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Identification of culturable endophytes in 'Champaka' pineapple grown in an organic system

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The objective of the study was to characterize culturable diazotrophic endophytic bacteria from 'Champaka' pineapple plants during the fruiting period in an organic system. Micropropagated plants were inoculated with the diazotrophic endophytic bacterium, strain AB 219a, during acclimatization phase. In the field, the plants were fertilized with different dosages and sources of organic compost. After 17 months of field cultivation, roots and stems were collected for the isolation of endophytes, DNA extraction, 16S rDNA amplification and molecular analysis. A total of nine bacterial groups were identified, and species *Burkholderia silvatlantica, Azorhizobium caulinodans, Pantoea eucrina, Erwinia* sp. and unculturable bacterium occurred in inoculated plants. In contrast, non-inoculated plants were associated endophytes related to *Burkholderia cenocepacia*, *Azospirillum brasilense*, *Enterobacter oryzae*, *Erwinia* sp. and *Sphingobium yanoikuyae*. Segments of 360 base pair from nifH gene were amplified from representative endophytes within identified species, except from the *Pantoea eucrina* strain AB 295. 16S rDNA phylogenetic analysis of endophytes isolated from inoculated plants revealed distinct families: Xanthobacteraceae, Burkholderiaceae and Enterobacteriaceae; and from non-inoculated plants, Rodospirillaceae and Sphingomonadaceae families were also identified. The 'Champaka' pineapple plants at fruiting stage associate with endophytes related to α, β and *γ Proteobacteria*, after plantlets inoculation or not with the diazotrophic bacterium *Burkholderia silvatlantica* (AB 219a). Most of those endophytes present *nifH* gene, a characteristic for nitrogen-fixing bacteria.

Key words: Biodiversity, nitrogen-fixing bacteria, *Ananas comosus,* microbial ecology.

INTRODUCTION

The association of diazotrophic bacteria with fruit plants has been described by many different researchers. In 1980, bacteria from the *Azospirillum* genus were initially identified in the rhizosphere of tropical fruits (Subba-Rao, 1983; Ghai and Thomas, 1989). Later, several bacteria belonging to the genera *Azospirillum, Herbaspirillum* and *Burkholderia* have been isolated from the roots and aerial parts of banana and pineapple plants (Weber et al., 1999). *Gluconoacetobacterdiazotrophicus* (Tapia-Hernández et al., 2000), and bacteria from the genera *Klebsiella, Bradyrhizobium* and *Serratia* (Ando et al., 2005) have been isolated from pineapple plants.

In pineapple, the benefits of plant-diazotrophic bacteria associations have been documented. The growth of seedlings micropropagated from the cultivar 'Champaka' was enhanced in the presence of strain AB 219, which was isolated from the 'Smooth Cayenne' pineapple and related to *Asaia bogorensis* (Weber et al., 2003). The induction of plant growth was reported for 'Victoria' cultivar, after the seedlings inoculation with several

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endophytic diazotrophic and epiphytic bacteria (Baldotto et al., 2010).

The better performance of pineapple plants in the presence of diazotrophic endophytic bacteria may be due primarily to the production of phytoregulators, but the biological nitrogen fixation associated with diazotrophic bacteria in the fruit crop may also be a contributing factor. Baldotto et al. (2010) observed an increase in leaf nitrogen (up to 193%) in 'Vitoria' cultivar seedlings inoculated with diazotrophic bacteria. In addition, Ando et al. (2000) described a lower abundance of natural $\delta^{15}N$ in the leaves of the 'Pattavia' cultivar when compared to other pineapple plants in the same growing area. Later, Ando et al. (2005) reported sequences of the *nifH* gene similar to those of the genera *Klesiella* and *Serratia* in leaves of 'Pattavia' and 'Smooth Cayenne' cultivars.

Considering the economic and social importance of the pineapple cultivation in Brazil, one of the leading producers (FAO, 2012), particularly in the Northeast region, where small farmers account up to 40% of the national fruit production (IBGE, 2012), the development of agro-technology to reduce production costs and to improve fruit quality is needed. Some problems are associated with cultivation of 'Pérola', 'Smooth Cayenne' and 'Champaka' (Cayenne Champac, Golden or MD2) suckers, when directly obtainned form fields. These cultivars are susceptible to fusarium wilt, a disease caused by *Fusarium subglutinans*, which is devastating (Reinhardt et al., 2002) and can leaded to impracticability of some production areas.

Biofertilizers containing plant growth promoting microorganisms increase the production of fruit plants (Mia et al., 2010). Besides, seedling inoculation with growth-promoting diazotrophic bacteria is environmental friendly. Inoculants containing *Azospirillum* spp. (Hungria et al., 2010) and other N_2-fixing bacteria (Moreira et al., 2010) have been tested in graminous plants. According to Abreu-Tarazi et al. (2010), micropropagated 'Gomo de Mel' pineapple seedlings contain bacteria related to *Actinobacteria*, α and β *Proteobacteria*. Also, in fruit crops it could become a profitable technology; so more detailed studies are needed on the identification and selection of fruit diazotrophic bacteria and their interactions with other endophytes. Knowledge of structures of endophytic bacterial populations in plants can help us to understand the plant-bacterium relationships. In this study we characterize culturable endophytes from the 'Champaka' pineapple plants at fruiting stage after seedlings inoculation with diazotrophic bacterium and cultivation in an organic irrigated orchard intercropped with sapota.

MATERIALS AND METHODS

Isolation and quantification of culturable endophytes

The 'Champaka' pineapple plants were inoculated or not with a a diazotrophic bacterium, 10^8 cells of the strain AB 219a, during acclimatization phase, and evaluated for root and stem endophytic colonization at fruiting stage. In the field, both plant groups were fertilized with three doses (40.17, 80.35 and 120.5 m^3 ha^{-1}) of three sources of composts; a) bovine manure, shredded leaves of wax palm (*Copernicia cerifera*) and sugar cane bagasse, b) plant debris and phosphate rock powder and c) sugar cane bagasse, coconut fiber, bovine manure, phosphate rock powder, rock powder (MB-4) and fruit residues of West Indian cherry (*Malpighia emarginata* DC), in an irrigated orchard intercropped with Sapota. Representative plants (54) from field treatments in three replicates were selected and fresh roots and stems were collected (taken after fruit harvest at 17 months), and these samples were taken to the Embrapa Tropical Agroindustry in Fortaleza, Brazil, for processing and isolating the culturable endophytes.

Root fragments (< 2 mm in diameter) and stems (1 g of fresh mass) without spots or necrosis were superficially sterilized in a solution of 1% Chloramine-T (monochloramine), washed three times in sterile water, macerated and serially diluted with sterile saline (up to 10^{-7}). The diluted suspensions were inoculated into penicillin-type vessels containing 5 ml of semi-solid JNFb N-free medium, according to Döbereiner et al. (1995). The vessels were incubated for five days in a BDO chamber regulated for 30°C, and the most probable number (MPN) of endophytes was determined for all treatments.

The MPN of endophytes from roots and aerial parts were submitted to analysis of variance, using the GLM procedure (General Linear Model) of the SAS® System (SAS Institute, 2000). The comparison of average values obtained from field treatments was achieved by using the F-test for contrasts. After bacterial growth in flasks was observed, the cultures were transferred to new vessels containing JNFb semi-solid medium. Typical bacterial growth, forming subsurface pellicles in the semi-solid medium were streaked onto Petri dishes containing solid Dygs medium (g $liter^{-1}$) 10 glucose, 2 malic acid, 1.5 peptone, 2 yeast extract, 0.5 K_2HPO_4, 0.5 $MgSO_4$ $7H_2O$, 1.5 glutamic acid, 15 agar) and the pH was adjusted to 6.5 (Weber et al., 1999). Cells from isolated colonies on solid medium were again inoculated into semi-solid JNFb medium, and the process was repeated until pure cultures were obtained and stoked in freezer (50% glycerol, at -18°C). Culturable endophytes from roots and stems of the 'Champaka' pineapple plants, and the strain AB 219a, used for plantlets inoculation can be found in the culture collections at Embrapa Tropical Agroindustry http://www.cnpat.embrapa.br) and/or at Embrapa Agrobiology (http://www.cnpab.embrapa.br).

Physiological characterization of culturable endophytes

Endophytes from different field treatments, and the strain AB 219a were activated in liquid Dygs medium and after reaching exponential growth; they were evaluated for their ability to use different carbon sources: D-fructose, sucrose, mannitol, inositol, D-galactose, succinate, oxalate, citrate, (D+) rhamnose, (L+) tartrate, and D, L-malate in semi-solid media containing salts of JNFb (pH 5.8), according to Weber et al. (1999). Also, the strain AB219a, used for plantlets inoculation, was evaluated for its ability to use different organic substrates under nitrogen fixation conditions.

DNA extractions, 16S rDNA amplification, sequencing and phylogenetic analysis

Representative culturable endophytes from field treatments, and the strain AB 219a were grown on solid Dygs medium and then used to total DNA extraction. The 16S rDNA gene was amplified by polymerase chain reaction (PCR) with primers Y1 and Y3 (Young et al., 1991). For sequencing, the PCR product was precipitated with 5M NaCl and 70% ethanol and sequenced using the MegaBACE1000 automated DNA sequencer (Amersham Bioscience)

Table 1. Population of endophytic bacteria detected within JNFb semi-solid medium (log g^{-1}MPN) from the 'Champaka' pineapple plants after fruit harvesting, as a function of the absence or presence of inoculation with the strain AB 219a and the sources and dosages of compost.

Plant	Compost	Root[1]			Stem		
		Dosage (m^3 ha^{-1})					
		40.17	**80.35**	**120.5**	**40.17**	**80.35**	**120.5**
	A	6.089 ± 0.053	6.082 ± 0,055	5.930 ± 0.219	2.424 ± 0.717	2.608 ± 0.399	2.561 ± 0.721
Control	B	5.941 ± 0.666	5.945 ± 0.793	6.342 ± 0.416	2.223 ± 0.482	2.305 ± 0.506	3.460 ± 0.529
	C	6.170 ± 0.744	6.149 ± 0.509	6.721 ± 0.407	2.603 ± 0.510	2.414 ± 0.085	2.616 ± 0.472
Average		6.067	6.059	6.331	2.417	2.442	2.879
	A	6.089 ± 0.014	6.005 ± 0.030	5.849 ± 0.362	2.221 ± 0.194	3.522 ± 0.105	2.750 ± 0.328
Inoculated	B	6.408 ± 0.898	6.102 ± 0.368	6.717 ± 0.296	2.270 ± 0.314	2.912 ± 0.707	2.919 ± 0.215
	C	6.759 ± 0.434	6.873 ± 0.225	6.148 ± 0.664	3.290 ± 0.937	2.085 ± 0.238	3.086 ± 0.234
Average		6.419	6.327	6.238	2.594	2.840	2.918

with the Y1 and Y3 primers and five others (16S362f, 16S786f, 16S1203f, 16S110r, and 16S850r) (Soares-Ramos et al., 2003). After 16S sequence assembly with approximately 1,500 base pairs, each sequence was subjected to similarity analysis using the BLAST algorithm (Altschul et al., 1997) and the non-redundant NCBI database (http://www.ncbi.nlm.nih.gov/). The sequences with the greatest similarity to each isolate were selected for alignment using Clustal W (Thompson et al., 1994). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and the program MEGA4 (Tamura et al., 2007) based on distance calculations according to Kimura (1980). Bootstrap analysis was conducted with 1,000 replicates (Felsenstein, 1985).

Polymerase chain reaction amplification of *nifH* gene

The *nifH* gene (360 base pair) from representative culturable endophytes from filed treatments, and from the strain AB 219a were amplified using primers PolF (5'-TGC GAY CCS AAR GCB GAC TC-3') and PolR (5'-ATS GCC ATC ATY TCR CCG GA-3') (Poly et al., 2001). The PCR mix reactions were performed in a final volume of 25 µl containing: 0.2 mM of each dNTP; buffer 1x; 3 mM $MgCl_2$; 0.5 µM of each primer; DMSO 5%; 1 U GoTaq DNA polymerase and 25-50 ng genomic DNA. The touchdown PCR protocol was used for increased specificity and sensitivity in PCR amplification. The cycling conditions were as follow: denaturation step at 95°C for 10 min followed by 16 touchdown cycles involving denaturation at 95°C for 45 s, annealing for 45 s, and primer extension at 72°C for 45 s, with a 0.5°C decrease in annealing temperature per cycle starting at 72°C. Additional 5 cycles were performed at annealing temperature of 57°C for 45 s and one cycle of final extension at 72°C for 10 min. The reactions were carried out in an Eppendorf Mastercycler® thermocycler and amplified fragments were analyzed by horizontal electrophoresis on a 1.5% agarose gel at 100 V for 2 h. Gels were stained with ethidium bromide, visualized under UV light and photographed using Kodak® Gel Logic 100 Imaging System.

RESULTS

The endophyte AB 219a used for seedlings inoculation was a stocked subculture of diazotrophic bacteria. It was previously named as AB 219 (Weber et al., 2010), and 16S rDNA gene had been partially sequenced and had been related to *Asaia bogorensis* (Weber et al., 2003). Here the strain AB 219a was subjected to complete sequencing of the ribosomal gene, these sequences were submitted to NCBI database (accession HQ 706106), and could be now identified as *Burkholderia silvatlantica*.

The 'Champaka' pineapple plants presented endophytic bacterial colonization at fruiting stage, regardless of seedlings inoculation and field treatments, and higher population densities was detected in roots when compared to plant stems (Table 1). Field compost treatments influenced the MPN of endophytes in roots, as could be observed with contrasts of organic sources A and C (p>0.0112); and in stems by contrasts with the compost dosages 40.17 and 120.5 m^3 ha^{-1} (p>0.0353); as so with the seedlings inoculation (p >0.0001). The strong evidence of beneficial endophytic association in inoculated pineapple stem may be due to the preferential site colonization of the strain AB 219a.

Culturable endophytes were obtained from inoculated (12 strains) and non-inoculated plants (13 strains) (Table 2), and they are able to grow within different organic substrates under nitrogen-fixing conditions (Table 3). The consequent bacterial growth in semi-solid N-free media allows establishing nine groups: first three bacterial groups (I to III), from control plants; other three

Table 2. Endophytes isolated from the 'Champaka' pineapple plants, as a function of the absence or presence of inoculation with the strain AB 219a and the sources of compost.

Plant	Source of compost	Strains (nº)	Root endophytes	Stem endophytes
Control	A	4	AB 280 (*_a_)	AB 294 (_a_), AB 291 (_a_), AB 301 (_c_)
	B	5	AB 287 (_a_), AB 288 (_b_)	AB 300 (_b_), AB 296 (_c_), AB 297 (_c_)
	C	4	AB 285 (_b_)	AB 305 (A), AB 292 (_a_), AB 293 (_c_)
Inoculated	A	3	AB 281 (_a_), AB 282 (_a_)	AB 290 (_b_)
	B	3	AB 286 (_b_), AB 289 (_c_)	-
	C	4	AB 284 (_a_)	AB 295 (_a_), AB 304 (_a_), AB 299 (_c_)

Dosage of compost used (*_a_ = 40.17 m^3 ha^{-1}, _b_ = 80.35 m^3 ha^{-1} and _c_ = 120.5 m^3 ha^{-1}). Selected strains: AB 286 (=BR12271), AB 287 (=BR12272), AB 299 (=BR12217), AB 292 (=12274), AB 280 (=BR12268), AB 281 (=BR12269), AB 284 (=BR12279), AB 285 (=BR12270), AB 290 (=BR12273), AB 294 (=BR12275), AB 295 (=BR12276), AB 301 (=BR12278) and the strain used for the plantlet inoculation (AB 219a = BR12266) were submitted to the culture collection of Embrapa Agrobiology (BR) in Seropédica, Brazil; and all other strains were maintained in the culture collection of Embrapa Tropical Agroindustry in Fortaleza.

Table 3. Ability of grouped endophytes from the 'Champaka' pineapple inoculated with the strain AB 219a, non-inoculated (Control) and both (inoculated or not inoculated), to use carbon sources in semi-solid media containing salts of JNFb (pH 5.8).

Group	Plant treatment	Carbon sources									
		(D+) Fructose	Sucrose	Mannitol	Inositol	(D+) Galactose	Succinate	Oxalate	Citrate	(L+) Rhamnose	(L+) Tartrate
I	Control	+	++	+	+	++	+	-	++	-	++
II	Control	+	-	+	+	+	+	++	++	+	++
IIII	Control	+++	++	+	+	++	+++	++	+	++	++
IV	Inoculated	++	++	+++	++	++	++	++	++	++	+
V	Inoculated	+	+	+	+	+	+	++	++	+	+
VI	Inoculated	-/+	-/+	+	+	+	+	+	+	+	-
VII	Both	-/+	-/+	-/+	+	+	++	-	+	+	-/+
VIII	Both	+	-/+	+	+	+	++	-/+	+	+	++
IX	Both	+	++	++	+	+	+	-/+	++	++	+

*Strains from groups: I (AB 280, AB 288), II (AB 291, AB 292), III (AB 293, AB 297, AB 301, AB 305), IV (AB 284, AB 304), V (AB 282), VI (AB 281, AB 286), VII (AB295, AB 300), VIII (AB 294, AB 299), and IX (AB 285, AB 287, AB 290, AB 296). **Good (+++), medium (++), poor (+), or null (-) growth after five days of incubation at 30 ºC. All isolates grew within JNFb medium.

Bacterial groups (IV to VI), from inoculated plants; and last three endophyte groups (VII to IX), from both plant treatments, with or without bacterial inoculation. We should mention that strains AB 284 and AB 304 (group IV) isolated from root and from stem of inoculated plants that received compost C (Table 2), exhibited growth in semi-solid media (Table 3) similar to growth we observed for the bacterium AB 219a. Also, a higher

Table 4. Analyses of sequence similarity of the 16S rDNA gene in selected endophytes from the 'Champaka' pineapple plants inoculated or not with the strain AB 219a and grown in an organic system.

Strain	Bacterial group	Treatment	Plant part	16S base pairs	Proximity of bacterial group BLASTn	E-value	Coverage (%)	ID (%)
AB 280	I	Control	Root	1507	CP000959.1 *Burkholderia cenocepacia* (MC0-3)	0.0	100	99
AB 292	II	Control	Steam	1471	AB120764.1 *Sphingobium yanoikuyae*	0.0	98	99
AB 301	III	Control	Steam	1447	DQ288687.1 *Azospirillum brasilense* (MTCC4036)	0.0	99	99
AB 294	VIII	Control	Steam	1522	EF522135.1 *Erwinia* sp. (CU208)	0.0	100	99
AB 285	IX	Control	Root	1504	EF488760.1 *Enterobacter oryzae* (Ola 01)	0.0	97	99
AB 287	IX	Control	Root	1500	EF488758.1 *Enterobacter oryzae* (Ola 50)	0.0	98	99
AB 284	IV	Inoculated	Root	1522	AY965240.1 *Burkholderia silvatlantica* (SRMrh-20)	0.0	98	99
AB 281	VI	Inoculated	Root	1485	AP009384.1 *Azorhizobium caulinodans* (ORS 571)	0.0	95	99
AB 286	VI	Inoculated	Root	1362	AP009384.1 *Azorhizobium caulinodans* (ORS 571)	0.0	100	99
AB 295	VII	Inoculated	Steam	1519	HQ455824.1 *Pantoea eucrina* (CT194)	0.0	99	99
AB 299	VIII	Inoculated	Steam	1503	EF522135.1 *Erwinia* sp. (CU208)	0.0	100	99
AB 290	IX	Inoculated	Steam	1505	FM872505.1 Uncultured bacterium (clone FA01C07)	0.0	99	98

* Strain AB 219a presented 1492 16S base pairs, E value 0.0 and coverage 100%, and ID 99%, **GenBank accession sequences of selected endophytes: AB 299 (=BR12217) HQ706104, AB 292 (=12274) HQ706105, AB 280 (=BR12268) HQ706107, AB 281 (=BR12269) HQ706108, AB 284 (=BR12279) HQ706109, AB 285 (=BR12270) HQ706110, AB 290 (=BR12273) HQ706111, AB 294 (=BR12275) HQ706112, AB 295 (=BR12276) HQ706113, AB 301 (=BR12278) HQ706114; and the AB 219a (=BR12266) HQ 706106used for plantlet inoculation.

endophytes MPN was detected in roots of plants from plant treatment receiving bacterial incoculum and compost C in comparison to the control plants receiving just that organic fertilizer ($p > 0.012$), and the differences between those treatments (Table 1) may be related to organic constituents of the compost C and soil nutrient balance.

Representative culturable endophytes from all bacterial groups (Table 3) were submitted to DNA extraction and the 16S rRNA gene was sequenced (Table 4), with identification of different bacterial species (Figure 1). From stems of inoculated pineapple plants, endophytes closely related to *Erwinia* sp. (strain AB 299), *Pantoea eucrina* (strain AB 295) and uncultured bacterium (strain AB 290), also, species belonging to Enterobacteriaceae (γ *Proteobacteria*) were obtained; from roots of the inoculated plants we isolated endophytes related to *Azorhizobium caulinodans* (AB 281 and AB 286), *Xanthobacteraceae* (α *Proteobacteria*), to *Burkholderia silvatlantica* (strain AB 284), Burkholderiaceae (β *Proteobacteria*). Plants association with endophytic bacteria may persist in an organic cropping system, so the diazotrophic bacterium (strain AB 219a) used for plantlets inoculation (Weber et al., 2010), also was closely related to *B. silvatlantica* (Table 4). From stems of the control plants we obtained endophytes closely related to *Azospirillum brasilense* (strain AB 301), belonging to *Rodospirillaceae,* to *Sphingobium yanoikuyae* (strain AB 292)*,* belonging to *Sphingomonadaceae,* these both species are from α*Proteobacteria*, and to *Erwinia* sp. (strain AB 294), γ *Proteobacteria*; from roots of the control plants were obtained endophytes closely related to *B. cenocepacia* (strain 280) and *Enterobacter oryzae* (strains AB 285 and AB 287), positioned into Burkholderiaceae and Enterobacteriaceae families, respectively.

Endophytes from different groups (Table 3) and closely related to different bacterial species (Table 4) were evaluated for *nifH* gene, using the PolF/PolR primers (Poly et al., 2001) for *nifH* fragment amplification. Also, the AB 219a, used for plantlets inoculation (Weber et al., 2010), was tested. Employed primers amplified a single and correct sized band in eleven of twelve DNA strains tested (Figure 2). The gene presence was a strong evidence of bacterial nitrogen-fixing capacity, so only for the strain AB 295, related to *Pantoea eucrina*, the *nifH* gene fragment was not detected by using that primer combination. Also, we should observe that AB 295 and AB 300 grew

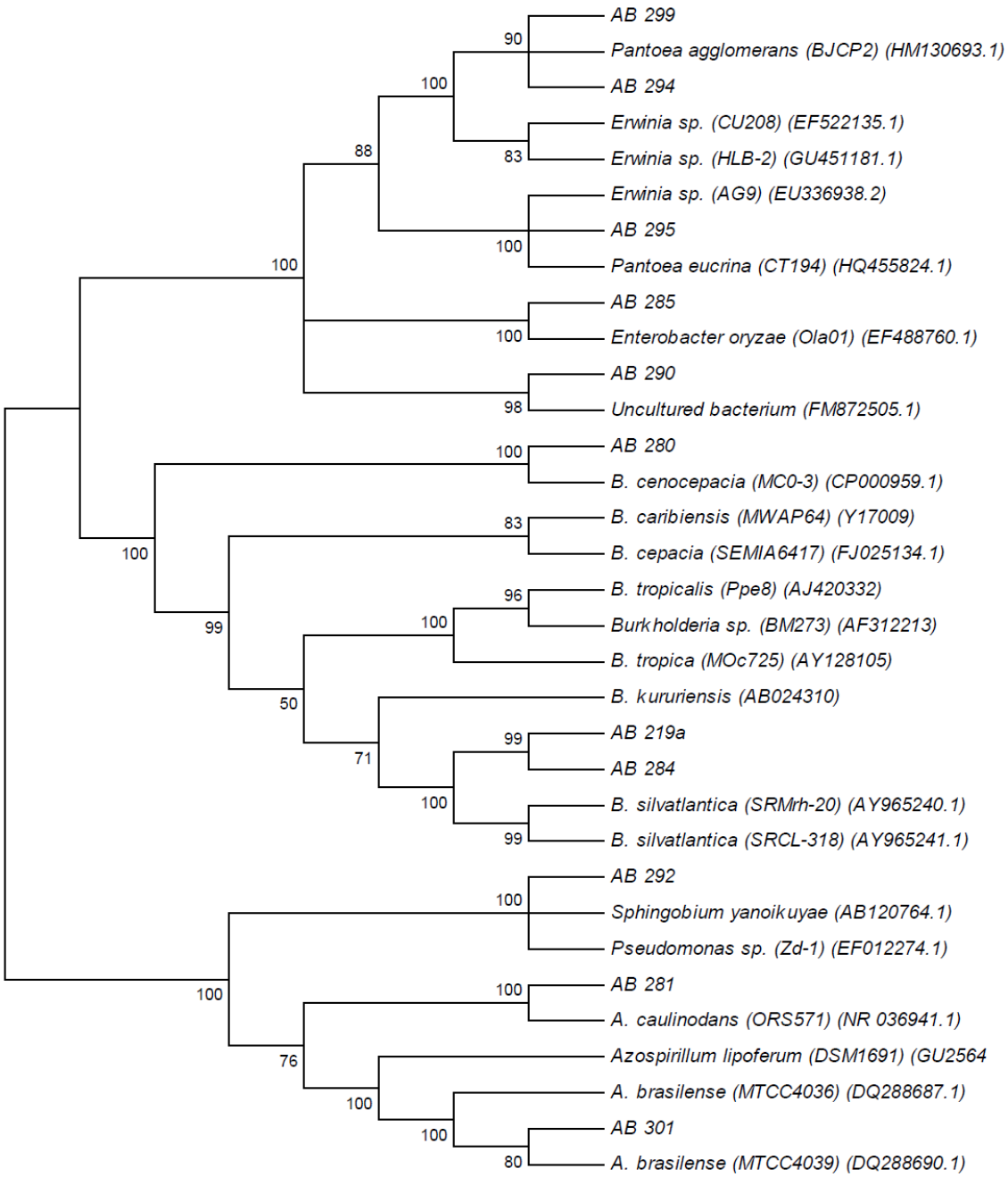

Figure 1. Phylogenetic tree based on 16S sequencing of selected culturable endophytes isolated from the 'Champaka' pineapple plants inoculated or not with the strain AB 219a and grown in an organic system. The program MEGA 4 and the neighbor-joining method were used to construct the tree.

poorly in semi-solid media (Table 3), under nitrogen-fixing conditions.

DISCUSSION

The 'Champaka' pineapple plants at fruiting stage, regardless of the seedlings inoculation with *B. silvatlantica* (AB 219a) and the organic fertilization in field (Weber et al., 2010), presented endophytic bacterial colonization in roots and stems. The density of endophytes was higher in plants that received the bacterial inoculum, suggesting that association with the AB219a may persist during a plant cycle. In the presence of that bacterial strain and the treatment with low dosage of organic compost (40.17 m^3ha^{-1}) the MPN of endophytesreached10^6 cells g^{-1} of fresh root. The higher population density in roots (1.7 10^6 cells g^{-1} of fresh material) in comparison with stems

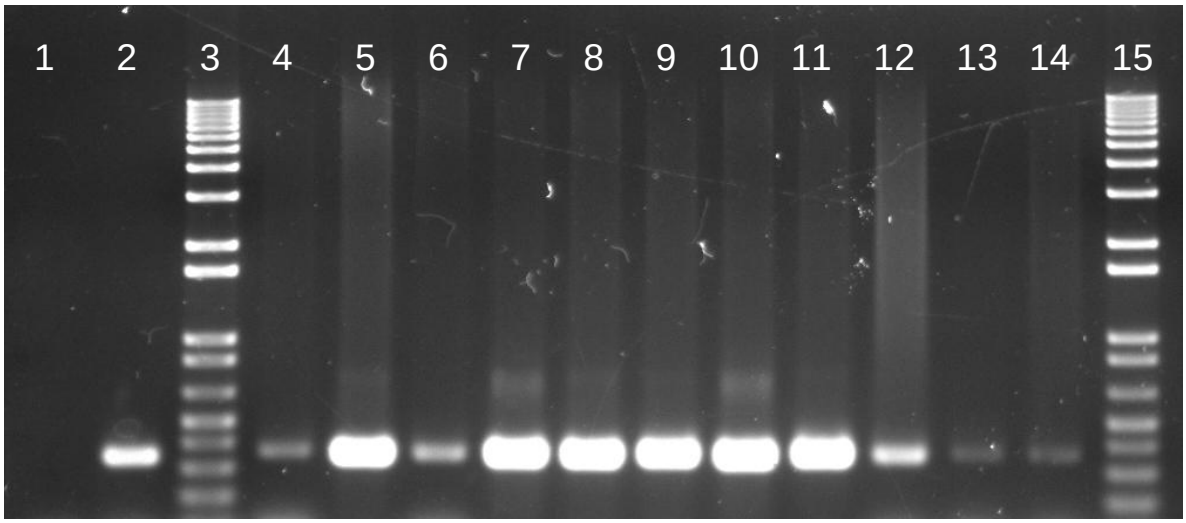

Figure 2. Agarose gel electrophoresis analysis of *nif*H gene(360 base pair) of selected endophytes from the 'Champaka' pineapple plants, and the fragment amplification by using primers PolF/PolR. Numbers: 1 negative control; 2 positive control (*Azospirillum brasilense*, strain SP245); 3 and 15 molecular marker; and 4 to 14 representing the following strains: AB 299, AB 219a, AB 280, AB 281, AB 284, AB 285, AB 286, AB 287, AB 290, AB 292, AB 294, respectively.

(4.8 10^2 cells g^{-1} of fresh biomass) is common in plants, and has been observed in pineapple cultivars: 'Vitória' (Baldotto et al., 2010) and "Champaka' (Weber et al., 2010).

The endophytic association in uninoculated pineapple plants was also confirmed, and it is related to the natural presence of endophytes in plants and to their distribution in field conditions. The presence of *Actinobacteria*, *α and β Proteobacteria* was previously observed in the 'Gomo de Mel' pineapple explants under axenic conditions (Abreu-Tarazi et al., 2010). The positive effect of the compost C on the MPN endophytes in roots may be due to the plant health and nutrition, as a consequence of the organic fertilizer and nutrient equilibrium in the soil. Weber et al. (2010) analyzing the compost C observed higher organic carbon (321.0 g kg^{-1}) and Fe contends (12431.3 mg kg^{-1}), comparatively to other composts employed: A (292.6 g organic carbon kg^{-1}, and 2431.3 mg Fe kg^{-1}) and B (321.0 g organic carbon kg^{-1}, and 2431.3 mg Fe kg^{-1}); and these nutrients may affect the plant symbiotic association. Organic fertilizers benefits on population of the diazotrophic endophytic bacteria were demonstrated in sugarcane, in comparison to conventional fertilizer management (Pariona-Llanos et al., 2010). Also, Shu et al. (2012), investigatingthe structureofnitrogen-fixing bacteriain rhizosphere andpadysoil andanalyzing relative abundanceof*nifH*, observed greaterabundance and diversity ofnitrogen-fixing bacteriain the soils underorganic management in comparison to conventional cropping system.

Our results demonstrate that pineapple plants associate with culturable endophytes (strains AB 284 and AB 304) closely related to *B. silvatlantica*, after plantlets inoculation with the AB 219a.Persistent plant endophytic association with this bacterial specie can be considered, but also wild strains may colonize the 'Champaka' pineapple plants. It should be observed that in description of *B. silvatlantica*, Perin et al. (2006) reported strains isolated from grasses (corn and sugar cane) and from pineapple plants. So, that plant association with *B. silvatlantica* could be expected. Endophytes from inoculated plants were related to five species belonging to three bacteria families: Burkholderiaceae (β Proteobacteria), where the strain AB 284 was related to B. silvatlantica; Enterobacteriaceae (γ Proteobacteria), strains AB 299 (Erwinia sp.), AB 295 (P. eucrina) and AB 290 (Uncultivable bacterium); and Xanthobacteraceae (α Proteobacteria), where the strain AB 281 was related to *A. caulinodans*. The presence of these endophytes may be favored by inoculation of plantlets with the strain AB 219a, except *Erwinia* sp. (strain AB 294), which was also detected in non-inoculated plants. These control plants were associated with endophytes related to four other species: *E. oryzae* (strains AB 285 and AB 287), belonging to Enterobacteriaceae; A. brasilense (strains AB 301), Rodospirillaceae; Sphingobium yanoikuyae (strain AB 292), Sphingomonadaceae; and B. cenocepacia (strain AB 280), Burkholderiaceae. The 'Champaka' pineapple plant colonization by those different endophytes is an evidence of a non-specific plant-bacterial relation, and the microorganisms effectively contributing to plant-growth are difficult to determine, as reported by Moreira et al. (2010).

The *nifH* gene of grouped endophytes from the'Champaka' pineapple plants was detected, except for the strain AB 295 (*Pantoea eucrina*). This evidence is

characteristic of nitrogen-fixing bacteria (Marin et al., 2003; Ando et al., 2005; Luvizotto et al., 2010; Shu et al., 2012). Also, our results indicate that Poly PCR primers (Poly et al., 2001) were able to amplify *nifH* sequences of pineapple endophytes DNA; however, the amplification efficiency differed between them (Figure 2). Some information had been previously reported about the contribution of endophytes we identified in this work. Bacteria related to *A. brasilense*, are able to colonize bananas (Weber et al., 1999; Mia et al., 2010) and especially graminous plants, leading to an increase of 16 to 30% in the production of wheat and corn grains (Hungria et al., 2010). *A. caulinodans* strains promote growth in rice seedlings (Senthilkumar et al., 2008), and *Sphingobium yanoikuyae* strains have activity of indol acetic acid production (Poonguzhaly et al., 2006). Other endophytes related to *Enterobacter oryzae* (Peng et al., 2009), *Pantoea eucrina* and *Erwinia* sp. may also influence the association of selected strains in pineapple cultivation. In further work we should consider the interaction of those endophytes with pineapple plants and the genetic similarity of strains related to *B. silvatlantica*. Based on the results obtained in the present study we conclude that pineapple 'Champaka' plants at fruiting stage associate with endophytes related to α, β and *γ Proteobacteria*, after plantlets inoculation or not with the diazotrophic bacterium AB 219a. Most of those endophytes present *nifH* gene, a characteristic of nitrogen-fixing bacteria.

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