High amounts of agrochemicals are regularly used for increasing yields in cultivation of banana. The use of plant growth-promoting rhizobacteria (PGPR) could represent an environmentally friendly alternative that can improve productivity. In this study, we tested a funnel-like strategy to bioprospect aerobic endospore-forming bacteria (AEFB) that could be useful to develop a biotechnological product to promote the growth of banana plants. First, 837 aerobic endospore-forming bacteria were obtained from the rhizosphere of banana and plantain. Then, the isolates were assessed for both: their capacity to promote growth of maize seedlings (used as a model plant) and to display specific biochemical PGPR-associated traits. Twenty-two of these strains significantly increased the dry weight of maize seedlings, some of them showing *in vitro* PGPR traits. Based on this screening, four isolates were selected to conduct evaluations on banana plants, from which, the bacterial strain *Bacillus subtilis* EA-CB0575 was chosen as a promising plant growth-promoting isolate. Further studies with this strain showed that the application of either spores, vegetative cells (both at concentrations 1×10^7 and 1×10^8 CFU/mL), or the cell-free supernatant (CFS) of its fermentation significantly increased the dry weight of banana plants, compared with the non-treated control. Our results suggest that both cellular structures of *B. subtilis* EA-CB0575 and the metabolites and/or elements contained in its CFS enhance the growth and development of banana plants.

1. Introduction

Current global trends in agriculture call for sustainability. This encourages the search of beneficial microorganisms, so that bioprospecting projects are requiered. The use of plant growth-promoting rhizobacteria (PGPR) to develop plant biostimulants that can be implemented in commercial agriculture has gained interest (Calvo et al., 2014). Specifically, those PGPR with capacity to form endospores (*i.e.* aerobic endospore-forming bacteria, AEFB), which mostly belong to the genus *Bacillus,* have mainly been studied for their potential to be formulated in stable commercial products, with low requirements for storage (Pliego et al., 2011; Setlow, 2006) As for other PGPR, isolates in this group produce biologically-active metabolites with capacity to promote plant growth, such as lipopeptides, siderophores, organic acids, and phytohormones among others (Bhattacharyya and Jha, 2012; Kumar et al., 2011).

The response to PGPR inoculation on different crops has been largely evaluated, showing significant increases in growth and yields (Bhattacharyya and Jha, 2012). However, most of these studies have been performed on annual plants such as rice, tomato, corn, or wheat (Adesemoye and Kloepper, 2009; Hayat et al., 2010), while a lack of knowledge exists for perennial plants such as citrus, apple, banana, cocoa, pine, among others (Jaizme Vega et al., 2004; Karlidag et al., 2007; De Vasconcellos Figueiredo and Bran Nogueira, 2009). Banana is the world's most popular fruit and the third most important crop in Colombia (FAO, 2013; Arias et al., 2004) In order to reduce the intensive use of agrochemicals, this crop is demanding implementation of sustainable practices, such as the use of PGPR (Mia et al., 2010a). Inoculation of rhizobacteria on banana has shown to promote early growth and improve nutrient uptake, which has been correlated with an enhanced growth at latter stages of development and better yields (Jaizme Vega et al., 2004; Kavino et al., 2010; Mia et al., 2010b). However, those studies have not

* Corresponding author.
E-mail address: vvilleg2@eafit.edu.co (V. Villegas-Escobar).

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provided strategies for prospecting of new effective isolates nor have explored the real potential of those strains to be formulated in commercial products. Indeed, difficulties are expected at this latter aspect, given that many of the strains included in those studies are non-sporulating and, therefore, hard to stabilize.

Although PGPR represent and alternative for growth promotion of perennial crops, such as banana, testing large numbers of isolates directly *in planta* results unfeasible. Hence, different strategies of prospecting have been used, such as the evaluation of *in vitro* PGPR traits, followed by greenhouse and field experiments (Dinesh et al., 2015; Walia et al., 2014; Zahid et al., 2015). Based on this strategy, Penrose and Glick (2003) used a rapid procedure for the isolation of 1-aminocyclopropane-1carboxylate (ACC) deaminase-containing bacteria, which were then evaluated in a root elongation assay. The authors showed that *Pseudomonas putida* isolates with the capacity of producing ACC deaminase promoted root growth in canola plants. However, strains exclusively selected on their performance *in vitro* often fail to prove effective when tested *in planta* (Ahmad et al., 2008; Agaras et al., 2015). Thus, an approach for selecting potential candidates, involving extensive evaluation *in planta*, is still required (Cattelan et al., 1999; Ahmad et al., 2008; Hynes et al., 2008; Thokchom et al., 2014).

To obtain a PGPR-based product useful for growers, in addition to reach a stable product with a shelf life long enough to be commercialized, there are other aspects to be considered. For instance, determination of the active ingredient and its proper concentration to promote plant growth is highly important (Bai et al., 2002; Jetiyanon et al., 2008; Kloepper et al., 2000; Ramírez and Kloepper, 2010). For PGPR-based products, the active ingredient could be associated with the type of cell structure and/or the metabolites produced by microbial cells. In the specific case of products based on AEFB, the majority of commercial products use spore cells, given that they provide long shelf life, discarding vegetative cells and the cell-free supernatant (CFS) (Buensanteai et al., 2013). However, these two fermentation by-products contain metabolites and nutrients that could also promote plant growth and, if so, along with spore cells, increase effectiveness and/or more reproducible effects in the field (Strigul and Kravchenko, 2006). Likewise, most of the biostimulants commercialized today usually use a standard inoculum concentration (10^8 CFU/mL) (Kloepper et al., 2007; Kumar et al., 2010), but when developing of biotechnological products it is always necessary to stablish an effective and precise dose, that give optimal results (Strigul and Kravchenko, 2006).

This study shows a bioprospecting strategy by which we found different AEFB with the potential to promote the growth of banana plants, when inoculated at early stages of development. The biotechnological potential of these strains was further studied by determining their effective inoculum concentration and the contribution of each component of their fermentation process to the plant growth promoting effect.

2. Materials and methods

2.1. Microorganisms

AEFBs (837 isolates) were isolated from the rhizosphere of banana and plantain plants in Uraba Antioquia in 2009, stored in TSB (Tripticase Soy Broth, Merck) plus 20% v/v glycerol at −80 °C (Humboldt Institute Collection No. 191) and activated in TSA (Merck) for 24 h at 30 °C before any experimental use. *B. subtilis* EA-CB0575, was isolated from the rhizosphere of a banana plant (cv. Valery) and identified by analysis of 16S rDNA gene sequencing (Genbank Accession Number KC170988). *Ralstonia solanacearum* EAP-009 and *Serratia marcescens* EAD-005 were isolated from infected corm tissue of a banana plant, stored in BG medium (Clough et al., 1994) with 20% glycerol at −80 °C and identified by analysis of 16S rDNA gene sequence (Genbank Accession Number KU603426 and KU603427 respectively). *Fusarium oxysporum* EAP-004 was isolated from infected tissue of carnation, identify by analysis of the internal transcribed spacer region-ITS and stored in filter papers (Schleicher & Schuell, No. 595, 4 μm) at 4 °C.

2.2. Isolation of aerobic endospore-forming bacteria

Samples were collected from roots of three *Musa* spp. cultivars (banana cv. Grand Naine and banana cv. Valery which belongs to AAA genome group and subgroup Cavendish, and plantain cv. Harton which belongs to the AAB genome group) in Urabá, Colombia (Northeastern region). These were taken from 3 banana plantations: Augura experimental field station (Plantation 1, 07°46′46″N, −76°40′0.2″W), La Navarra (Plantation 2, 07°51′58.6″N, −76°37′39″W), and El Aserrío (Plantation 3, 07°52′25.2″N, −76°38′0.26″W). For banana cv. Grand Naine, root samples were obtained from plantations 1, 2, and 3; for cv. Valery, from plantations 1 and 4; and for plantain cv. Harton, from plantations 1 and 5. At each plantation, composite root samples of three adjacent plants (at pre-flowering stage) were collected at 5–10 cm depth, stored in propylene bags at 4 °C, and processed within 24 h. Root sampling was performed in 2009.

Isolation of AEFB was carried out by suspending 40 g of roots with adhering rhizosphere soil in 400 mL of sterile sodium phosphate buffer (0.1 M, pH 7.0) and Tween 80 (Panreac, 0.1% v/v), sonicating the suspension for 10 min in an ultrasound cleaner (Bransonic 52, 20 Hrz). Suspensions were then placed on an orbital shaker (200 rpm, 28 °C) for 1 h and homogenized in a blender for 15 s. Suspensions were serially diluted and heat treated (80 °C, 20 min), and then, 100 μL of each dilution were plated on Trypticase Soy Agar (TSA, 50%). Different morphotypes were selected, purified, and stored at −80 °C in Trypticase Soy Broth (TSB) with glycerol (20% v/v). Before experimental use, each isolate was first grown on 50% TSA (48 h, 30 °C).

2.3. Screening for PGPR-associated biochemical traits and growth promotion on maize seedlings

Given that massive *in planta* tests on banana are unfeasible, a funnel-like strategy of selection was outlined. The first phase included the screening of the entire collection of AEFB (837 isolates) for production of indolacetic acid (IAA) and antagonism against frequent root pathogens (in our experience, two *in vitro* traits commonly associated with plant growth promotion). Production of IAA was determined by the colorimetric technique using Salkowski's reagent as described by Patten and Glick (2002), while antagonism was evaluated against *Fusarium oxysporum* EAP-004 and *Serratia marscesens* EAD005 by using the dual-culture plate method previously described by Lemessa and Zeller (2007). Those strains that were antagonist of *S. marcensens* were also tested against *R. solanacearum* EAP-009 by the same methodology.

In addition, the whole collection of isolates was tested *in planta*, using maize (*Zea mays* ICA V-109) as a model plant (a monocot, like banana). Seeds were surface-sterilized by immersion in 70% ethanol for 1 min and 1% NaOCl for 10 min, followed by five rinses with sterile distilled water. Next, seeds were incubated for pre-germination on sterile damp paper for 3 days. Pre-germinated seeds were soaked in the corresponding bacterial suspension (1×10^8 CFU/mL), according to the treatments, and transplanted into plastic containers with 500 g of substrate (a mixture of soil, sand, compost, and rice husk in a ratio of 1:1:0.5:0.5, Tahamí-Cultiflores S.A.), with 6 replicates per treatment. Containers were taken to the greenhouse and, 15 days after planting, shoot length was assessed

visually, identifying those strains for which it was evidently higher than that of the non-inoculated control.

A second phase of selection included the evaluation of 94 outstanding isolates in a new maize test, evaluating 12 to 16 strains at a time, with 9 replicates per treatment. This time, plant growth was determined by measuring the shoot and root dry weight, also at 15 days after planting, and ANOVA tests were conducted. From the latter evaluation, 22 isolates were selected and subjected to one additional test on maize (third phase of evaluation), in order to confirm the reproducibility of their effect. Here, the same number of replicates, time of growth, response variables, and data analysis were used. In addition, some other *in vitro* PGPR-related traits (not determined in the first phase) were tested on these 22 strains: solubilization of phosphate (performed on phosphate rock medium; Kim et al. (1997), modified by Osorio (2008)), production of siderophores (colorimetric determination using CAS solution; Schwyn and Neilands, 1987), and nitrogen fixation (growth on semisolid medium NFb; Dobereiner, 1998). From these last tests, 4 isolates were selected for further studies on banana plants.

2.4. 16S rDNA identification

The 22 AEFB isolates tested in the third phase were identified according to their 16S rDNA sequence. For this, total DNA was isolated from each strain by using the UltraClean® Microbial DNA Isolation Kit (MO BIO laboratories, Inc), and a ≈1400-bp fragment of the 16S rDNA gene was amplified by PCR with the universal bacterial primers 8F/1492R (Hendrickson et al., 2002). PCR amplification was performed with GoTaq® Flexi DNA polymerase (Promega, Madison WI, 53711), as recommended by the manufacturer (0.25 μM of each primer). The conditions for PCR included an initial denaturation at 94 °C for 3 min, followed by 31 cycles of 94 °C for 1 min, 57 °C for 45 s, and 70 °C for 8 min, with a final extension step at 70 °C for 8 min. Purification of PCR products and sequencing was performed by Macrogen Inc. Geumchun-gu (Seoul, South Korea). The sequences were manually edited and aligned, and the consensus sequence was computed using Clustal W. Then, the consensus sequence was submitted for (nucleotide) BLAST analysis at the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), determining the bacterial species as the best match.

2.5. Growth promotion evaluations on banana plants

Tests on banana plants included 3 different experiments. The first experiment served as final phase in our funnel-like strategy, being conducted to determine which of the 4 selected strains from the maize tests promoted growth of banana plants. On the other hand, the second and third experiments aimed at determining the active components of a PGPR-based fermentation product for plant growth promotion. More precisely, the second experiment was done to determine the effect of concentration and type of cellular structure of *B. subtilis* EA-CB0575 on growth promotion, while the third experiment aimed at testing if the CFS of a culture of such strain could promote plant growth. In each of these experiments, roots of 8-week old banana plants cv. Williams were submerged in the different treatments, transplanted into 3 kg pots filled with substrate mixture and kept in greenhouse conditions for 4 months. Plants were fertilized monthly with NPK and foliar fertilizer (Wuxal®).

2.5.1. Experiment 1

A complete randomized design with 30 plants per treatment was set up. The bacterial strains *B. cereus/thuringiensis* EA-CB0131, *B. subtilis* EA-CB0575, *B. megaterium* EA-CB0784, and *B. subtilis* EA-CB1121 were mixed with a talc-based formulation (Saharan et al., 2011) or with pesta-based formulation (Connick et al., 1993) at a concentration of 1×10^9 CFU/g, and then diluted in water (1:10 w/v) to reach a concentration of 1×10^8 CFU/mL. Application of sterile distilled water and talc formulation (with no bacteria) were used as controls. Roots of 8-week old banana plants cv. Williams were submerged in the different treatments, transplanted into 3 kg pots filled with substrate mixture (1 sand: 0.5 soil: 0.5 rice husk), kept in greenhouse conditions (29–33 °C, watered every other day) and re-inoculated monthly by applying as a drench 20 mL of the corresponding treatment for 3 months.

Four months after inoculation, 10 randomized plants per treatment were harvested to determine the effect of each bacterial inoculum on plant growth by determining shoot length (distance from the base of the corm to the intersection of leaves 1 and 2), as well as fresh and dry weights of shoots and roots. The other 20 plants were submerge in the corresponding treatment for 1 h and then transplanted to the field (Cenibanano Experimental Field). In the field, the experiment was conducted using a completely randomized design with 4 plots per treatment, and each plot with 5 plants. The treated plants were located in the middle of banana plants that were used as border plants. The border plants were not treated to avoid mixing effects. The shoot length, the harvesting time for bunch production as well as the weight, number of hands and number of fingers of the bunch were determined. All plants were subject to the fertilization and fumigation program routinely used in the experimental field.

2.5.2. Experiment 2

Here, a complete randomized design, with 10 plants per treatment, was followed to determine the effect of the application of two different cell structures of *B. subtilis* EA-CB0575 (vegetative cells and spores) at 4 different concentrations (1×10^5, 1×10^6, 1×10^7, and 1×10^8 CFU/mL) on banana growth. Roots of 8-week old banana plants cv. Williams were submerged in the different treatments, transplanted into 3 kg pots filled with substrate mixture (1 sand: 0.5 soil: 0.5 rice husk), kept in greenhouse conditions (29–33 °C, watered every other day) and re-inoculated monthly by applying as a drench 20 mL of the corresponding treatment for 3 months. Pots were sowed and treated as described in experiment 1. The growth-promoting effect of the bacterial inoculation was determine by measuring shoot length (cm) and total dry weight (g). This experiment was conducted twice, but only concentrations 1×10^7 and 1×10^8 CFU/mL of both cell structures of *B. subtilis* EA-CB0575 were evaluated in the second test. Each experiment was independently analyzed by analysis of variance (ANOVA) and LSD multiple comparison tests.

2.5.3. Experiment 3

For this, a complete randomized design with 11 plants per treatment was used. Four treatments were tested: 1) CFS from TSB culture (CFS-V), 2) CFS from SBM culture (CFS-S), 3) fresh sterile broth TSB (TSB), and 4) fresh sterile broth of Sporulation *Bacillus* Medium (SBM) (Posada Uribe et al., 2015). Application of sterile distilled water was used as a control. Roots of 8-week old banana plants cv. Williams were submerged in the different treatments, transplanted into 3 kg pots filled with substrate mixture (1 sand: 0.5 compost: 1 soil: 0.5 rice husk) and kept in greenhouse conditions (22–33 °C, 12 h light/12 h dark, 80% of maximum water-holding capacity) for 4 months. The growth-promoting effect of the CFS was performed as described above for experiment 2.

2.6. Preparation of cell suspensions and cell-free supernatants of B. subtilis *EA-CB0575*

Briefly, 50 mL of an overnight culture of *B. subtilis* EA-CB0575 was inoculated in a 5000 mL Erlenmeyer flask containing 550 mL of either SBM (Sporulation *Bacillus* Medium) medium for spore

production (Posada Uribe et al., 2015) or TSB medium for vegetative-cell production (Trypticase Soy Broth, Merck) and incubated at 30 °C and 150 rpm. For spore-cell suspension, cells were recovered by centrifugation (3.170 × *g*, 20 min) after 72 h of growth, and the pellet was washed twice with distilled water and resuspended in sterile distilled water. Then, the spore-cell suspension was heat treated (80 °C, 20 min) and its concentration adjusted with sterile distilled water. For the vegetative-cell suspension, cells were recovered by centrifugation (3.170 x *g*, 20 min) after 24 h of incubation, the pellet was washed twice with sterile distilled water and the concentration was adjusted with sterile distilled water. On the other hand, to obtain the CFS, the supernatants obtained after centrifugation of each bacterial culture were filtrated twice through 0.2 μm cellulose acetate filters (Sartorius Biolab). CFS obtained from the production of vegetative cells in TSB was denoted CFS-V and that from the production of spores in SBM was denoted CFS-S.

2.7. Statistical analysis

Analysis of variance (ANOVA) by fitting a linear model was used to analyze each experiment; in all cases, as a complete randomized design. For this, StatGraphics Centurion XVI Version 16.1.18 (Statpoint Technologies Inc., Virginia; USA) and R (function aov), were used. Normality and homocedasticity of residuals from all response variables were ensured by testing them using Kolmogorov ($P > 0.05$) and Levene tests ($P > 0.05$), and checking their graphical distributions by the function plot of R. In case of significant P value ($P < 0.05$), means were compared by using Dunnett and LSD multiple comparison tests.

3. Results

A funnel-like strategy was successful as a bioprospecting tool to select PGPR in banana plants

A collection of 837 isolates was obtained from the three *Musa* spp. cultivars (banana cv. Grand Naine, banana cv. Valery, and plantain cv. Harton). From these isolates, 67% (562 strains) produced IAA at concentrations between 1.9 and 60.0 μg/mL, while only 18.3% (153 isolates) and 3.2% (27 isolates) inhibited the growth of *F. oxysporum* EAP-004 and *S. marcescens* EAP-005 *in vitro,* respectively (Supplementary material, Table A.1). Likewise, the *in planta* test using maize as a model plant, showed that 94 isolates, out of the 837 tested, seemed to promote plant growth as plant inoculated with them were visually bigger than those not inoculated (Supplementary material, Table A.1).

As for the second phase of selection, 22 isolates, out of the 94 selected from phase 1, promoted growth of maize ($P < 0.05$; data not shown). Those 22 isolates were confirmed to promote maize growth in the third phase of selection, as all of them significantly increased the shoot and/or root dry weights (9–45% with respect to the non-inoculated control; Table 1). These strains displayed a range of features commonly suggested to contribute to plant growth promotion (Table 2). All of them produced IAA (ranging from 1.9 to 48.0 μg/mL), 55% (12 strains) produced siderophores, 23% (5 strains) solubilized phosphate and 59% (13 strains) were potential nitrogen fixers. Only one strain (*B. amyloliquefaciens* EA-CB0158) showed antibacterial activity against *S. marcescens* and *R. solanacearum*, whereas all *B. subtilis* and *B. amyloliquefaciens* strains inhibited the growth of *F. oxysporum*. Based on their 16S rDNA sequence, 40.9% (9 strains) were identified as *B. pumilus*, 13.6% (3 strains) as *B. cereus/thuringiensis*, 13.6% (3 strains) as *B. subtilis*, 9.1% (2 strains) as *B. megaterium*, 9.1% (2 strains) as *B. stratosphericus*, 4.5% (1 strain) as *B. amyloliquefaciens*, 4.5% (1 strain) as *B. altitudinis*, and 4.5% (1 strain) as *B. gibsonii* (Table 2).

In the final (fourth) phase of selection, strains *B. cereus/thuringiensis* EA-CB0131, *B. subtilis* EA-CB0575, *B. megaterium* EA-CB0784, and *B. subtilis* EA-CB1121, selected based on their performance on maize and their biochemical traits, were tested on banana plants under greenhouse conditions. All 4 strains promoted at least one growth variable, when compared with the controls (water, talc-based formulation and pesta-based formulation) (Table 3 and 4). Nonetheless, strains EA-CB0575 and EA-CB0784 had a greater effect, as they significantly increased between 2 and 4 out of the 5 plant variables evaluated in Greenhouse. Specifically, significant increases were found in fresh and dry weights of root and in dry weight of shoots for *B. subtilis* EA-CB0575 (Tables 3 and 4), while length, dry weights of shoot and root, and fresh weigh of root showed significant increases for *B. megaterium* EA-CB0784 compared with the formulated control (Tables 3 and 4). In field conditions, strains *B. subtilis* EA-CB1121 in talc-based formulation (Table 3) and *B. subtilis* EA-CB0575 in pesta-based formulation (Table 4) reduced the harvesting time for bunch production in approximately 9.0% in comparison with the formulated control. Additionally strain EA-CB0575 in talc-formulation (Table 3) and strain EA-CB1121 in pesta-based formulation (Table 4) increased significantly the shoot length of banana plants in comparison with the formulated control. The other field variables: bunch weight, number of hands and number of fingers were not affected by any strain in any type of formulation (data not shown). Based on its biochemical traits, *B. subtilis* EA-CB0575 was selected for further analysis, given that it produced less IAA (an excessive production of this phytohormone has been linked to deleterious root effects; Nehl et al., 1997). Moreover, this strain produced greater amounts of siderophores and had antagonist activity against *F. oxysporum*, a trait that EA-CB0784 did not display.

B. subtilis *EA-CB0575 cells and the cell-free supernatant are potentially active ingredients for a biotechnological product*

Both spores and vegetative cells significantly increased shoot length and total dry weight of banana plants, in a concentration-dependent manner (Fig. 1). Vegetative cells at a concentration of 1×10^7 CFU/mL increased shoot length of banana plants by 31% (Fig. 1A), whereas concentrations of 1×10^7 and 1×10^8 CFU/mL increased the dry weight of plants by 75 and 41% respectively (Fig. 1B). For spores, those increases were 16, 17, and 20% for shoot length at concentrations of 1×10^6, 1×10^7 and 1×10^8 CFU/mL respectively (Fig. 1A), and 37% on average for dry weight (Fig. 1B). Neither spores nor vegetative cells at 1×10^5 CFU/mL affected shoot length and total dry weight of the plants. Consistently, when this experiment was repeated for concentrations of 1×10^7 and 1×10^8 CFU/mL of both vegetative and spore cells, shoot length and total dry weight also increased (data not shown).

Application of both CFS (CFS-V and CFS-S) and fresh culture SBM medium significantly increased the shoot length and total dry weight of banana plants, when compared to the non-treated plants (control) (Fig. 2). In average CFS-V, CFS-S and SBM increased shoot length of banana plants by 20% with no significant differences between them (Fig. 2A); whereas SBM had the highest effect on total dry weight, showing increments of 27% when compared to non-treated plants followed by CFS-V and CFS-S which both showed a 15% increment (Fig. 2B).

4. Discussion

Here, we present a successful strategy for prospecting PGPR suitable for developing products to promote growth of banana. This contributes not only to increase the number of biofertilizers avail-

Table 1
Effect of 22 potential PGPR strains on shoot and root dry weight of maize seedlings in greenhouse 15 days after sowing.

Strain	Bacterial species[a]	Shoot		Root	
		Dry weight (g)	Increase (%)	Dry weight (g)	Increase (%)
Trial 1 Maize					
EA-CB0009	*B. pumilus*	0.48 ± 0.05[b] bc[c]	16	0.34 ± 0.06 ef	20
EA-CB0012	*B. cereus/thuringiensis*	0.43 ± 0.02 c	9	0.36 ± 0.04 ef	25
EA-CB0070	*B. pumilus*	0.48 ± 0.02 bc	16	0.42 ± 0.03 c	36
EA-CB0083	*B. cereus/thuringiensis*	0.49 ± 0.02 b	18	0,43 ± 0.03 c	37
EA-CB0131	*B. cereus/thuringiensis*	0.44 ± 0.03 c	11	0.34 ± 0.02 f	20
EA-CB0158	*B. amyloliquefaciens*	0.45 ± 0.06 c	11	0.33 ± 0.11 f	18
EA-CB0177	*B. pumilus*	0.53 ± 0.01 b	26	0.47 ± 0.03 a	42
EA-CB0185	*B. megaterium*	0.50 ± 0.02 b	21	0.44 ± 0.02 ab	38
EA-CB0309	*B. altitudinis*	0.46 ± 0.02 c	13	0.39 ± 0.03 de	31
EA-CB0312	*B. pumilus*	0.57 ± 0.03 a	33	0.46 ± 0.04 a	41
EA-CB0336	*B. pumilus*	0.43 ± 0.11 c	9	0.38 ± 0.08 de	29
EA-CB0570	*B. stratosphericus*	0.53 ± 0.04 b	31	0.44 ± 0.02 ab	38
Control[d]		0.39 ± 0.07 d	0	0.27 ± 0.04 g	0
Trial 2 Maize					
EA-CB0575	*B. subtilis*	0.38 ± 0.01[b] g[c]	16	0.31 ± 0.12 e	15
EA-CB0579	*B. gibsonii*	0.50 ± 0.02 de	36	0.34 ± 0.03 d	20
EA-CB0586	*B. subtilis*	0.46 ± 0.03 f	30	0.39 ± 0.03 c	31
EA-CB0686	*B. pumilus*	0.54 ± 0.03 bc	41	0.49 ± 0.02 a	45
EA-CB0784	*B. megaterium*	0.55 ± 0.04 bc	42	0.49 ± 0.08 a	45
EA-CB1077	*B. pumilus*	0.55 ± 0.03 bc	42	0.42 ± 0.03 b	36
EA-CB1121	*B. subtilis*	0.58 ± 0.01 a	45	0.48 ± 0.05 a	44
EA-CB1134	*B. stratosphericus*	0.56 ± 0.02 bc	43	0.47 ± 0.01 a	42
EA-CB1177	*B. pumilus*	0.48 ± 0.01e	33	0.35 ± 0.03 d	23
EA-CB1397	*B. pumilus*	0.52 ± 0.02 cd	38	0.39 ± 0.02 c	31
Control		0.32 ± 0.1 h	0	0.27 ± 0.07 f	0

[a] Identification according to the best match analysis with BLAST of the 16S rDNA sequence using the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
[b] Standard error intervals (n = 9).
[c] Different letters indicate significant differences ($P < 0.05$) by LSD multiple range test. (Trial 1. Shoot dry weight *P-value* < 0.001, root dry weight *P-value* < 0.001); (Trial 2. Shoot dry weight *P-value* < 0.001, root dry weight *P-value* < 0.001; LSD *P-value* < 0.001).
[d] Control: water.

able for such crop, but also to prospect and develop biofertilizers for other perennials. The high demand of resources, time, and space to conduct tests on perennial plants significantly limits the number of bacterial isolates that can be tested *in planta*. However, finding one single PGPR strain usually requires testing hundreds of bacterial isolates (Dinesh et al., 2015). Then, having a strategy that allows for a high chance of finding successful strains, while having a low demand of time and resources, is of high interest. In addition, our strategy allowed us to determine factors that are crucial for successful development and use of PGPR products for banana, such as the active components of the fermentation process and the effective bacterial concentration. This type of factors, even though of high practical importance, is still poorly explored in the literature and empirical tests are yet needed.

Traditionally, prospecting of PGPR is primarily based on either of these two strategies: 1) Mass screening of rhizobacterial isolates for *in vitro* traits believed to be associated with plant-growth promotion, followed by greenhouse and field experiments only with those isolates considered promising, and 2) Mass screening of isolates directly *in planta*, with subsequent experiments to confirm positive effects (Dinesh et al., 2015; Walia et al., 2014; Zahid et al., 2015). However, isolates selected on the base of their *in vitro* traits often fail to prove effective during *in planta* evaluations (Ahmad et al., 2008; Agaras et al., 2015). Likewise, direct *in planta* screenings of large numbers of isolates result frequently unfeasible for perennial plants, given the costs, time, and labor required. In consequence, our approach combined both strategies by massively testing the bacterial isolates for *in vitro* PGPR-associated traits and also testing them all for their direct *in planta* effect, but using maize as a model monocotyledonous plant. These tests were conducted in a funnel-like manner, testing the reproducibility of the effects in repeated cycles of evaluation, each time with a fewer number of isolates, until reaching a number low enough to make experiments on banana plants feasible and cost-effective.

Our approach showed to be highly effective, as all 4 strains selected to be tested on banana increased at least one plant variable both in the greenhouse and the field. Specifically, strains like *B. subtilis* EA-CB0575 and *B. megaterium* EA-CB0784 displayed a strong effect on plant growth, increasing shoot and root weight and shoot length by up to 36 to 92% in the greenhouse. Indeed, yield-related variables, such as the harvesting time, showed statistically significant improvements in the field, which is clearly important for commercial production. In contrast, only 2.6% of the 837 AEFB isolates consistently promoted growth of maize in the greenhouse, and this number could be expected to be even lower under field conditions. Likewise, biochemical traits determined *in vitro* did not directly correlate with plant growth, as shown by the fact that 67% of all the isolates produced IAA, but only few actually promoted maize growth. These facts together indicate that both traditional approaches would have been inappropriate (and maybe unfeasible) for our interest on banana, having low effectiveness and involving high costs and resources.

In our study, we focused on aerobic endospore-forming bacteria in order to ensure the feasibility of formulation in commercial products with low requirements of storage and long shelf life (Omer, 2010; Young et al., 1995). This was accomplished in our prospecting process by selectively isolating bacteria of this group by heat treating the field samples used as a source of bacteria. Such advantage of endospore-forming PGPR has indeed led to the fact that the vast majority of PGPR-based products available in the market today contain bacteria of this group, even though reports of PGPR belonging to many different genera are abundant (Borriss, 2011). Strains identified in our collection, according to their 16S rDNA sequences, belonged to different species of *Bacillus*, with *B. pumilus* as the most frequent, followed by *B. cereus/thuringiensis, B. sub-*

Table 2
In vitro PGPR traits displayed by the bacterial strains that promoted maize growth in the third phase of selection.

Strain	Sample		Bacterial species[c]	IAA[d] (μg/mL)	Siderophores[e] (mM)	Phosphate solubilization[f]	Nitrogen fixation[g]	Growth inhibition		
	Cultivar[a]	Plantation[b]						*F. oxysporum*[h] (%)	*S. marscesens*[i] (mm)	*R. solanacearum*[j] (mm)
EA-CB0009	BG	Augura	*B. pumilus*	2.4 ± 0.2[k]	–	–	+	–	–	–
EA-CB0012	BV	Augura	*B. cereus/thuringiensis*	5.5 ± 0.4	–	–	–	–	–	–
EA-CB0070	BG	Augura	*B. pumilus*	2.7 ± 0.4	–	–	–	–	–	–
EA-CB0083	BV	La Navarra	*B. cereus/thuringiensis*	11.6 ± 0.5	–	–	–	–	–	–
EA-CB0131	BV	Augura	*B. cereus/thuringiensis*	3.0 ± 0.7	19.7 ± 0.2	+	–	–	–	–
EA-CB0158	PH	El Aserrio	*B. amyloliquefaciens*	11.6 ± 1.3	4.0 ± 0.9	–	+	67.0 ± 0.0	4.2 ± 0.18	9.0 ± 0.3
EA-CB0177	BV	La Navarra	*B. pumilus*	41.4 ± 3.6	–	–	+	–	–	–
EA-CB0185	PH	Augura	*B. megaterium*	39.6 ± 1.5	7.5 ± 0.2	–	+	–	–	–
EA-CB0309	BG	Augura	*B. altitudinis*	14.0 ± 1.7	–	–	–	–	–	–
EA-CB0312	BG	Augura	*B. pumilus*	6.5 ± 0.6	5.1 ± 0.3	+	+	–	–	–
EA-CB0336	BV	La Navarra	*B. pumilus*	16.1 ± 0.3	–	–	–	–	–	–
EA-CB0570	BV	La Navarra	*B. stratosphericus*	22.3 ± 0.5	6.7 ± 1.1	–	–	–	–	–
EA-CB0575	BV	La Navarra	*B. subtilis*	19.7 ± 0.1	15.4 ± 0.6	–	+	38.0 ± 0.2	–	–
EA-CB0579	BG	Augura	*B. gibsonii*	19.9 ± 0.2	–	–	–	–	–	–
EA-CB0586	BV	Augura	*B. subtilis*	6.2 ± 0.4	5.9 ± 0.4	+	+	51.0 ± 0.1	–	–
EA-CB0686	PH	El Aserrio	*B. pumilus*	1.9 ± 0.2	3.0 ± 0.8	–	+	–	–	–
EA-CB0784	BV	La Navarra	*B. megaterium*	30.4 ± 1.0	7.1 ± 0.5	+	+	–	–	–
EA-CB1077	BV	La Navarra	*B. pumilus*	21.3 ± 2.4	–	–	–	–	–	–
EA-CB1121	BG	Augura	*B. subtilis*	11.1 ± 0.7	2.0 ± 0.2	–	+	47.0 ± 0.0	–	–
EA-CB1134	PH	El Aserrio	*B. stratosphericus*	9.0 ± 0.8	4.6 ± 0.7	+	+	–	–	–
EA-CB1177	BV	La Navarra	*B. pumilus*	48.0 ± 0.1	–	–	+	–	–	–
EA-CB1397	BG	Augura	*B. pumilus*	9.8 ± 1.2	4.7 ± 0.9	–	+	–	–	–

[a] Plant cultivar from which the isolate was obtained: banana cv. Grand Naine (BG), banana cv. Valery (BV), or plantain cv. Harton (PH).
[b] Name of the plantations from where the isolate was obtained.
[c] Identification according to the best match analysis with BLAST of the 16S rDNA sequence using NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
[d] Indol production determined by colorimetric method (Patten and Glick, 2002),. *B. pumilus* UA567 was used as the positive control (60 ug/mL total indol production) and TSB medium supplemented with 500 μg/mL tryptophan was used as a negative control. Values represent the average of three replicates for different assays.
[e] Siderophore production determined by cultivation in broth and detection by the CAS method (Schwyn and Neilands, 1987) using EDTA (0–20 uM) as standard for the calibration curve.
[f] Solubilization of mineral phosphate. Positive (**+**): Growth of bacterial strain on medium containing rock phosphate as the only source of P and formation of transparent halo due to phosphate consumption.
[g] Nitrogen fixation determined by bacterial growth in NFb medium (free of nitrogen). Positive (**+**): Indicates bacterial growth and color change of the medium.
[h] Activity of the strain against *F. oxysporum*, determined by the agar-diffusion method. Data showed correspond to the percentage of inhibition in those treatments for which it was significant, compared with the fungus growing alone.
[i] Activity of the isolate against *S. marscesens* EAD005. Data showed correspond to the size of the inhibition zone.
[j] Activity of the strain against *R. solanacearum* EAP009. Data showed correspond to the size of the inhibition zone.
[k] Standard error intervals.

Table 3
Growth promoting effect of potential PGPR strains in talc-based formulation on banana plants in greenhouse and field conditions.

Strain	Greenhouse										Field			
	Shoot length (cm)		Shoot				Root				Bunch harvesting time (months)		Shoot length (cm)	
			Fresh weight (g)		Dry weight (g)		Fresh weight (g)		Dry weight (g)					
B. cereus/thuringiensis EA-CB0131	28.0 ± 4.1[a]	abc[b]	124.5 ± 25.8	ab	8.6 ± 0.5	b	41.4 ± 4.9	b	9.6 ± 0.2	ab	7.1 ± 0.4	bc	188.4 ± 5.2	c
B. subtilis EA-CB0575	31.5 ± 1.1	ab	138.8 ± 4.1	a	10.6 ± 0.3	a	68.2 ± 2.9	a	8.9 ± 0.3	abc	7.3 ± 0.3	bc	210.6 ± 6.4*	a
B. megaterium EA-CB0784	33.0 ± 2.4	a	118.0 ± 13.8	abc	10.5 ± 0.3	a	50.6 ± 3.2	ab	10.3 ± 0.2	a	7.1 ± 0.4	bc	204.3 ± 3.8*	ab
B. subtilis EA-CB1121	30.5 ± 2.1	ab	86.3 ± 16.8	bc	11.1 ± 0.8	a	32.2 ± 2.7	b	8.5 ± 0.9	bc	6.9 ± 0.2*	c	193.2 ± 4.3	bc
Control (Talc formulation)	26.0 ± 2.0	bc	93.5 ± 14.1	abc	8.9 ± 0.2	b	46.8 ± 7.4	b	7.7 ± 0.7	c	7.6 ± 0.3	b	186.2 ± 3.5	c
Control (Water)	21.6 ± 1.0	c	72.3 ± 12.5	c	7.7 ± 0.3	b	41.0 ± 8.9	b	7.5 ± 0.2	c	8.3 ± 0.3	a	193.0 ± 5.7	bc

* Indicate significant differences ($P < 0.05$) by Dunnett multiple comparison test against control talc formulation (EA-CB1121 bunch harvesting time $P = 0.0523$, EA-CB0575 shoot length $P = 0.00391$; EA-CB0784 $P = 0.05394$).

[a] Standard error intervals ($n = 10$ Greenhouse, $n = 20$ Field).

[b] Different letters indicate significant differences ($P < 0.05$) by LSD multiple comparison tests (Greenhouse: Shoot length $P = 0.024$, fresh and dry shoot weights $P = 0.0304$ and 0.001, fresh and dry root weights $P = 0.016$ and 0.002. Field: bunch harvesting time $P = 0.0001198$, shoot length $P = 0.00421$).

Table 4
Growth promoting effect of potential PGPR strains in pesta-based formulation on banana plants in greenhouse and field conditions.

Strain	Greenhouse										Field			
	Shoot length (cm)		Shoot				Root				Bunch harvesting time (months)		Shoot length (cm)	
			Fresh weight (g)		Dry weight (g)		Fresh weight (g)		Dry weight (g)					
B. cereus/thuringiensis EA-CB0131	32.0 ± 1.5[a]	ab[b]	149.9 ± 9.2	a	8.1 ± 0.3	de	73.7 ± 4.8	a	8.7 ± 0.4	b	7.1 ± 0.3	cd	194.7 ± 6.2	ab
B. subtilis EA-CB0575	30.3 ± 2.4	b	127.2 ± 19.3	ab	10.7 ± 0.2	b	78.7 ± 5.7	a	12.6 ± 0.3	a	6.7 ± 0.2*	d	206.4 ± 3.3	a
B. megaterium EA-CB0784	37.5 ± 2.2	a	167.4 ± 10.6	a	11.9 ± 0.4	a	75.4 ± 6.0	a	12.8 ± 0.4	a	7.6 ± 0.1	b	187.4 ± 6.5	bc
B. subtilis EA-CB1121	31.3 ± 2.3	b	93.8 ± 22.8	bc	9.7 ± 0.6	bc	51.4 ± 4.9	b	8.7 ± 0.6	b	7.3 ± 0.1	bc	178.3 ± 6.6*	c
Control (Pesta formulation)	27.8 ± 1.9	b	157.0 ± 11.3	a	8.8 ± 0.5	cd	42.4 ± 9.6	b	9.1 ± 0.4	b	7.4 ± 0.1	bc	200.8 ± 3.6	ab
Control (Water)	21.6 ± 1.2	c	72.3 ± 17.6	c	7.7 ± 0.3	e	41.0 ± 9.9	b	7.5 ± 0.3	c	8.3 ± 0.3*	a	193.0 ± 3.8	ab

[a] Standard error intervals ($n = 10$ Greenhouse, $n = 20$ Field).

[b] Different letters indicate significant differences ($P < 0.05$) by LSD multiple comparison tests (Greenhouse: Shoot length $P = 0.0003$, fresh and dry shoot weights $P = 0.0043$ and 0.0000, fresh and dry root weights $P = 0.0017$ and 0.0000. Field: bunch harvesting time $P = 1.394e\text{-}06$, shoot length $P = 0.003672$).

* Indicate significant differences ($P < 0.05$) by Dunnett multiple comparison test against control pesta formulation (Control water and EA-CB0575 bunch harvesting time $P = 0.00609$ and $P = 0.03159$ respectively, EA-CB1121 shoot length $P = 0.0116$).

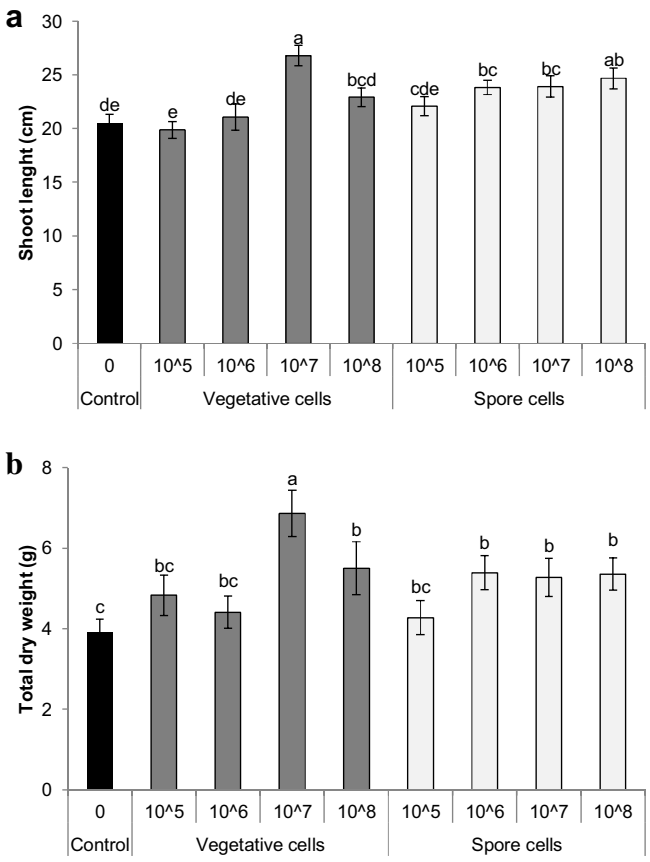

Fig. 1. Effect of different concentrations of spores and vegetative cells of *Bacillus subtilis* EA-CB0575 on shoot length (A) and total dry weight (B) of banana plants in greenhouse. Different letters indicate significant difference between the treatments (LSD: Shoot length $P = 0.0067$, Total dry weight $P = 0.0011$).

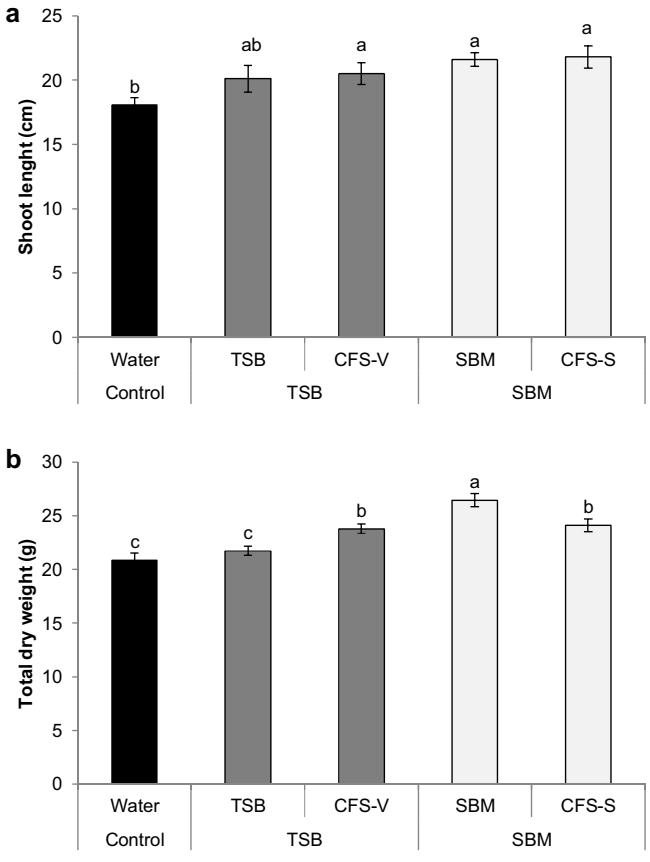

Fig. 2. Effect of cell free supernatants obtained from *Bacillus subtilis* EA-CB0575 culture and fresh culture media on shoot length and total dry weight of banana plants in greenhouse. A) Shoot length (cm), B) Total dry weight (g). Different letters indicate significant difference between the treatments (LSD: Shoot length $P = 0.0135$, Total dry weight $P < 0.0001$).

tilis, *B. megaterium*, and *B. amyloliquefaciens*. Strains of these species have previously been reported as plant growth promoters (Kumar et al., 2011; Porcel et al., 2014; Walia et al., 2014; Yuan et al., 2013), with *B. pumilus* and *B. amyloliquefaciens* specifically mentioned to promote growth of banana plants (Jaizme Vega et al., 2004; Kavino et al., 2010; Mia et al., 2010a,b). Strains selected for evaluation on banana plants in the greenhouse and the field proved to belong to the species *B. cereus/thuringiensis* (EA-CB0131), *B. subtilis* (EA-CB0575), *B. megaterium* (EA-CB0784), and *B. subtilis* (EA-CB1121). For these, species *B. subtilis* and *B. megaterium* did not represent any risk for human health and safety (HSE, 2013) and, in fact, strains of these two genera are included in a number of PGPR-based products. However, *B. cereus* is a bacterial species included in the Hazard Group 2 by the Health and Safety Executive of the United Kingdom (HSE, 2013), which restricts its inclusion in biotechnological products.

Currently, fundamentally applied aspects such as the actual active components of PGPR fermentation products and the effective cell concentration are deeply unexplored. This lack of knowledge limits practical implementation of PGPR in agriculture and increases variability of field performance. Only few studies have determined the actual biological components that cause the plant growth effect in PGPR-based products, which leaves a significant gap of knowledge for manufacturing companies (Bai et al., 2002; Jetiyanon et al., 2008; Ramírez and Kloepper, 2010; Buensanteai et al., 2013; Idris et al., 2007). In our study, we found that both vegetative cells and spores increased the growth of banana plants in a concentration dependent manner. These results suggest two alternative hypotheses. On the one hand, both cellular structures could be able to initiate bacterial colonization of banana roots, for which spores would first need to germinate, so that bacterial populations could establish and promote plant growth. In contrast, an alternative hypothesis is that root colonization is not needed for promotion of banana growth and only an initial increase of populations on the roots could be sufficient to increase the levels of extracellular metabolites (*e.g.* phytohormones) in the rhizosphere that elicit plant growth. These two hypotheses are currently being tested in our lab by using bioreporters and biomarkers that will allow us to directly test root colonization.

We also found that the cell-free supernatant (CFS) from the culture of either vegetative or spore cells significantly promoted plant growth. This fact is highly important for the manufacturing process of PGPR-based products, as supernatants are frequently discarded, so that active components are unnecessarily left out of the product. Such growth-promoting effect could be associated with either/both 1) active compounds present in the supernatants (*i.e.* secondary metabolites) or/and 2) unconsumed nutrients that can be used by the plant or by other beneficial microorganisms inhabiting the rhizosphere (Buensanteai et al., 2013). Different authors have reported that some of the components produced during the fermentation process, such as secondary metabolites, extracellular proteins, and phytohormones, could be associated with the observed growth enhancement (Idris et al., 2007). In our case, active compounds such as lipopeptides and siderophores have been found to be produced by the strain we tested (data not shown), so they could be responsible for the enhancement of growth observed. In addition, nutrients present in the CFS could directly benefit the plant or indirectly promote plant growth by benefiting beneficial microor-

ganisms naturally present in the rhizosphere of banana. From our experiments it is not possible to differentiate the effect caused by the medium components and that by the metabolites, given that the CFS has a reduced nutrient concentration, compared with the fresh culture medium. This fact prevents us from making any valid inference based on the direct comparison between the effect of the fresh medium and that after bacterial fermentation.

Lastly, the effect of the cell concentration was determined. This factor is usually overlooked in investigations, but it is crucial for obtaining optimal and effective results. For both cellular structures (vegetative and spore cells), concentrations between 1×10^7 to 1×10^8 CFU/mL had the greatest effect, which is in agreement with previous reports in other plant species (Bai et al., 2002; Ramírez and Kloepper, 2010). Probably, a minimum of 10^7 CFU/mL is needed so that the bacterial cells can outcompete the diverse microbial populations that exist in the rhizosphere and, therefore, express its mechanism of action.

Overall, the results obtained in this study show a promising strategy for the development of a biotechnological product based on endospore-forming PGPR. First, the funnel-like approach followed to prospect for PGPR isolates in nature was both effective and efficient, leading to finding plant growth-promoting strains with reasonable time and resource investments. This was especially significant in our study, given the importance of banana for the economy of developing countries and the challenge that this plant species implies because of its tropical and perennial nature. In addition, in our case, both spores and vegetative cells, and the fermentation supernatant showed to be active ingredients in the product, which would increase its efficacy in the field by providing different components able to promote plant growth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scienta.2016.09.040.

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