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Growth-promoting potential of bacterial biomass in the banana micropropagated plants

Christiana de F. B. da Silva[1], Thaís L. de Brito[2], Carlos A. K. Taniguchi[3], Larissa A. Lopes[2], Gustavo A. S. Pinto[4] & Ana C. P. P. de Carvalho[5]

[1] Embrapa Agroindústria Tropical/Laboratório de Patologia Pós-colheita. Fortaleza, CE. E-mail: christiana.bruce@embrapa.br (Corresponding author) - ORCID: 0000-0002-1794-8581
[2] Universidade Federal do Ceará. Fortaleza, CE. E-mail: thaislima.biotecnologia@gmail.com - ORCID: 0000-0002-1541-2230; larissalveslopes@gmail.com - ORCID: 0000-0002-6967-4930
[3] Embrapa Agroindústria Tropical/Laboratório de Solos. Fortaleza, CE. E-mail: carlos.taniguchi@embrapa.br - ORCID: 0000-0002-1280-8678
[4] Embrapa Agroindústria Tropical/Laboratório de Bioprocessos. Fortaleza, CE. E-mail: gustavo.saavedra@embrapa.br - ORCID: 0000-0002-3672-8795
[5] Embrapa Agroindústria Tropical/Laboratório de Cultura de Tecidos e Genética Vegetal. Fortaleza, CE. E-mail: cristina.carvalho@embrapa.br - ORCID: 0000-0002-4562-8999

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Bacillus spp.
Musa spp.
plant growth-promoting rhizobacteria

ABSTRACT
In the banana production system, a sustainable alternative for producing quality plantlets would be inoculation with plant growth-promoting bacteria (PGPB). Therefore, this study aimed to evaluate the growth-promoting potential of a bacterial biomass in micropropagated banana plantlets cultivar Prata Catarina, and to identify the mechanisms involved in plant-microorganism interactions. In vitro, the biochemical assays tested were the solubilisation of phosphates, production of enzymes, production of ammonia, siderophores, and indole acetic acid. In the in vivo tests, the plants were bacterised (10^9CFU mL^{-1}) in two phases: acclimatisation, and cultivation in plastic bags. The design was a randomised block with 9 and 7 repetitions per treatment, which were: T1: control; T2: plants treated with isolate E2 (*Bacillus pumilus* group); T3: plants treated with RAB9 isolate (*B. pumilus*) for each phase. Bacterial isolates were capable of producing cellulases, amylases, pectinases, lipases, proteases, and siderophores. The plants gained in height, root length, root dry mass, pseudostem diameter, and leaf area. It is concluded that the PGPB can promote the growth of micropropagated banana plantlets through the production of enzymes and siderophores.

Palavras-chave:
Bacillus spp.
Musa spp.
rizobactéria promotora de crescimento em planta

Potencial da biomassa bacteriana promotora de crescimento no desenvolvimento de mudas micropropagadas de bananeira

RESUMO
Na bananicultura, uma alternativa sustentável para produção de mudas de qualidade seria a inoculação com as bactérias promotoras de crescimento de plantas (BPCP). Portanto, objetivou-se avaliar o potencial da biomassa bacteriana promotora de crescimento em mudas micropropagadas de bananeira cultivar Prata Catarina e identificar quais os mecanismos envolvidos nas interações planta/microrganismo. As provas bioquímicas testadas in vitro foram solubilização de fosfatos, produção de enzimas, produção de amônia, sideróforos e ácido indolacético. Nos ensaios in vivo, as plantas foram bacterizadas (10^9UFC mL^{-1}) em duas fases: na aclimatização e no cultivo em sacos plásticos. O delineamento experimental foi em blocos casualizados, com 9 e 7 repetições, por tratamento, dos quais foram: T1: controle; T2: plantas tratadas com isolado E2 (*Bacillus pumilus* group); T3: plantas tratadas com isolado RAB9 (*B. pumilus*), para cada uma das fases. Os isolados bacterianos foram capazes de produzir celulases, amilases, pectinases, lipases, proteases e sideróforos. Foram observadas nas mudas ganhos em altura, comprimento da raiz, massa seca da raiz, diâmetro do pseudocaule e área foliar. Conclui-se que as BPCP são capazes de promover o crescimento de mudas micropropagadas de bananeira, através da produção de enzimas e sideróforos.

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Introduction

The banana crop (*Musa* spp.) has an important socioeconomic role, and the Northeast region of Brazil is the second major producer, contributing 2.233.7 t in 2017 (Agrianual, 2017). In the banana production system, seedlings are produced through micropropagation. A sustainable alternative for the reduction of pesticides and soluble fertilisers in the production of these plants would be inoculation with beneficial microorganisms, such as Plant Growth-Promoting Rhizobacteria (PGPR) (Arturson et al., 2006; Romeiro, 2007; Glick, 2012). PGPR promote nutritional growth, antagonism to pathogens, and stimulation of plant host defences (Choudhary & Johri, 2009; Hayat et al., 2010) and several studies have highlighted the growth-promoting activity of these microorganisms in banana plants. The rhizobacteria present positive responses to physiological growth characteristics, as well as to nutritional parameters of banana plants (Baset Mia et al., 2010; Souza et al., 2016).

To promote growth, PGPR use different mechanisms of action such as the production of phytohormones, antibiotics, hydrocyanic acid, lytic enzymes, siderophores, phosphate solubilisation, and nitrogen fixation (Glick, 2012; Souza et al., 2015). Identification of the compounds produced by the bacteria is fundamental since it permits selection of the most efficient isolates in the colonisation process (Bernardes et al., 2010). The use of PGPR is an important tool in agricultural production, primarily due to the demand for a decrease in the dependence on soluble fertilisers and defensives within the context of sustainable agriculture (Kumar et al., 2012; Ahemad & Kibret, 2014).

Therefore, the objective of this study was to evaluate the growth-promoting potential of a bacterial biomass on micropropagated plants of the banana cultivar Prata Catarina, as well as to identify the mechanisms involved in this interaction.

Material and Methods

The experiments were conducted in the year of 2015 in the Laboratories of Post-harvest Pathology, Bioprocess and under greenhouse conditions at Embrapa Agroindústria Tropical (Fortaleza, CE) (3° 45' 1.4" S, 38° 34' 30.9" W). The greenhouse had the following climatic variables: minimum and maximum temperatures of 27 and 40 °C, respectively, and 80% mean relative humidity.

The bacterial isolates used were RAB9 (*Bacillus pumilus* Meyer & Gotttheil) and E2 (*Bacillus pumilus* group) obtained from the Collection of Cultures of the Laboratory of Phytobacteriology of the Federal Rural University of Pernambuco. The isolates were preserved in NYD broth (dextrose 10 g L^{-1}, yeast extract 5 g L^{-1}, meat extract 3 g L^{-1} and meat peptone 5 g L^{-1}) with 15% glycerol in an ultrafreezer (-85 °C). For activation, the isolates were transferred to NYDA medium (NYD broth with 18 g L^{-1} of agar) and incubated at 30 °C in a BOD incubator for 24 h. The bacterial biomass was produced in a 3 L New Brunswick model BioFlo 115 bench, with submerged fermentation and a maximum working volume of 2.2 L, containing 1 L of NYD medium. Rushton impellers with 6 flat blades were used to improve agitation of the reaction medium.

The reactor was fed by filtered compressed dry air at its inlet and outlet through filters (0.20 µm PTFE) and the flow rate used was 6 L min^{-1}. The water for cooling was fed through a Thermo Scientific Chiller System (model Thermo Flex 1400) at 15 °C. After stabilisation of the system, the medium was aseptically inoculated via the septum with a 60 mL syringe fitted with a hypodermic needle containing 50 mL of the inoculum prepared in NYD medium and containing the isolates in the growth logarithmic phase at a concentration of 0.01 g L^{-1}. A further 0.3% of mineral oil was added to prevent foaming. After 24 h of fermentation, the medium was centrifuged at 3500 rpm (Biofuge Stratus, with rotor 15000) for 15 min. The pellets were washed with 30 mL of distilled water and centrifuged again. They were resuspended in distilled water and the concentration of the solution was adjusted to 10^9CFU mL^{-1} (according to the McFarland scale). Tween 80 (0.05%) was added to the suspensions to avoid cell aggregation.

For growth promotion, 180 micropropagated plants of the banana cultivar Prata Catarina were used, and were supplied by Bioclone Seedling Production S.A. The plants were removed from flasks, washed, and excessive roots were cut. The plantlets were transferred to expanded 162-cell polystyrene trays containing 5 L of the planting formulation. The formulation consisted of autoclaved soil, dry coconut shell powder, and washed sand (6% HCl solution, for 36 h followed by washing with distilled water until pH = 7) in a ratio of 1:1:1 by volume, and slow-release fertiliser (5 kg m^{-3}) (adapted from Lédo et al., 2008).

The experiments were conducted in two phases: acclimatisation in trays, and cultivation in 1.5 L plastic bags. In each experiment, the isolates were inoculated by spraying on the aerial part of the plants until the inoculum was completely drained (10^9 CFU mL^{-1}). In the first experiment, the plants were evaluated 60 days after inoculum spraying in the acclimatisation phase. In the second experiment, the plants were evaluated after 60 days of acclimatisation + 60 days of cultivation in plastic bags.

The variables analysed were plant height (mm), number of leaves, leaf area (cm^2), measurement of the length and width of the two largest leaves of the plant, root dry mass (g), diameter of the pseudostem (mm), and length of the root system (mm). For the plants grown in plastic bags, the survival rate was also calculated. The experimental design was in randomised blocks with the following treatments: T1: control, T2: plants treated with isolate E2, and T3: plants treated with RAB9 isolate. For the first and second experiments, 9 replicates (each replicate = one plant) and 7 replicates (each replicate = 4 plants), respectively, were used. The data were submitted to variance analysis and the means were compared by the Tukey test, at 5% probability, using SISVAR software. All data were transformed to $\sqrt{x + 1}$.

To characterise the growth-promoting mechanisms of the isolates, the following tests were performed: a) indolacetic acid (AIA) production: King B medium (Romeiro, 2007) containing tryptophan (5 mM) was used. Ehrlich reagent was added, with

the formation of a pink ring being considered positive (adapted from Whitman, 2009); b) phosphate solubilisation: Mandels and Weber medium (Mandels & Weber, 1969) rich in calcium phosphate (1%) was used. The formation of a clear zone around the colonies was considered positive (adapted from Cattelan, 1999); c) production of siderophores: the isolates were grown in King B medium, then centrifuged and Chromium Azurol S (CAS) indicator solution was added to the supernatant. The conversion of the blue color from CAS to yellow indicated production of siderophores (adapted from Cattelan, 1999); d) ammonia production: a filter paper tape was soaked with an acidity indicator, and the filter tapes were then added to tubes containing the isolates grown in King B medium. Ammonia production was indicated by a pink color in the paper tape (Romeiro, 2007); e) cellulose utilisation: red congo was added to the isolates grown in Mandels and Weber medium rich in carboxymethylcellulose (CMC). The formation of a clear zone around the colonies was considered positive (Romeiro, 2007); f) use of chitin: for isolates grown in Mandels and Weber medium rich in chitin, the formation of a light zone around the colonies was considered positive (adapted from Cattelan, 1999); g) protease production: the isolates were cultured in YMA medium (0.5 g L^{-1} of K_2HPO_4, 0.2 g L^{-1} of $MgSO_4.7H_2O$, and yeast extract 0.5 g L^{-1}, pH 7) containing skimmed milk (10%). The formation of a clear zone around the colonies was considered positive (adapted from Souza et al., 2008); h) use of starch: lugol was added to isolates grown in Mandels and Weber medium rich in soluble starch (1%). The formation of a clear zone around the colonies was considered positive (adapted from Rodrigues, 2006); i) production of pectinase: red congo was added to the isolates grown in Mandels and Weber medium rich in citrus pectin (1%). The formation of a clear zone around the colonies was considered positive (adapted from Beg et al., 2000); j) production of lipase: the isolates were cultured in lipase detection medium (10 g L^{-1} of peptone, 5 g L^{-1} of NaCl, 0.1 g L^{-1} of $CaCl_2.2H_2O$, and 18 g L^{-1} of agar). The presence of halos around the colonies formed by crystals indicated the secretion of lipase (Sierra, 1957). All the assays were carried out in triplicate, using the media tested without the addition of bacterial isolates as a control.

Results and Discussion

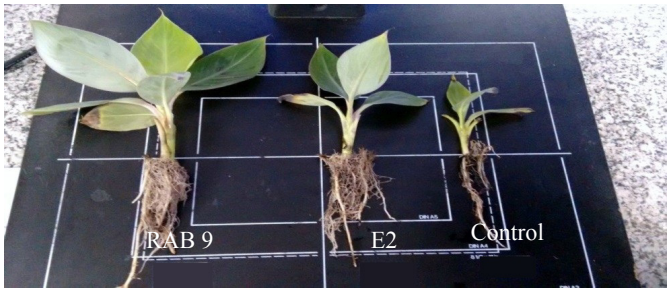
The biomasses of RAB9 and E2 obtained in the bioreactor were 4.8 and 3.8 g L^{-1}, respectively, in 24 h, under agitation and aeration conditions. The increase in biomass can be attributed to the controlled conditions of pH, temperature, agitation, and aeration of the bioreactor. However, this does not occur when Erlenmeyer flasks are used (Schmidell et al., 2001).

Regarding aeration, Mantzouridou et al. (2002) have suggested that it contributes to better microbial development, promoting better homogenisation and oxidation reactions of biomolecules for energy production. Submersed fermentation for large-scale biomass production is considered more adequate due to the ease of control of process parameters (Sousa, 2013). Therefore, this type of fermentation was adopted in the above-mentioned study.

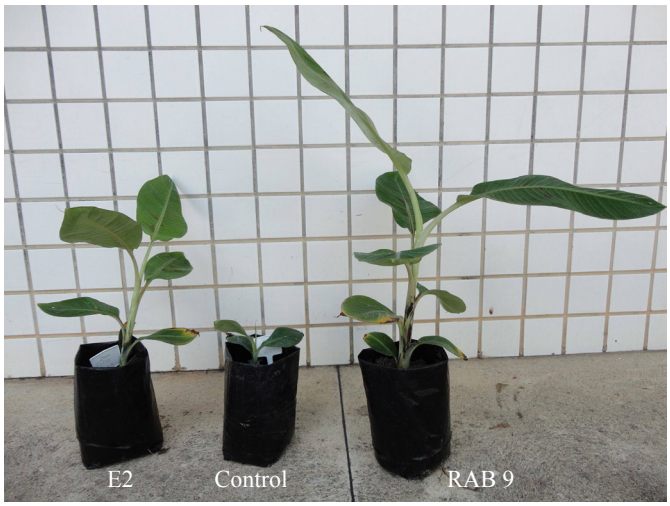
After treatments with the bacterial biomass, variation in the development of the micropropagated banana plants was observed both in the acclimatisation and cultivation in plastic bags phases (Figures 1A and B). However, the distinctions in height, leaf area, dry mass, and diameter of the pseudostem were only significantly evident in the culture in bags phase when compared to the control (Tables 1 and 2). In addition, the plants transplanted to bags presented abiotic stress with the presence of burning at the leaf edges, likely due to the high temperatures in the greenhouse. In the plants inoculated with the bacterial isolates, however, a higher survival rate was observed when compared to the uninoculated plants (Table 3).

PGPB are known to have significant protection against abiotic stress (Glick, 2012; Vurukonda et al., 2016) and, in the present study, the bacterial isolates used may have conferred

A.



B.



Treatments: E2 (*Bacillus pumilus* group), control, and RAB9 (*Bacillus pumilus* Meyer & Gotttheil)

Figure 1. Micropropagated plantlets of the banana cultivar Prata Catarina treated and untreated with bacterial biomass after 60 days of acclimatisation (A); after acclimatisation and cultivation in plastic bags for 60 days (B)

Table 1. Development of micropropagated plantlets of the banana cultivar Prata Catarina in the acclimatisation phase, after inoculation with the bacterial biomasses (E2 and RAB9)

Treatment	H (mm)	NL	RL (mm)	PD (mm)	LA (cm^2)	RDM (g)
Control	131.95 a	2.66 b	69.72 b	5.58 a	44.12 a	0.78 a
E2	148.14 a	3.88 a	114.69 a	6.34 a	88.25 a	0.73 a
RAB9	151.94 a	3.88 a	121.87 a	7.04 a	91.85 a	1.32 a
CV (%)	24.55	26.07	32.56	24.1	59.16	67.56

CV - Coefficient of variation; Means followed by the same lowercase letter do not differ ($p \geq 0.05$). H - Height; NL - Number of leaves; RL - Root length; PD - Pseudostem diameter; LA - Leaf area; RDM - Root dry mass

Table 2. Development of micropropagated plantlets of the banana cultivar Prata Catarina in the cultivation in plastic bags phase, after inoculation with bacterial biomasses (E2 and RAB9)

Treatment	H (mm)	NL	RL (mm)	PD (mm)	LA (cm^2)	RDM (g)
Control	28.30 b	0.57 b	12.86 c	1.22 c	6.49 b	0.09 b
E2	295.73 a	5.43 a	115.00 b	10.44 b	106.21 a	3.63 b
RAB9	383.42 a	6.53 a	193.82 a	17.22 a	169.42 a	11.58 a
CV (%)	34.27	27.55	41.13	31.08	63.43	60.47

CV – Coefficient of variation; Means followed by the same lowercase letter do not differ ($p \geq 0.05$). H - Height; NL - Number of leaves; RL - Root length; PD - Pseudostem diameter; LA - Leaf area; RDM - Root dry mass

Table 3. Survival of micropropagated plantlets of the banana cultivar Prata Catarina in the cultivation in plastic bags phase, after inoculation with the bacterial biomasses (E2 and RAB9)

Treatment	Survival (%)
Control	3.57 a
E2	32.14 b
RAB9	96.43 c
CV (%)	19.31

CV – Coefficient of variation; Means followed by the same lowercase letter do not differ ($p \geq 0.05$)

protection against adverse factors to the banana cultivar Prata Catarina.

For all the evaluated variables during the cultivation in plastic bags phase, the isolates RAB9 and E2 were prominent in the promotion of growth of the banana plants. The results obtained in the present study are similar to those reported by Mello et al. (2002), when a RAB9 isolate was used in micropropagated plants of the pineapple cultivar Pérola. The authors observed increases of 163 and 107% in leaf and root dry matter, respectively, and 87% in leaf area, when compared with uninoculated plants.

The bacterial isolates from the present study presented several mechanisms of action which may be related to the growth promotion seen in the micropropagated plantlets of the banana cultivar Prata Catarina (Table 4).

However, none of the isolates was able to produce IAA, chitinase, and ammonia, or to solubilise phosphates. Similar results were found by Mello et al. (2002) in which none of the isolates presented positive results for the above mentioned biochemical tests. In contrast, in another study, Vardharajula et al. (2011) associated the growth promotion of maize seedlings with isolates of *Bacillus* sp. that could produce ammonia, indoleacetic acid, and phosphate solubilisers. Therefore, it is likely that other mechanisms are involved in promoting growth in micropropagated plants of the banana cultivar Prata Catarina.

Table 4. Mechanisms of action of the bacterial isolates RAB9 and E2 for growth promotion of the micropropagated plantlets of the banana cultivar Prata Catarina

Biochemical tests	Bacterial isolates	
	E2	RAB9
Production of IAA	-	-
Solubilisation of phosphates	-	-
Production of siderophores	+	+
Production of ammonia	-	-
Production of cellulases	-	+
Production of chitinases	-	-
Production of proteases	+	+
Production of amylases	+	+
Production of pectinases	+	+
Production of lipases	+	+

Positive results of the tests (+), negative results of the tests (-)

All bacterial isolates from the present study were able to sequester iron and, therefore, were capable of producing siderophores. Ribeiro & Cardoso (2012) tested several mechanisms of growth promotion and observed that 37 isolates produced siderophores, including isolates of *Bacillus* spp.

In terms of the enzymes tested, the RAB9 and E2 isolates could produce amylases (Figures 2A and B), lipases (Figures 2C and D), proteases and pectinases.

Dinesh et al. (2015) evaluated rhizobacteria associated with ginger rhizosphere in relation to the capacity for growth promotion. All isolates of *Bacillus amyloliquefaciens* obtained were able to produce proteases, pectinases, cellulases, and α-amylases. In addition, Szilagyi-Zecchin et al. (2014) showed that *Bacillus* spp. isolates produced pectinases and cellulases, but not chitinase and β 1,3 glucanase.

In the present study, only the RAB9 isolate was able to produce cellulases, by degrading the carboxymethylcellulose (CMC) present in the medium. In another study, Kavamura et al. (2013) evaluated the relationship between growth promotion under water stress in native cacti from Caatinga. These authors verified that most of the isolates identified belonged to the genus *Bacillus* and 79 and 71% of these isolates produced enzyme cellulase and ammonia, respectively, under dry conditions. These results are similar to the results presented in this study since the plants inoculated with the RAB9 isolate (cellulase producer) showed higher survival under greenhouse conditions (temperatures between 27-40 °C).

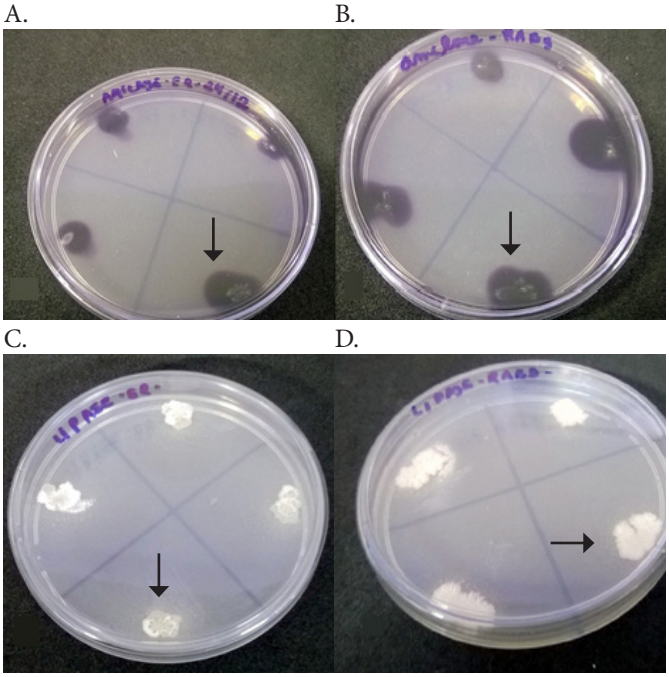


Figure 2. Detection of the growth-promoting mechanisms of the bacterial isolates E2 and RAB9. Production of amylases: the presence of a halo lighter than the culture medium (indicated by the arrows) (A-B); Production of lipases: halos formed by crystals (indicated by the arrows) (C-D)

Our results showed that the use of growth-promoting bacteria inoculated in the phases of acclimatisation and cultivation in plastic bags present a sustainable alternative in the banana production system. The rhizobacteria used in the present study were efficient in promoting the growth of the plants, as evidenced by gains in height, root length and dry weight, pseudostem diameter, and leaf area.

Conclusions

1. The bacterial biomass promoted increases in the development of the micropropagated banana cultivar Prata Catarina plants.

2. The mechanisms that may be involved in growth promotion are the production of siderophores and enzymes (proteases, amylases, pectinase, lipases and cellulases).

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