

Interaction of endophytic diazotrophic bacteria and *Fusarium oxysporum* f. sp. *cubense* on plantlets of banana ‘Maçã’

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Abstract The objective of this work was to evaluate the effect of endophytic diazotrophic bacteria *Herbaspirillum* and *Burkholderia* on the *Fusarium oxysporum* f. sp. *cubense* (Foc) establishment and on the plantlets growth of the ‘Maçã’ banana (*Musa* spp., group ABB). Two assays were carried out in a greenhouse at the National Center of Tropical Agroindustry in Fortaleza city, Ceará State (Brazil), using randomized block designs in the factorial arrangements 4×2 and 8×2. On the first trial plantlets were inoculated with the *Burkholderia* sp. AB202; *Herbaspirillum* sp., BA227, both of strains and controls bacteria, with and without Foc and cultivated in pots filled with an autoclaved mixture of washed sand and vermiculite (ratio 3:2 v v⁻¹), during 4 months. On a second assay, plants were subjected to following conditions: absence and presence of strains AB202, AB213 (*Burkholderia* spp.), BA227, BA234

(*Herbaspirillum*-like), AB202 plus BA227, AB213 plus BA234, the mixture of the four bacterial strain, absence and presence of the Foc; and cultivated in pots filled with autoclaved Haplic Arenosol, during 2 months. The plant association with diazotrophs and the Foc was confirmed, and factors interacted significantly on the most probable number of bacteria and the colony forming units of the pathogen on roots and plant rhizomes. The potential of the endophytes on the inhibition of Foc propagate units and on the plant growth promotion was demonstrated. The higher biomass was observed four and 2 months after plant inoculation with AB202 and BA234. Results showed that these endophytes may be used as potential biofertilizer and biocontrol agents.

Keywords *Burkholderia* · *Herbaspirillum* · *Fusarium oxysporum* · *Musa* spp. · Biocontrol

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Introduction

Banana trees are widely cultivated in tropical countries. Brazil, in 2005, produced 6,802.99 Tons on 499.23 ha (FAO 2007). It has a high consumer demand worldwide, being an important source of nutrients. Although Brazil detains a high crop production, it has a low efficiency, mainly in the Northeast region, where only 12 Tons per ha is harvested. A low technological level is adopted by farmers and diseases are frequently uncontrolled on

production areas. *Fusarium* wilt disease is a serious constraint to many cultivars grown in different regions, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), Smith (Cordeiro 1999).

The pathogen Foc is widely spread in different producing regions, infecting many genotypes, including the cultivar ‘Maçã’ from subgroup ‘Prata’, which represents 95% of the whole national banana plantations (Cordeiro 1999; Silva et al. 2003). In some plantations of the Brazilian Northeastern region Foc is so destructive that farmers obtain no more than one harvest, turning the area unsuitable to new crop (Weber and Freire 2003).

Substitution of the susceptible cultivar by resistant hybrids is effective against *Fusarium* wilt (Cordeiro 1999; Silva et al. 2003). However, consumers become displeased due to their ‘Maçã’ banana preference (Matsuura et al. 2004). Thus that kind of banana has a higher local cost when compared to the others and the farmers search for new areas to continue planting that cultivar. As chemical control presents low effectiveness (Campanioni et al. 2005; Nel et al. 2006), biological control is an attractive option, mainly during plantlet formation.

Considering the soil antagonists, such as *Pseudomonas fluorescens* (Saravanan et al. 2003), *P. aeruginosa* (Ayyadurai et al. 2006), *Burkholderia cepacia* (Pan et al. 1997), *Trichoderma harzianum* (Thangavelu et al. 2003), *Trichoderma* sp. (Nel et al. 2006) and *Streptomyces* sp. (Getha et al. 2005), which reduce symptoms of *Fusarium* wilt, screening of plant growth-promoting rhizobacteria (PGPR) would contribute to optimise the yield. The PGPR competitively colonize plant roots, and stimulate plant growth and/or reduce the incidence of plant diseases (Haas and Défago 2005). *Pseudomonas* strains 84 and 4B when introduced to banana roots of tissue-cultured plants at de-flasking stage improved plant growth and reduced infection of Foc in the rhizome under green house conditions (Smith et al. 2003). Recently, Jaizme-Vega et al. (2004) found that bacterization of banana cv. Grand Nain and ITC-1297 plantlets with *Bacillus* spp. significantly improved plant growth and foliar mineral contents.

Endophytic diazotrophic bacteria have been isolated from banana plants (Weber et al. 1999) and some strains are plant growth-promoting (Weber et al. 2000) and also are able to inhibit Foc growth *in vitro* (Muniz et al. 2007). Since micropropagated plantlets

are sensitive to soil pathogens (Ayyadurai et al. 2006), inoculation with selected microorganisms would be desirable. Hence, the present investigation was undertaken to evaluate the effect of endophytic diazotrophic bacteria *Herbaspirillum* and *Burkholderia* on the Foc establishment and on the plantlets growth of the ‘Maçã’ banana.

Material and methods

Plant material

The banana ‘Maçã’ plantlets were obtained from shoot tip culture. The proliferation medium was MS added with 30 g l⁻¹ of sucrose and 2.5 mg l⁻¹ of 6-benzylaminopurine. The shoots were elongated on same medium without growth regulators, at Embrapa Tropical Agroindustry and Biotece Tissue Culture Laboratories, both located in Fortaleza, Ceará, Brazil. The explants used were 8 to 10 cm height, with three to four leaves.

Microbial isolation and characterization

Endophytic diazotrophic bacteria were isolated from root and stem samples of pineapple (*Ananas comosus* L. Merrill) cultivar ‘Cayenne Champac’ (Champaka) (AB202) collected in Ceará, variety ‘Pérola’ (AB213) from Paraíba State, banana (*Musa* spp.) cultivar ‘Pacovan’ (BA227) and ‘Prata’ (BA234) from Ceará State. Following the purification steps, bacterial strains were characterized by the morphology of colonies on solid JNFb medium and by their ability of using different carbon sources in semi-solid and semi-specific media, according to Weber et al. (1999), and by sequencing and phylogenetic analysis of 16S rRNA genes. The molecular data based-method was performed at the Center of Chemical, Biological and Agricultural Research (CPQBA, Campinas State University, São Paulo State, Brazil). Briefly, total genomic DNA of the bacteria was purified according to Young and Blakesley (1991) and the fragments of the 16S rRNA genes were amplified and sequenced following Rainey et al. (1996), using an automatic system MegaBace 500 (GE Healthcare). The data obtained were compared with 16S rRNA sequence data from type strains available in the public databases GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>) and RDP (Ribosomal Database Project, Wisconsin, USA,

<http://www.cme.msu.edu/RDP/html/index.html>); using nucleotide blast and RDP sequence match routines. All bacterial strains were stored at the laboratory of Soil Microbiology of Embrapa Tropical Agroindustry, Fortaleza, Ceará, at -18°C in liquid Dygs medium (Rodrigues Neto et al. 1986) with glycerol (50%). The Foc was isolated from rhizome tissue of symptomatic plants of the cultivar ‘Maçã’ obtained from local production areas. Typical structures of that pathogen were confirmed during the purification step on potato dextrose agar (PDA), and plates were maintained in a refrigerator at $5\pm 1^{\circ}\text{C}$ before preparing the fungal inoculum.

The bacterial strains were deposited at CBMAI (Brazilian Collection of Environmental and Industrial Microorganisms, Campinas, São Paulo State), under the accession numbers CBMAI 255 (= AB202), CBMAI 256 (= AB213) and CBMAI 258 (= BA227), except for BA234, which was deposited at Embrapa Agrobiologia, in Seropédica, Rio de Janeiro State.

Microbial inoculation

Bacterial strains were grown in liquid Dygs medium on a rotary shaker (125 rpm) for 24 h, at 30°C , whilst the *Fusarium* strain was grown in the medium with same formulation on a rotary shaker (125 rpm) for 48 h, at 30°C . A final concentration of 5×10^7 colony forming units (CFU) per ml of the bacterial suspensions was obtained as measured by optical densities (OD) of 0.7, using a Genesys 336002 Spectrophotometer ($\lambda=550\text{ nm}$).

Cultures of different bacterial strains were used to inoculate the banana plantlets. Groups of four uniform size seedlings were transferred into tubes containing 10 ml of sterilized saline solution (1 mg K_2HPO_4 , 0.5 mg MgSO_4 , 0.2 mg NaCl , 0.5 mg $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 0.8 μg $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 24 μg $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 28 μg H_3BO_3 , 20 μg $\text{NaMoO}_4\cdot 2\text{H}_2\text{O}$, 23.5 μg $\text{MnSO}_4\cdot \text{H}_2\text{O}$ and 10 μl FeEDTA 1.64% solution), adjusted to pH 6.5. Aliquots of 4.0 ml of respective strain suspension were added to each tube. Plants immersed only in the media served as control. Plants were maintained at 28°C in growth chamber for 24 h. After bacterial suspensions discharge, each treatment tube was added with Foc spores suspension. Another part of the treatment tubes was added by saline suspensions without Foc to comparison. The plantlets were

then maintained at 28°C in growth chamber for another 24 h. Plants were used for pots greenhouse experiments.

Banana pots experiment

The experiment was conducted at the greenhouse comprising two factorial assays, arranged in a randomized complete block design. The first assay occurred from February to May 2006 and had eight treatments with six replicates. Soil used in plastic pots (2.5 l) was autoclaved washed sand plus vermiculite (3:2, v v⁻¹) with trace nutrients composition. The treatments were:

- Four levels of bacterial inoculation: *Burkholderia* sp. AB202 (=CBMAI 255), *Herbaspirillum* sp. BA227 (=CBMAI 258), a combination of them, and uninoculated control;
- Two levels of fungal inoculation: Foc and uninoculated control

The second assay occurred from May to July 2006 and had 16 treatments and six replicates. It was carried out in pots containing 2.5 l of an autoclaved Haplic Arenosol soil, characterized according to van Raij et al. (2001), and presenting pH in CaCl_2 (0.01 M)=7.0; Organic Matter=8.5 mg dm^{-3} ; P (resin)=7.0 mg dm^{-3} ; K+ (resin)=1.9 $\text{mmol}_c \text{dm}^{-3}$; Ca+2 (resin)=19.5 $\text{mmol}_c \text{dm}^{-3}$; Mg+2 (resin)=3.5 $\text{mmol}_c \text{dm}^{-3}$; Na+ (Merlich)=3.0 $\text{mmol}_c \text{dm}^{-3}$; Cu (DTPA)=0.2 mg dm^{-3} ; Fe (DTPA)=9 mg dm^{-3} ; Mn (DTPA)=11.6 mg dm^{-3} and Zn (DTPA)=1.1 mg dm^{-3} . The treatments were as follows:

- Eight levels of bacterial inoculation: *Burkholderia* strains AB202 (=CBMAI 255) and AB213 (=CBMAI 256), *Herbaspirillum* strains BA227 (=CBMAI 258) and BA234 (=BR12141), a combination of AB202 + BA227 strains, a combination of AB213 + BA234 strains, a combination of all of them, uninoculated control;
- Two levels of fungal inoculation: Foc and uninoculated control

The soil was maintained humid in the pots by a nebulisation system, which was daily activated for four or five times for every 5 min. Fortnightly, each vase received 50 ml of Hoagland solution (Hoagland and Arnon 1950), modified by N content (4.2 $\text{mg NH}_4\text{NO}_3$).

Plant surviving and diazotrophic bacteria and Foc colonization

Sampling occurred 120 days after cultivation for the first assay and after 75 days for the second assay. Plants were divided into roots (main and lateral roots) and aerial (basal stem and leaves) pieces. Samples for bacterial density determination were thoroughly washed with water, cut into small pieces from 1 g, and transferred to sterile tubes containing 10 ml of 1% T chloramine for 5 min. The samples were then washed with distilled water, macerated with mortar and pestle, and the homogenate was serially diluted (until 10^{-7}) and inoculated on vials containing JNFb medium (Döbereiner et al. 1995), using three replicates and incubated at 30°C. Dilutions were also plated on PDA medium and incubated at 30°C. After 72 h of incubation, vials were evaluated to determine the most probable number (MPN) of diazotrophic bacteria. Plates were counted for CFU Foc determination. Remaining plant portions were dried for biomass measuring using the formula: $[100 (X-Y) Y^{-1}]$, where X corresponded to inoculated plant weight and Y, control plant weight.

Statistical analysis

Bacteria MPN data were transformed to Bacteria MPN and Foc CFU data, to $\log x+1$, prior statistical analysis. Data were subjected to ANOVA at the significant level and means were compared by Duncan's multiple range test ($P<0.05$).

Results

Characterization of diazotrophs

The bacteria tested were able to use malate, succinate, mannitol and sorbitol as sole carbon source in semi-solid N-free media. Phenotypic data of carbon source utilization (Table 1) combined with 16S rRNA gene analysis (Fig. 1) allowed the identification of the strains AB202 and AB213 as *Burkholderia* sp. and *Burkholderia anthina*, respectively. Strain AB202 presented high similarity values with *B. cepacia* and *B. vietnamiensis* (99%) in the BLASTn search, and was recovered in a tight cluster with *Burkholderia*

cepacia in the phylogenetic tree (Fig. 1). However, this grouping was not supported by high bootstrap value, indicating that this closer relationship is not reliable. In that sense, identification of strain AB202 was not conclusive at the species level. Both strains presented medium or good growth on the sugar substrates D-raffinose, sucrose, D-fructose, D-glucose, L-arabinose and N-acetyl-glucosamine. The two other strains, BA227 and BA234, exhibited none or poor growth with D-raffinose, sucrose and D-fructose semi-solid N-free media. Strain BA234 exhibited blue centered colonies on solid NFb medium, forming veil-like membranes near to the surface of semi-solid N-free JNFb medium, typical characteristics of *Herbaspirillum* genera, as described by Döbereiner et al. (1995). 16S rRNA sequence analysis indicated that strain BA227 was phylogenetically related to different species of *Herbaspirillum* (Fig. 1); however a conclusive identification was not achieved.

Greenhouse experiment

Colonization of banana plants was successfully accomplished by *Burkholderia* spp. and by *Herbaspirillum* sp., as well as by Foc (Tables 2 and 4). Bacteria isolated from the inoculated plants showed the same physiological characteristics as those we observed prior inoculation (Table 1).

First assay ANOVA showed a significant factor interaction on bacteria MPN and on Foc CFU from roots and rhizomes of the inoculated plants, except for bacteria MPN from root plants ($P<0.05$) (Table 2). Bacterial population within those root plants was compared aiming to verify the *Burkholderia* sp. (AB202) inoculation benefit on the plants in the absence of the pathogen. Rhizome plants did not show variation between bacteria population in the absence of Foc, except for *Herbaspirillum* sp. (BA227) and the combination of AB202+BA227.

In the second assay, where the substrate was a Haplic Arenosol, largest diazotrophic bacteria populations were detected on roots in the presence of AB213, BA227, BA234, plus the Foc, when compared to the control samples (Table 4). Increments on the bacteria populations were detected after inoculations with AB202, BA227, the combination of all of them, in the absence of Foc. In the presence of Foc, larger bacteria populations were found out at the rhizomes of banana plants inoculated with AB202, BA234,

Table 1 Growth characteristics of the diazotrophic bacteria used to inoculate ‘Maçã’ banana plantlets

Strains	Carbon sources in semi-solid media							
	D-Raffinose	D-Sucrose	D-Fructose	D-Glucose	L-Arabinose	L-Rhamnose	Mesoerythritol + NH ₄	N-Acetyl glucosamine
AB202	++	+++	++	++	++	++	–	+++
AB213	++	+++	++	++	+++	–	++	+++
BA227	–	+	–	+	+	+	++	++
BA234	–	+	+	++	++	+	+++	++

Growth: good (+++), medium (++), weak (+) not observed (–).

AB213+BA234, AB202+BA227+AB213+BA234, when compared to control samples. These results indicate the preference of the bacteria to colonize specific plant tissues.

Bacteria inoculation also influenced Foc propagules. In the first assay, a reduction of the pathogen propagules was detected on roots of plants bacterized with AB202 and the combination of AB202 plus BA227 strains, when compared to control plants (Table 2). This reduction was also observed in rhizomes of plants bacterized with BA227 and the combination of all the strains. Also, survival rate was higher in the presence of AB202 strain and for the combination of all of them (Table 3), indicating the Foc infection process was blocked. The Foc detection on control plants in the second assay (Table 4) is probably due to the short distance between the pots inside the greenhouse (20 to 40 cm). Even so, roots presented a lowest Foc CFU, when BA227 strain was introduced. There was a reduction of the Foc CFU in plantlets inoculated with BA234 strain and the

combination of AB202 plus BA227, when compared to the control. Surviving plant rates were maximal when AB202, AB213 and the combination of AB213 plus BA234 strains were introduced (Table 4). These inhibition and survival values may be due to *Herbaspirillum*-like bacteria action against Foc infection.

Plantlets also showed a better growth, with largest amount of dry root biomass after inoculation of AB202 in the first assay (Table 3). In the presence of that bacterial strain a relative increment of 35.6% in the root biomass and 23.1% in the total plant biomass were observed. In the second assay (Table 5), an increase on the dry root biomass was observed with BA234 strain inoculation, but those medium values didn't differ of AB213, BA227 and combination of all strains inoculated. Contrary to the first assay, AB202 strain was less effective on root growth induction, may be due to soil substrate and shorter cultivation period. Accumulated total biomass didn't vary significantly between the strains, although a

Fig. 1 Phylogenetic analysis based on partial 16S rRNA gene sequences of the endophytic diazotrophic bacteria and related microorganisms using the Kimura 2p evolutionary model and the neighbor joining method for tree reconstruction. Bootstrap values (1000 replicate runs) greater than 70% are listed. GenBank accession numbers are listed after species names. *Thiobacillus denitrificans* NCIMB 9548^T was used as outgroup

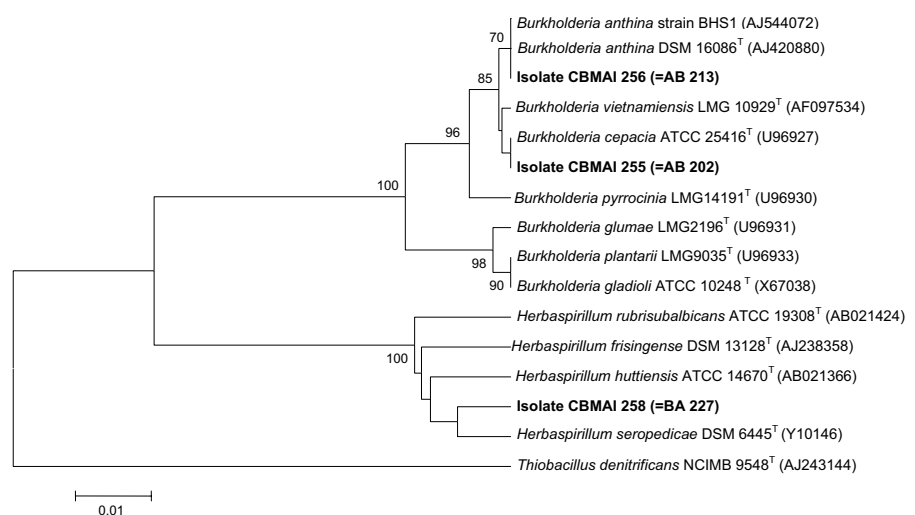


Table 2 Populations of diazotrophic bacteria and Foc unit propagate on roots and rhizomes of ‘Maçã’ banana plants inoculated with *Herbaspirillum* sp. (BA227), *Burkholderia* sp. (AB 202) and the Foc and cultivated on sand and vermiculite

Pathogen	Bacteria				Mean
	Control	AB202	BA227	AB202 + BA227	
MPN diazotrophic bacteria on roots ($\times 10^3 \text{ g}^{-1}$)					
Control	0.74bA	10.58aA	3.69bA	1.75bB	4.19
Foc	2.52aA	5.73aA	2.76aA	5.03aA	4.01
Mean	1.63	8.16	3.23	3.39	
MPN diazotrophic bacteria on rhizomes ($\times 10^2 \text{ g}^{-1}$)					
Control	2.79bA	2.94bA	6.89aB	6.79aA	4.85
Foc	0.36bB	0.36bB	0.29bA	0.76aB	0.44
Mean	1.58	1.64	3.56	3.77	
CFU Foc on roots ($\times 10^2 \text{ g}^{-1}$)					
Control	0.0aB	0.0aB	0.0aB	0.0aB	0.0
Foc	11.07aA	3.31bcA	11.81abA	0.95cA	6.78
Mean	5.53	1.65	5.91	0.48	
CFU Foc on rhizomes ($\times 10^2 \text{ g}^{-1}$)					
Control	0.0aB	0.0aB	0.0aB	0.0aB	0.0
Foc	0.91bA	4.09aA	0.71bcA	0.48cA	1.54
Mean	0.45	2.04	0.36	0.24	

Analysis of three plants with transformed data ($\log x+1$); results followed by the same letter, minuscule in horizontal lines and large in columns, indicate not significant differences at $P<0.05$, using Duncan’s multiple range test.

relative increment of 26.7% was observed in the presence of BA234 strain and 18.3% with AB213 strain.

Discussion

Previous studies of endophytic bacteria have relied on phenotypic and genotypic characterizations for

their identification (Mao et al. 2006; Lodewyckx et al. 2002). Comparative sequence analysis of 16S rRNA genes provides a reliable approximation to species level and cultivation studies help to enhance tentative allocation. Patterns of carbon assimilation in *Herbaspirillum* sp. were similar to the observations made by Weber et al. (1999). Affiliation to this genus was confirmed by BA227 16S rRNA sequences. Carbon assimilation patterns for strains BA227 and BA234

Table 3 Surviving rates and dry biomass of ‘Maçã’ banana plants inoculated with *Herbaspirillum* sp. (BA227), *Burkholderia* sp. (AB 202) and the Foc and cultivated on sand and vermiculite

Treatment	Survival rate		Dry biomass (g plant^{-1})		RI ² (%)
	(%)	Root	Aerial part	Total	
Bacteria					
Control	79.1b	0.662b	1.436a	2.098b	0.0
AB202	100.0a	0.898a	1.684a	2.582a	23.1
BA227	83.3b	0.736ab	1.80a	2.115ab	0.8
AB202+ BA227	100.0a	0.725ab	1.466a	2.191ab	4.7
Pathogen					
Control	95.8 ^a	0.773a	1.540a	2.313a	0.0
Foc	83.3b	0.738a	1.443a	2.181a	-5.7

Analysis of six plants with transformed data (\times^{-2}); results followed by the same letter, within the columns, indicate not significant differences at $P<0.05$, using Duncan’s multiple range test.

²Relative increment= $[100 (X-Y) Y^{-1}]$, where X is the inoculated plant and Y the control plant.

Table 4 Populations of diazotrophic bacteria and Foc unit propagates on roots and rhizomes of ‘Maçã’ banana plants inoculated with *Herbaspirillum* sp. (BA227, BA234), *Burkholderia* sp. (AB 202, AB213) and Foc and cultivated on Haplic Arenosol

Bacteria	Pathogen		Mean
	Control	FOC	
MPN diazotrophic bacteria on roots ($\times 10^3 \text{ g}^{-1}$)			
Control	0.70aA	0.58bB	0.64
AB202	5.24aA	0.74abB	2.98
AB213	3.90aB	39.54aA	21.72
BA227	15.99aB	3.65aA	9.82
BA234	2.48aB	5.47aA	3.96
AB202 + BA227	3.89aA	1.13abB	2.51
AB213 + BA234	7.14aA	6.37aB	6.75
AB202+BA227+AB213+ BA234	18.84aA	6.66aB	12.75
Mean	7.56	8.02	
NMP diazotrophic bacteria on rhizome ($\times 10^2 \text{ g}^{-1}$)			
Control	0.55bA	0.78bA	0.66
AB202	18.95aA	32.88aA	25.91
AB213	5.42abA	8.79abA	7.11
BA227	20.99aA	6.00abA	13.50
BA234	4.55abA	37.19aA	41.34
AB202 + BA227	9.73aA	15.59abA	12.66
AB213 + BA234	14.51aA	55.28aA	34.89
AB202+BA227+AB213+ BA234	146.63aA	27.33aA	86.98
Mean	34.76	22.98	
CFU Foc on root ($\times 10^2 \text{ g}^{-1}$)			
Control	0.17aB	392.74aA	196.45
AB202	0.0aB	26.37abA	13.19
AB213	0.0aB	46.07abA	23.04
BA227	0.0aA	0.79bA	0.39
BA234	0.0aB	11.27abA	5.63
AB202 + BA227	0.0aB	9.20abA	4.60
AB213 + BA234	0.0aB	43.95abA	21.98
AB202+BA227+AB213+ BA234	0.0aB	219.33abA	109.67
Mean	0.02	93.71	
CFU Foc on rhizome ($\times 10^2 \text{ g}^{-1}$)			
Control	0.17aB	92.92aB	46.54
AB202	0.0aB	8.19abA	4.10
AB213	0.0aB	10.00abA	5.00
BA227	0.0aB	50.11abA	25.05
BA234	0.0aB	7.02bA	3.51
AB202 + BA227	0.0aB	2.62bA	1.31
AB213 + BA234	0.0aB	15.67abA	7.83
AB202+BA227+AB213+ BA234	0.0aB	21.78abA	10.89
Mean	0.02	26.04	

Analysis of three plants with transformed data ($\log x+1$); results followed by the same letter, minuscule in lines and large in columns, indicate not significant differences at $P<0.05$, using Duncan's multiple range test.

strains differed from species *H. seropedicae* (Baldani et al. 1986), *H. rubrisubalbicans* (Baldani et al. 1996) and *H. frisingense* (Kirchhof et al. 2001) and as such they may be included in a new diazotrophic species.

Bacterial colonization of plants was effective, with some variations in populations. These bacteria MPN variations in both assays show a trend of the colonization site within plants and the strain AB202

Table 5 Surviving rates and dry biomass of ‘Maçã’ banana plants inoculated with *Herbaspirillum* sp. (BA227, BA234), *Burkholderia* sp. (AB 202, AB213) and Foc and cultivated on Haplic Arenosol

Treatment	Survival rate		Dry biomass (g plant ⁻¹)		RI ^a (%)
	(%)	Root	Aerial part	Total	
Bacteria					
Control	66.7b	0.850b	1.586a	2.436a	0.0
AB202	100.0a	0.878b	1.779a	2.658a	9.1
AB213	100.0a	0.988ab	1.893a	2.881a	18.3
BA227	91.7ab	1.014ab	1.918a	2.705a	11.0
BA234	91.7ab	1.215a	1.870a	3.086a	26.7
AB202 + BA227	83.3ab	0.928ab	1.889a	2.817a	15.6
AB213 + BA234	100.0a	0.916ab	1.934a	2.850a	17.0
AB202 + BA227 + AB213 + BA234	91.7ab	0.922ab	1.510a	2.432a	-0.2
Pathogen					
Control	97.6a	1.140a	2.055a	3.196a	0,0
Foc	83.3b	0.787b	1.540b	2.327b	-27.2

Analysis of six plants with transformed data (\times^{-2}); results followed by the same letter, within the columns, indicate not significant difference at $P < 0.05$, using Duncan's multiple range test.

^aRelative increment = $[100 (X - Y) Y^{-1}]$, where X is the inoculated plant and Y the control plant.

seems to be greatly adapted to roots in plants grown using poor fertile soil. *B. vietnamiensis* has been reported from the rhizosphere of rice plants (Gillis et al. 1995) and bacteria associated with *B. cepacia* have been found in larger numbers on banana roots (Weber et al. 1999). According to these authors, growth of *Burkholderia*-like bacteria prefers substrates with higher sugar concentrations, in comparison to other endophytic bacteria, making them more competitive for colonization sites.

In this work, diazotrophic bacteria were also detected in roots and rhizomes of control banana plants (Tables 2 and 4), in accordance with Weber et al. (2000) and Weber et al. (2003), who isolated diazotrophic bacteria from roots and rhizomes of micropropagated banana plantlets cv. ‘Prata Anã’ and ‘Caipira’, and from pineapple, respectively. Considering that micropropagated banana plantlets are sensitive to soil pathogens (Ayyadurai et al. 2006), we suggest the inoculation of plantlets with selected diazotrophic bacteria.

Bacterial–fungal pathogen interactions have been extensively reported, including studies with *Burkholderia* sp. strains against *Fusarium* sp. (Whipps 2001). In this work we observed a reduction of Foc unit propagules, suggesting that endophytic colonization reduces *Fusarium* wilt severity in the cultivar ‘Maçã’

plantlets. Since the first experimental evidence for the involvement of bacteria-mediated induced resistance against *Fusarium* wilt (Van Peer et al. 1991), several studies have suggested that treatments with selected endophytic bacteria could induce plants to defend themselves against pathogen attack (Kloepper 1993). Induced resistance has also been associated to bacterial presence in roots (Loon et al. 1998). Few plantlets showed internal symptoms of *Fusarium* wilt in this study, although this characteristic could not be differentiated between treatments. Determination of propagules in roots and rhizomes is therefore a practical measure and can be employed as a technique for banana genotypes to obtain a degree of sensibility to Foc colonization. This antagonism was also detected via an *in vitro* assay, and Muniz et al. (2007) observed antagonism of AB202, AB213, BA234 and BA227 strains against Foc with inhibition values corresponding to 78, 78, 74 and 21 cm² of culture radial growth, respectively. Previous data obtained from *Herbaspirillum* species (Baldani et al. 1986, 1996; Kirchhof et al. 2001) and other endophytic diazotrophic bacteria from grasses in Brazil (Baldani and Baldani 2005) did not show antagonism against fungi, indicating a possibility of strains BA227 and BA234 representing different bacterial species.

Gillis et al. (1995) observed production of siderophores by *B. vietnamiensis* TW75^T and antagonist effect against *Fusarium* and other soil fungi (Bevivino et al. 1994). In addition, Weber and Freire (2003), using the cultivar ‘Maçã’ plantlets colonized by AB202, observed reduction of *Fusarium* wilt in field banana. Increments in biomass may be also related to endophytic bacterization. Larger biomass increments were detected by Weber et al. (2000) working with cv. ‘Prata Anã’ and ‘Caipira’ after inoculation of diazotrophic bacteria isolated from fruit plants.

Based upon the results obtained we suggest the inoculation of endophytic diazotrophic bacteria as biofertilizers and antagonists to Foc on ‘Maçã’ banana plantlets. This should be considered as an additional option in the integrated management of *Fusarium* wilt, considering that *B. cepacia* (Pan et al. 1997), *P. fluorescens* (Saravanan et al. 2003), *P. aeruginosa* (Ayyadurai et al. 2006), *T. harzianum* (Thangavelu et al. 2003), *Trichoderma* sp. (Nel et al. 2006), non-pathogenic *F. oxysporum* (Forsyth et al. 2006) and *Streptomyces* sp. (Getha et al. 2005) have been indicated for biocontrol of *Fusarium* wilt in banana.

Conclusions

1. The diazotrophic endophytic bacteria *Burkholderia* and *Herbaspirillum* characterized in this study showed the potential to reduce Foc attack and to promote the growth of banana ‘Maçã’ plants.
2. Foc propagules can be determined directly from banana plant roots and rhizomes, aiming at the establishment of the resistance degree to Foc.
3. Strains of *Burkholderia* sp. (AB202) and *Herbaspirillum-like* (BA234) induce increments on banana plant biomass production, after four and 2 months of cultivation, respectively.

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