ANTIFUNGAL COMPOUND PRODUCED BY THE CASSAVA ENDOPHYTE *Bacillus pumilus* MAIIIM4A

Flávia Mandolesi Pereira de Melo[1,2]*; Marli Fátima Fiore[1]; Luiz Alberto Beraldo de Moraes[3]; Maria Estela Silva-Stenico[1]; Shirlei Scramin[2]; Manoel de Araújo Teixeira[2,4]; Itamar Soares de Melo[2]

[1]*USP/CENA - Lab. de Biologia Celular e Molecular, Av. Centenário, 303, C.P. 96 - 13400-970 - Piracicaba, SP - Brasil.*
[2]*Embrapa Meio Ambiente - Lab. de Microbiologia Ambiental, Rod. SP 340, km 127,5, C.P. 69 - 13820-000 - Jaguariúna, SP - Brasil.*
[3]*USP/FFCLRP - Depto. de Química, Av. Bandeirantes, 3900 - 14040-901 - Ribeirão Preto, SP - Brasil.*
[4]*UNIVÁS/MG - Lab. de Microbiologia, Av. Prefeito Tuany Toledo, 470 - 37550-000 - Pouso Alegre, MG - Brasil.*
**Corresponding author <flamelo@cnpma.embrapa.br>*

ABSTRACT: In the search for new organisms and new secondary metabolites, a study was conducted to evaluate the diversity of endophytic bacteria from ethnovarieties of cassava cultivated by Brazilian Amazon Indian tribes and also to study the secondary metabolites produced by a *Bacillus pumilus* strain. Sixty seven cassava endophytic bacteria were subjected to 16S rRNA sequencing and FAME analysis. The bacterial profile revealed that 25% of all endophytic isolates belonged to the genus *Bacillus*. The isolate *B. pumilus* MAIIIM4a showed a strong inhibitory activity against the fungi *Rhizoctonia solani*, *Pythium aphanidermatum* and *Sclerotium rolfsii*. Secondary metabolites of this strain were extracted using hexane, dichloromethane and ethyl acetate. Extracts were subjected to bioautography and LC/MS analysis, which allowed the identification of pumilacidin, an antifungal compound produced by *B. pumilus* MAIIIM4a. The bacterial endophytic localization was confirmed by cassava cell tissue examination using scanning electron microscopy.
Key words: ESI-MS/MS, pumilacidin, endophytic bacteria, bioautography

COMPOSTO ANTIFÚNGICO PRODUZIDO PELO ENDÓFITO DE MANDIOCA *Bacillus pumilus* MAIIIM4A

RESUMO: Na busca de novos organismos e novos metabólitos secundários, um estudo foi conduzido visando avaliar a diversidade de bactérias endofíticas de etnovariedades de mandioca cultivadas por tribos indígenas da Amazônia brasileira e também para estudar metabólitos secundários produzidos por *Bacillus pumilus*. Sessenta e sete bactérias endofíticas de mandioca foram identificadas através do seqüenciamento do gene 16S rRNA e por meio da análise de ácidos graxos (FAME). Essas análises revelaram que 25% de todos os endofíticos pertenciam ao gênero *Bacillus*. O isolado *Bacillus pumilus* MAIIIM4a apresentou forte ação inibitória contra os fitopatógenos *Rhizoctonia solani, Pythium aphanidermatum* e *Sclerotium rolfsii*. Os metabólitos secundários deste isolado foram extraídos do sobrenadante usando-se hexano, diclorometano e acetato de etila. Esses extratos foram utilizados nas análises de bioautografia e LC-MS, as quais permitiram a identificação do composto pumilacidina, um antifúngico produzido por *B. pumilus* MAIIIM4a. A localização das bactérias endofíticas foi confirmada examinando-se o tecido celular da mandioca através de microscopia eletrônica.
Palavras-chave: ESI-MS/MS, pumilacidina, bactéria endofítica, bioautografia

INTRODUCTION

Endophytes are microorganisms (bacterial and fungal) that inhabit a wide variety of plant tissue types without causing any apparent harm to the host (Hallmann et al., 1997). In order to colonize the plant and compete with other microorganisms, they produce many enzymes and toxins (Lima et al., 2005). Also, a variety of secondary metabolites, including enzymes, antibiotics (Pleban et al., 1997), anticancer (Stierle et al., 1993), anti-inflammatory (Trischman et al., 1994), antifungal (Korzybski et al., 1978), and biological control agents (Hallmann et al., 1997) have been isolated from endophytic microorganisms. To date, many promising endophytic bacteria have been reported as biocontrol candidates against plant pathogens. Most of them produce enzymes and antibiotics as the main action mechanisms. However, their frequent occurrence

in agricultural crops and subsequent relevance to crop production systems is yet to be explored widely (Loeffler et al., 1986; Krebs et al., 1998). Cassava (*Manihot esculenta* Crantz ssp. *esculenta*) is one of the main species cultivated by farmers of tropical areas and it is thought to be originated from Brazilian Amazon basin, whereas many ethnovarieties have been cultivated by Indian tribes.

The cassava roots are the major carbohydrate source throughout the humid tropics for over 500 million people (FAO, 2000). Despite its global importance as a subsistence crop, cassava has not received major attention in crop research programs. In this context, studies about cassava endophytic bacteria may bring important contribution to the knowledge of the crop disease control mechanisms and the beneficial relationships between plants and bacterial endophytes communities (Teixeira et al., 2007). The aim of the present study was to provide a survey of bacterial endophytic population in cassava plants from an Indian tribe plantation of Brazilian Amazon, characterize the isolated strains and identify the antifungal metabolites produced by the isolates.

MATERIAL AND METHODS

Isolation and identification of endophytic bacteria

Six healthy cassava plants were collected from different Indian tribes plantation located in the Autazes region (03°18'003" S - 60°37'15,5" W), Amazonas State, Brazil. Freshly collected plants were placed in plastic bag, maintained in low temperature (±10ºC) and transported to the laboratory where they were separated into stems, root and leaf and cut into sections about 1 cm^2. A vigorous rinsing with sterilized distilled water and neutral detergent was used to remove adhering microorganisms. All the material (10 roots pieces, 20 stem pieces and 15 leaves pieces) were then surface disinfected using 70% ethanol for 1 min, 2% sodium hypochlorite for 6 min, again 70% ethanol for 30 s to remove sodium hypochlorite and finally rinsed with sterilized distilled water (Araújo et al., 2001). To confirm the surface disinfection process the tissue segments of the final rinse were plated out onto tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI, USA). Seven segments of each cassava tissue were inoculated in Petri dish containing TSA medium supplemented with 1 μL mL^{-1} of the fungicide benomyl, to inhibit fungal growth. The Petri dishes (replicated three times) were incubated at 27°C for 10 d. After this period, endophytic bacterial colonies were transferred to plates containing fresh TSA. Individual colonies without contaminants were kept in mineral oil at 4ºC.

Identification of bacteria by fatty acid methyl ester (FAME) analysis

Pure bacterial cultures were grown on TSA medium for 2 d at 28ºC. Triplicate of 40 ng wet mass of cells were harvested and placed into reaction tubes. One milliliter of methanolic sodium hydroxide solution (15% NaOH [m/v] in 50% methanol [v/v]) was added to the cells and the mixture was heated (100°C) in water bath for 30 min to saponify the cells. The fatty acids were methylated in 2.0 mL methanolic hydrochlorid acid solution (6N HCL in 46% methanol [v/v]) in a water bath at 80ºC for 10 min. The FAMEs were extracted from aqueous phase with 1.25 mL of methyl-tert-butyl ether: hexane (1:1, v/v). The FAME extracts were analyzed on gas chromatography (Hewlett Packard, Rolling Meadows, IL, USA). The FAME compounds were identified using the microbial identification software (Sherlock aerobe method and TSA library version 4.0) developed by MIDI Inc. (Newark, NJ, USA).

Identification of bacteria by sequencing the 16S rRNA gene fragment

Pure cultures of bacteria were cultivated in 10 mL of TSA liquid medium and incubated for 48 h at 28ºC at 120 rpm. Aliquots of 1.5 mL of culture were rinsed twice with 500 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). The cell pellets were resuspended in 500 μL of TE buffer plus 30 μL of 10% sodium dodecyl sulfate [m/v] and 0.5 g of 0.1-mm-diameter glass beads (BioSpect Products, Inc., Bartlesville, OK, USA). Each sample was shaken vigorously for 3 sec in a mini-bead beater (BioSpect Products, Inc). One milliliter of phenol was added; the solution was mixed well, and centrifuged at 12,000 × *g* for 10 min. The aqueous phase containing the DNA was extracted once with 1 mL of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with a 0.6 volume of isopropanol and a 0.1 volume of 5 M NaCl and washed with 70% ethanol. The pellet was washed with 70% ethanol, air dried and resuspended in 50 μL sterilized ultrapure water containing RNAse (10 mg mL^{-1}). The DNA from each sample was finally analyzed by agarose gel electrophoresis to estimate the yield.

The 16S rRNA partial sequence was amplified from the *B. pumilus* MAIIIM4a genomic DNA using the primers F968 (5'-AACGCGAAGAACCTT AC-3') (Nübel et al., 1996) and R1387 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (Heuer et al., 1997). Amplification was performed in a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) using 50 μL PCR mixture containing 10 ng of genomic DNA, 0.2 μM of each oligonucle-

otide primer, 200 μM dNTPs, 3.75 mM $MgCl_2$, 1 × PCR buffer and 5.0 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Thermal cycling was performed using a touchdown series as described by Araújo et al. (2002). PCR amplifications were then analyzed by electrophoresis using 1% agarose gel stained with ethidium bromide. Gel was viewed on an Alpha DigiDoc System Gel Documentation System (Alpha Innotech, San Leandro, CA, USA) and recorded.

The PCR fragments were purified using the Ultra Clean PCR clean-up kit (MO BIO Laboratories, Inc., Solana Beach, CA, USA) according to the manufacturer's instruction. Direct PCR sequencing reaction was performed using the purified PCR product, R1387 primer and BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). After the completion of the sequencing reaction, a 100% isopropanol wash followed by a 70% ethanol wash was performed to remove residual dye terminators. The purified reaction was then resuspended in HiDi formamide (Applied Biosystems) and the samples were placed in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The nucleotide fragment sequences of 16S rRNA obtained in this study were compared with sequence information available in the National Center for Biotechnology Information database using BLAST (www.ncbi.nlm.nih.gov/BLAST). Phylogenetic tree was constructed by multiple pair-wise alignments using the CLUSTAL W computer program, version 1.8 (Thompson et al., 1994). The neighbour-joining tree (Saitou & Nei, 1987) was built with BioEdit version 5.0.6 (Hall, 2001). The tree reliability was evaluated by bootstrap analysis with 1,000 replicates. The nucleotide sequence determined in this study has been deposited in GeneBank under accession number DQ011675.

Scanning electron microscopy of endophytic bacteria in cassava tissues

Microorganisms-free axenic seedlings of cassava were obtained by tissue culture procedures. Three flasks containing 400 mL of Murashige & Skoog medium (Murashige & Skoog, 1962) and three with sterilized distilled water were prepared. Cassava seedlings were transferred to the flasks and 10^8 CFU mL^{-1} of *B. pumilus* MAIIIM4a was inoculated. Non-inoculated media containing cassava seedlings was routinely used as control. After 5 d the cassava plants were separated into stems, roots and leaves and each tissue was cut into sections of approximately 2 cm^2. Each cassava tissue section was fixed in a modified Karnovksy solution (Kitajima & Leite, 1999) and the samples were kept at 8°C overnight. The samples were washed 3 times with cacodylate buffer (0.05 M) for 10 min and immersed in 1% OsO_4 (in 0.05 M cacodylate buffer, pH 7.2) for 1 h. The fragments were washed five times, 15 min changes in distilled water. Samples were dehydrated in an ethanol series (30, 50, 70, 90 and 100%) for 15 min per change. The latter phase was repeated three times. Ultimately, the cassava material were critically point dried, gold sputter coated with gold and examined using a field emission scanning electron microscope (SEM) (LEO Co-operation Zeiss/Leica, Oberkochem, Germany). All experiments were performed in triplicate.

Screening of bacteria for antagonism

All bacterial isolates were screened for their activity against the pathogenic fungi *Pythium aphanidermatum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. A plug of the fungus was placed on one side of the potato dextrose agar (PDA) plate, and a loop of bacteria was streaked down the opposite side of the plate. Control plates consisted of the fungus placed on PDA plates alone. Plates were incubated at 28°C for 48 h. Antifungal activity from each bacterial isolate was recorded as the size of the zone of inhibition.

Extraction of secondary metabolites

According to the antifungal screening, one bacterium *Bacillus pumilus* MAIIIM4a that showed strong antifungal activity was chosen for further studies, including the characterization of its metabolites. Thus this strain was cultivated in liquid PD (potato-dextrose) medium and incubated in a shaker (150 rpm) at 28°C for 72 h. At the end of this period, the cultures were centrifuged at 10,000 × *g* for 5 min. and 500 mL of the supernatant were used for successive extractions with hexane (200 mL), dichloromethane (200 mL) and ethyl acetate (200 mL). The respective organic phases were collected and dried with anhydrous sodium sulfate and evaporated and then the crude extracts were obtained: 7 mg from hexane extraction, 8 mg from dichloromethane extraction and 8 mg from ethyl acetate extraction.

Antimicrobial activity

The test-fungi described previously were grown in PDA medium at 25°C for 24 h and then 20 μL of the hexane, dichloromethane and ethyl acetate extracts were applied in sterile disks of 7.0-mm-diameter (Sensibiodisc, Cecon, São Paulo, SP, Brazil), previously inserted in Petri dishes inoculated with fungal strains. The controls consisted of 20 μL of solvent or the medium extracted in the same solvent. Zones of inhibition were recorded after two days. This experiment was performed in triplicate.

Thin layer chromatography and bioautography

Silica gel G60 F_{254} alumina backed plates (10 × 10 cm) were used for separation and identification of inhibitory fractions. Aliquots (10 µL) of each extract obtained as described above were applied and the layers developed with dichloromethane/ethyl acetate (9:1). The UV active absorbing spots were visualized at 254 and 366 nm. For bioautography experiments, chromatograms were placed in 9 × 9 cm sterile Petri dishes with covers. Overlay media (PDA, 20 mL) containing a suspension of 10^6 mL^{-1} cells of the fungal tests was sprayed over the developed TLC plates. After solidification of the media, the TLC plates were incubated at 25°C. The fungal growth was observed daily and the formation of inhibition zones was recorded.

GC-MS

Extracts were derivatized with diazomethane and subjected to gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS analysis was performed on a Shimadzu equipped with a 30 m × 0.25 mm × 0.25 µm capillary column. The following oven program was applied: the temperature was 40°C programmed up to 280°C at 8°C/min. The carrier gas He (research grade 99.95% purity) was used at a flow rate of 1 mL min^{-1}. A sample volume of 1 µL (10 mg mL^{-1}) was injected. The injector and the transfer lines were maintained at 240°C. The Mass Selective Detector (QP-5000) was operated in the Electron Ionization mode at 70 eV in the full scan range of *m/z* 100 to 2000. Nist 12 and Wiley library were used to identify metabolites in the extracts.

Mass spectrometry

MS was performed using a hybrid quadrupole time-of-flight (TOF) high resolution (7.000) and high accuracy (5 ppm) Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source (ESI). The working conditions for positive and negative ESI were as follows: desolvation gas (nitrogen) was heated at 250°C; capillary was held at a potential of 3.2 kV, and the cone voltage was set at 25 kV. MS/MS tandem mass spectra were acquired by mass-selecting the target ion using the quadrupole mass analyzer followed by 25eV, collision induced dissociation using argon in the quadrupole collision cell and mass analysis by TOF. The crude extracts were dissolved in a mixture of methanol and water 2:1 (v/v) and 10 mM ammonium acetate for analysis in both positive and negative ion mode. The sample was introduced into the source at 10 µL min^{-1} with a syringe pump.

RESULTS

Isolation and identification of endophytic bacteria

Sixty-seven strains of endophytic bacteria were isolated from six cassava plants. The taxonomic results revealed that out of 67 isolates subjected to 16S rRNA gene and FAME profile analysis, only 44 showed similarities comparing the two techniques. It was possible to identify 19 genera of endophytic bacteria in cassava plants (Table 1). More than 25% of the isolates were classified as *Bacillus* species. The bacteria identified in cassava were isolated from all colonizing parts of the plant. The percentage of colonization in roots was 41% of the total isolated bacteria, followed by the stem with 34% and leaves with 25%. Some of the strains were present in all tissues of the plant, while others colonized specific parts. The resulting profiles were identified with microbial identification software (MIDI) using the TSBA database, version 4.0 (MIDI, Newark, Del.) and a dendrogram can be visualized in Figure 1 (Teixeira, 2004). The 16S rRNA sequence of the *B. pumilus* MAIIIM4a compared favourably (98% identity) with several sequences of *B. pumilus* (AF526907, AY289549, AY496869, AY167886) from public database by BLAST analysis. Phylogenetic relationships were investigated with other bacteria from Genebank using the joining method (Figure 2). All the *B. pumilus* strains formed a monophyletic cluster in the tree. The bootstrap analysis based on 1,000 resamplings of the neighbour-joining data, used to test the robustness and stability of the branching, showed that the *B. pumilus* strains sequences clustered with high bootstrap value (98%) separated from the other species of *Bacillus*.

Scanning electron microscopy of cassava tissues

Scanning electron microscopy analysis demonstrated that *B. pumilus* MAIIIM4a beneficially infected

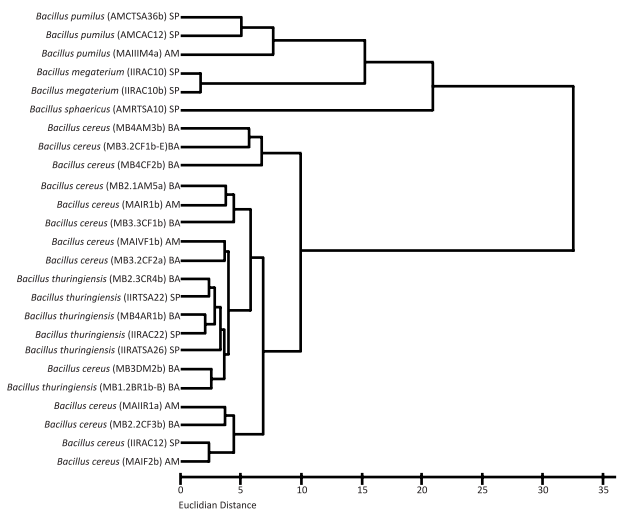


Figure 1 - Dendrogram obtained by FAME profile analysis of *Bacillus* strains (Teixeira, 2004).

Table 1 - Identification of endophytic bacteria using FAME and 16S rRNA gene. The fat acids profiles were obtained comparing the data from the TSBA4.0 library (reference strains). Isolates with similarity index (SI) of 0.6 or higher were considered positively identified.

Strain	Plant tissue	Identification	MIDIa (SI)	16S rRNA (% identity)
MAIIR2b	Root	*Kluyvera cryocrescens*	0.699	99
MAIVR1b	Root	*Bacillus cereus*	0.782	99
MAIVR2a	Root	*Streptomyces olivaceus*	0.606	96
MAIIIR1b	Root	*Bacillus thuringiensis*	0.855	100
MAIIIR3b	Root	*Stenotrophomonas maltophilia*	0.565	99
MAIR1b	Root	*Bacillus cereus*	0.789	99
MAIIR1a	Root	*Bacillus cereus*	0.854	98
MAIVR3b	Root	*Burkholderia cepacia*	0.785	99
MAIIIR4a	Root	*Enterobacter aerogenes*	0.791	99
MAIR2f	Root	*Burkholderia cepacia*	0.754	90
MAIIIR4a-B	Root	*Klebsiella penumoniae*	0.762	99
MAIR2b	Root	*Bacillus cereus*	0.756	100
MAIIIR4b	Root	*Acidovorax avenae*	0.584	99
MAIIIR2a	Root	*Stenotrophomonas maltophilia*	0.621	96
MAIIIR2b	Root	*Burkholderia cepacia*	0.733	94
MAIR2a	Root	*Bacillus cereus*	0.650	100
MAIIIR3a	Root	*Bradyrhizobium japonicum*	0.721	99
MAIVR2b	Root	*Microbacterium hominis*	0.628	98
MAIIIM2b	Stem	*Bacillus anthracis*	0.689	100
MAIM1b	Stem	*Brachybacterium paraconglomeratum*	0.601	99
MAIIIM2a	Stem	*Enterobacter aerogenes*	0.621	100
MAIVM3a	Stem	*Bacillus anthracis*	0.632	99
MAIIIM1a	Stem	*Salmonella enteritidis*	0.800	99
MAIM3a	Stem	*Brevibacillus brevi*	0.600	99
MAIVM2a	Stem	*Enterobacter cancerogenus*	0.751	100
MAIIM2b	Stem	*Salmonella bongori*	0.802	97
***MAIIIM4a**	**Stem**	***Bacillus pumilus***	**0.938**	**100**
MAIVM2b	Stem	*Salmonella choleraesius*	0.931	100
MAIM1b	Stem	*Escherichia coli*	0.871	99
MAIIM3a-B	Stem	*Salmonella bongori*	0.623	96
MAIIIM1b-A	Stem	*Serratia rubidae*	0.654	97
MAIIIM1b	Stem	*Escherichia coli*	0.878	98
MAIM1a	Stem	*Escherichia coli*	0.539	99
MAIF2b	Leave	*Bacillus cereus*	0.806	100
MAIIIF1a	Leave	*Stenotrophomonas maltophilia*	0.654	99
MAIF6b	Leave	*Clavibacter michiganensis*	0.848	99
MAIIIF2b	Leave	*Curtobacterium luteum*	0.659	100
MAIF1b	Leave	*Bacillus pumilus*	0.748	100
MAIF1a	Leave	*Microbacterium aerborescens*	0.610	99
MAIVR1f	Leave	*Pseudomonas rhodesiae*	0.784	98
MAIIIR1f	Leave	*Enterobacter cloacae*	0.655	99
MAIIF1a	Leave	*Microbacterium imperiale*	0.784	99
MAIF4b	Leave	*Ochrobactrum antropi*	0.890	98
MAIIF2a	Leave	*Microbacterium imperiale*	0.747	94

[a]FAME (Fatty acid methyl ester). [a]Source: Teixeira, 2004. *Strain selected for antimicrobial analysis

the tissues, established and colonized leaves, stems and roots of cassava plants. However, the distribution of this species was variable according to the colonization microhabitats. Populations of *B. pumilus* MAIIIM4a in the stem and root of cassava are shown in Figure 3. The majority of cells of *B. pumilus* MAIIIM4a invaded and colonized intracellular and intercellular sites of cassava tissues. It was not detected presence of any bacteria in tissues of the axenic plants.

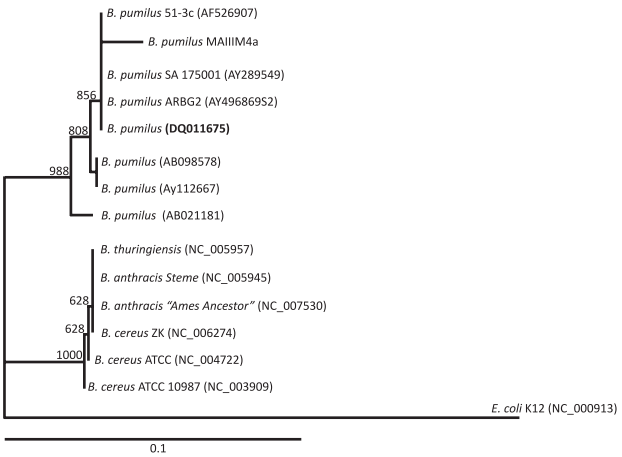


Figure 2 - A phylogenetic tree of the *B. pumilus* MAIIIM4a 16S rRNA gene sequence. The 16S rRNA gene sequence from *Escherichia coli* strain K12 was treated as the outgroup. Numbers at the nodes represent percentage bootstrap values of 1000 resamplings. Sequence shown in bold was generated during this study. The scale is the expected number of substitutions per position. Nucleotide sequence obtained in this study has been deposited in GeneBank under accession number DQ011675.

Screening of bacteria for antagonism against *P. aphanidermatum*

A total of 67 bacterial isolates were screened for their antagonistic ability to suppress the mycelial growth of *P. aphanidermatum* in an *in vitro* assay. Ten isolates showed strong antagonistic activity against the phytopathogen. These antagonists were isolated from all parts of the plants, that is, 30% from roots, 20% from leaves and 50% from stem. The percentage of inhibition against *P. aphanidermatum* found is presented as follow: *Enterobacter cancerogenus* MAIVM2a (33%), *Bacillus anthracis* MAIVM3a (26%), *B. anthracis* MAIIIM2b (48%), *Kluyvera cryocrescens* MAIIR2b (29%), *Bradyrhizobium japonicum* MAIIIR3a (35%), *B. pumilus* MAIIIM4a (54%), *B. cereus* MAIF4b (33%), *Clavibacter michiganensis isidiosum* MAIF6b (34%), *B. cereus* MAIVM1b (51%), *Burkholderia cepacia* MAIVR3b (35%). Due to the highest antifungal activity of *B. pumilus* MAIIIM4a, this strain was chosen for further analysis.

Antimicrobial activity

Dichloromethane and ethyl acetate extracts showed high bioactivity against the phytopathogens *R. solani*, *P. aphanidermatum* and *S. rolfsii*, while the hexane extract presented low bioactivity only against *P. aphanidermatum* (Table 2).

Table 2 - Bioactivity of different *B. pumilus* extracts against phytopathogenic fungi.

Extracts	*R. solani*	*P. aphanidermatum*	*S. rolfsii*
Hexane	-	+	-
Dichloromethane	+++	+ +	+ +
Ethyl acetate	+++	+ +	+ +

(-) Inactive (+) Active (++ or +++) Highly active.

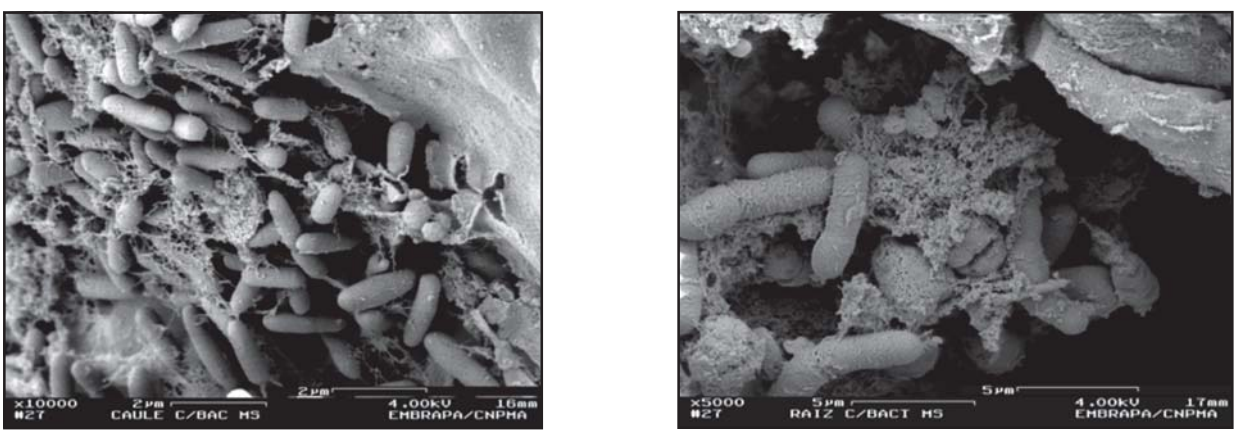


Figure 3 - Scanning electron micrographs of *B. pumilus* MAIIIM4a colonizing cassava tissues. a) Stem colonization; b) Root colonization.

TLC and bioautography

The choice of extraction conditions was very important, since different solvents showed different fractions of compounds. Preliminary TLC analysis of *B. pumilus* MAIIIM4a supernatant extracted into ethyl acetate indicated that 10 fractions were obtained, while hexane extraction presented only two fractions (Table 3). The bioautography was performed only for the ethyl acetate extract. Clear inhibition zones at R_f of 0.62, 0.56 and 0.42 for *B. pumilus* MAIIIM4a extracts were observed against *P. aphanidermatum*, showing that the compounds responsible for the antifungal activity are strongly apolar.

GC-MS

The results obtained by GC-MS are presented in Table 4. Mass spectra data were compared with Nist 12, Nist 62 and Wiley 139 libraries. These data suggested that hexane extracts were constituted of long chain hydrocarbons. Dichloromethane extract presented phthalate derivative, composed of aromatic and different classes of oxygenated compositions (esters, alcohols, diols, ketones and others), while ethyl acetate extract had phthalate, fatty ester, long chains of unsaturated hydrocarbons and diols.

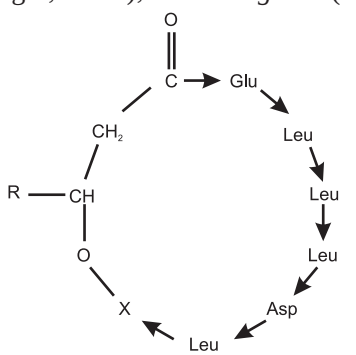
Mass Spectrometry

Mass peaks were assigned to lipopeptide species by comparison with reference data and by mass spectrometric *in situ* sequence analysis of selected compounds by ESI-MS/MS. These mass data correspond well to those determined by other authors indicating the presence of pumilacidin A-E series (Kalinovskaya et al., 2002; Pabel et al., 2003) (Figure 4).

The predominant ions in ESI mass spectrum in the positive ion mode were that of *m/z* 1036.7, 1050.7, 1064.7 and 1078.7 (Figure 5a) and the more abundant one was that of *m/z* 1050.7, which corresponds to the protonated pumilacidin A with a C_{12} lipid carbon chain and Ile7. In a similar manner, ESI mass spectrum in the negative ion mode showed the pumilacidin A-E series $[M-H]^-$ (Figure 5b). Different isoforms exist for each lipopeptide, which vary in the chain length of their fatty acid components and amino acid residues in their peptide rings, respectively.

DISCUSSION

A total of 67 endophytic bacteria were isolated from cassava plants collected in the Brazilian Amazon region. The isolation methodology used probably underestimates the real bacterial population, since it has been isolated only bacteria that could grow in culture conditions. Several isolates were identified by FAME profile analysis or 16S ribosomal RNA gene sequencing. The selected isolate MAIIIM4a was confirmed to belong to *B. pumilus* species and showed similarity with *B. pumilus* strains AMCTSA36b and AMCAC12. *Bacillus* species are found as the main endophytic bacteria in several plants, including *Zea mays* L. (Lalande et al., 1989), *Gossypium hirsutum* L. (Misaghi & Donndelinger, 1990), *Beta vulgaris* (Jacobs et al.,



	R	X	$[M+H^{1+}$	Formula
PB	C_{12}-*iso*- and *anteiso*	Val	1036	$C_{53}H_{93}N_7O_{13}$
PA	C_{12}-*iso*- and *anteiso*	Ile	1050	$C_{54}H_{95}N_7O_{13}$
PE	C_{13}-*iso*	Ile	1064	$C_{55}H_{97}N_7O_{13}$
PD	C_{14}-iso	Val	1064	$C_{55}H_{97}N_7O_{13}$
PC	C_{14}-*iso*- and *anteiso*	Ile	1078	$C_{56}H_{99}N_7O_{13}$

Figure 4 - Structure and mass data of lipopeptides pumilacidins. (PA-PE indicates pumilacidins series A-E).

Table 3 - R_f values for different solvent extracts using UV light to visualize spots (254 and 366 nm).

Extracts of *B. pumilus*	*Rf									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Control	0.89	0.62	0.53	0.23	-	-	-	-	-	-
Hexane	0.33	0.24	-	-	-	-	-	-	-	-
Dichloromethane	0.89	0.80	0.65	0.60	0.53	0.23	-	-	-	-
Ethyl acetate	0.89	0.78	0.71	0.67	0.62	0.56	0.49	0.42	0.29	0.25

*R_f is defined as the distance traveled by the compound divided by the distance traveled by the solvent

Table 4 - Compounds identified in the hexane extract using GC/MS.

Solvent	Peak number	Retention Time	Relative Abundance	Compound
		min.	%	
Hexane	1	3.663	1.16	a
	2	4.147	4.51	a
	3	4.183	1.85	*n*-propil acetate
	4	5.680	26.79	a
	5	13.556	2.18	*n*-dodecane
	6	15.480	5.10	*n*-tridecane
	7	17.298	24.15	*n*-tetradecane
	8	18.372	2.96	b
	9	18.997	12.35	*n*-pentadecane
	10	20.603	12.94	*n*-hexadecane
	11	22.105	3.19	*n*-heptadecane
	12	22.201	2.83	2,6-dimethyl octadecane
Dichloromethane	1	3.699	1.11	a
	2	4.250	41.64	3-hydroxy 2-butanone
	3	4.360	0.57	b
	4	5.284	25.64	2,3-butanediol
	5	5.383	1.03	b
	6	5.475	0.80	b
	7	5.602	5.76	methylpropenylcarbinol
	8	5.707	6.50	a
	9	5.796	2.13	isopropyl acetate
	10	9.089	1.78	2,3-butanedione monooxime
	11	10.197	4.27	benzyl alcohol
	12	12.065	1.20	2,6-dimethyl morfoline
	13	13.155	1.13	mMethylphenylacetate
	14	20.623	3.36	diethyl phthalate
	15	25.855	3.08	butyl phthalate
Ethyl acetate	1	3.824	0.28	a
	2	4.263	1.44	a
	3	5.547	46.77	b
	4	5.620	2.78	2,3-butanediol
	5	5.767	5.80	a
	6	17.163	8.32	1-pentadecene
	7	20.506	10.41	1-hexadecene
	8	23.450	5.64	1-octodecene
	9	23.789	0.81	b
	10	25.238	0.82	14 methyl-pentadecanoate
	11	25.896	6.19	a
	12	26.111	3.86	a
	13	26.402	4.23	b
	14	28.546	1.02	a
	15	33.053	1.36	dioctyl phthalate

a = Compounds present in the culture medium. b = Not identified.

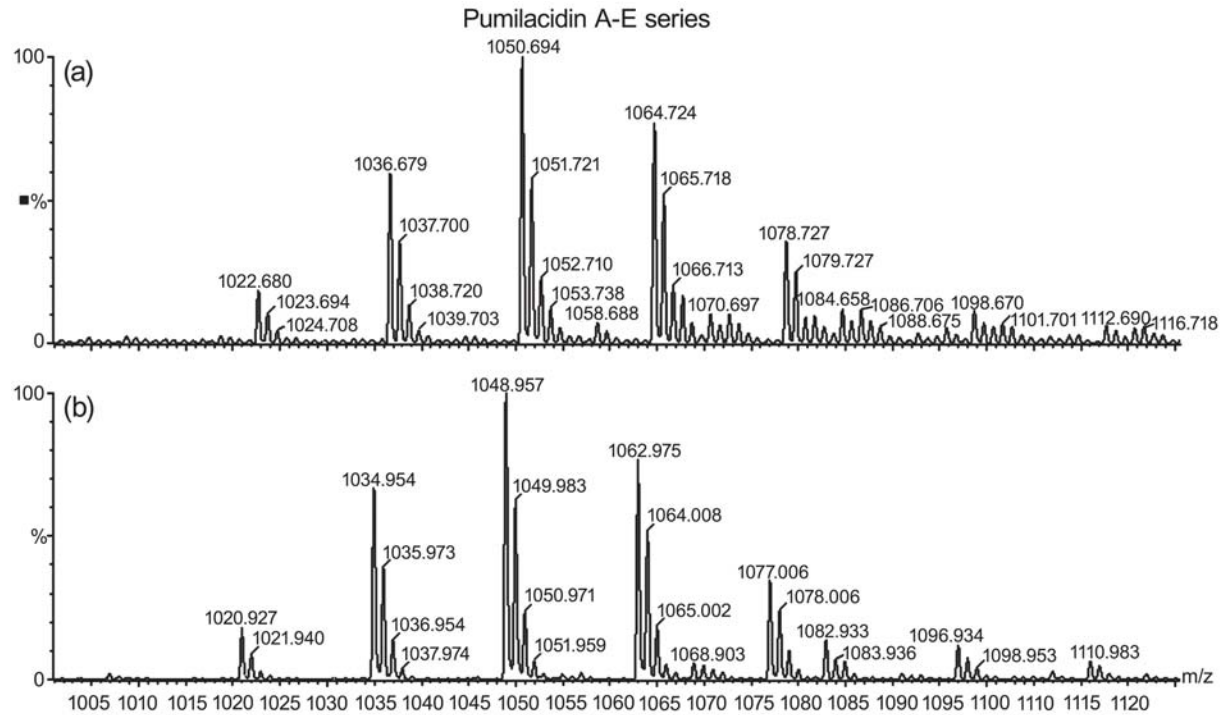


Figure 5 - ESI mass spectrum in the positive (a) and negative (b) ion mode of the pumilacidin A-E series.

1985), and *Solanum tuberosum* L. (Hollis, 1951), and the species that are often cited as endophytic are: *B. cereus* and *B. lentus* (Araújo et al., 2001), *B. subtilis* (Bai et al., 2002), *B. megaterium* (Elvira-Recuenco & Vuurde, 2000; Araújo et al., 2001), *B. insolitus* and *B. brevis* (Sturz et al., 1997) and *B. pumilus* (Araújo et al., 2002). This genera has been reported as a potent biological control agent (Cook, 1986), demonstrating a great potential in agriculture.

Scanning electron microscopy was able to detect this strain in inner tissues in all parts of the plant. *Bacillus pumilus* MAIIIM4a colonized roots and stem, indicating that this isolate is efficient and a good candidate as biological control agent.

With the increased occurrence of phytopathogens causing plant disease, the search for new antifungal bioagent has gained urgency. The production of bioactive compounds by *Bacillus* species is well established (Korzybski et al., 1978; Naruse et al., 1990; Munimbazi & Bullerman, 1998). These classes of bioactive compounds act as antifungal peptides, antifungal lipopeptides and antimicrobial polypeptides (Pabel et al., 2003). To characterize antimicrobial activity of *B. pumilus* MAIIIM4a, the isolate was tested against three phytopathogenic fungi: *R. solani*, *P. aphanidermatum* and *S. rolfsii*. The antifungal screening revealed that *B. pumilus* MAIIIM4a produced a compound with high bioactivity against *P. aphanidermatum*. The bioautography was considered an efficient test to determine antimicrobial activity, since less than 2.5 μg of extract was enough to form inhibition halo around the active fractions. LC-MS/MS was able to identify a compound assigned as pumilacidin by comparison with reference data. Pumilacidin is produced by *B. pumilus* and are cyclic acylheptapeptide composed of a beta-hydroxy fatty acid (Naruse et al., 1990). This lipopeptide has antimicrobial (Pabel et al., 2003), antiviral and antiulcer activity (Naruse et al., 1990).

Mass spectrometric data corroborate the identification of one bioactive compound as a lipopeptide, pumilacidin, a characteristic of *B. pumilus*. This is the first time that an endophytic *B. pumilus* was characterized as pumilacidin producer.

ACKNOWLEDGEMENT

Financial support was provide by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 99/09177-1). F.M.P.M. was supported by a graduate scholarship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior).

REFERENCES

ARAÚJO, W.L.; MACCHERONI JR, W.; AGUILLAR-VILDOSO, C.I.; BARROSO, P.A.V.; SARIDKIS, H.O.; AZEVEDO, J.L. Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. **Canadian Journal of Microbiology,** v.47, p.229-236, 2001.

ARAÚJO, W.L.; MARCON, J.; MACCHERONI JR, W.; ELSAS, J.D.van; VUURDE, J.W.L.van; AZEVEDO, J.L. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. **Applied and Environmental Microbiology**, v.68, p.4906-4914, 2002.

BAI, Y.; DÁOUST, F.; SMITH, D.L.; DRISCOLL, B.T. Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. **Canadian Journal of Microbiology,** v.48, p.230-238, 2002.

COOK, R.J. Plant health and the sustainability of agriculture, with special reference to disease control by beneficial microorganisms. **Biological Agriculture and Horticulture**, v.3, p.211-232, 1986.

ELVIRA-RECUENCO, M.; VUURDE, J.W.L.van. Natural incidence of endophytic bacteria in pea cultivars under field conditions. **Canadian Journal of Microbiology**, v.46, p.1036–1041, 2000.

FAO. Championing the cause of cassava. 2000. Available in www.fao.org/NEWS/2000/000405-e.htm. Accessed 28 Sept. 2000.

HALL, T. **BioEdit:** version 5.0.6**.** Raleigh: North Carolina State University, Department of Microbiology, 2001. 192p.

HALLMANN, J.; QUADT-HALLMANN, A.; MAHAFFEE, W.F.; KLOEPPER, J.W. Bacterial endophytes in agricultural crops. **Canadian Journal of Microbiology,** v.43, p.895-914, 1997.

HEUER, H.; KRSEK, M.; BAKER, P.; SMALLA, K.; WELLINGTON, E.M. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. **Applied and Environmental Microbiology**, v.63, p.3233-3234, 1997.

HOLLIS, J.P. Bacteria in healthy potato tissue. **Phytopathology**, v.41, p.350-366, 1951.

JACOBS, M.J.; BUGDEE, W.M.; GABRIELSON, D.A. Enumeration, location, and characterization of endophytic bacteria within sugar beet roots. **Canadian Journal of Botany**, v.63, p.1262-1265, 1985.

KALINOVSKAYA, N.I.; KUZNETSOVA, T.A.; IVANOVA, E.P.; ROMANENKO, L.A.; VOINOV, V.G.; HUTH, F.; LAATSCH, H. Characterization of Surfactin-like Cyclic Depsipeptides Synthesized by *Bacillus pumilus* from Ascidian *Halocynthia aurantium*. **Marine Biotechnology**, v.4, p.179–188, 2002.

KITAJIMA, E.W.; LEITE, B. **Curso introdutório de microscopia eletrônica de varredura.** 2. ed. Piracicaba: NAP/ MEPA-USP/ ESALQ, 1999. 46p.

KORZYBSKI, T.; KOWSZYK-GIFINDER, Z.; KURYTOWICZ, W. **Antibiotics:** origin, nature, and properties. Washington, D.C.: American Society for Microbiology, , 1978. p.501-534.

KREBS, B.; HÖDING, B.; KÜBART, S.; ALEMAYEHU-WORKIE, M.; JUNGE, H.; SCHMIEDEKNECHT, G.; GROSCH, R.; BOCHOW, H.; HEVESI, M. Use of *Bacillus subtilis* as biological control agent. I. Activities and characterization of *Bacillus subtilis* strains. **Journal of Plant Diseases and Protection**, v.105, p.181-197, 1998.

LALANDE, R.; BISSONNETTE, N.; COUTLEE, D.; ANTOUN, H. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. **Plant and Soil**, v.115, p.7-11, 1989.

LIMA, A.O.S.; QUECINE, M.C.; FUNGARO, M.H.P.; ANDREOTE, F.D.; MACCHERONI JR, W.; ARAÚJO, W.L.; SILVA FILHO, M.C.; PIZZIRANI-KLEINER, A.A.; AZEVEDO, J.L. Molecular characterization of a ß-1,4-endoglucanase from an endophytic *Bacillus pumilus* strain. **Applied Microbiology and Biotechnology**, v.68, p.57-65, 2005.

LOEFFLER, W.; TSCHEN, S.-M.; VANITTANAKOM, N.; KUGLER, M.; KNORPP, E.; HSIEH, T.-F.; WU, T.-G. Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F29-3: a comparison with activities of other *Bacillus* antibiotics. **Journal of Phytopathology**, v.115, p.204-213, 1986.

MISAGHI, I.J.; DONNDELINGER, C.R. Endophytic bacteria in symptom-free cotton plants. **Phytopathology**, v.80, p.808-811, 1990.

MUNIMBAZI, C.; BULLERMAN, L.B. Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. **Journal of Applied Microbiology**, v.84, p.959-968, 1998.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v.15, p.473-497, 1962.

NARUSE, N.; TENMYO, O.; KOBARU, S.; KAMEI, H.; MIYAKI, T.; KONISHI, M.; OKI, T. Pumilacidin, a complex of new antiviral antibiotics. Production, isolation, chemical properties, structure and biological activity. **Journal of Antibiotics,** v.43, p.267-280, 1990.

NÜBEL, U.; ENGELEN, B.; FELSKE, A.; SNAIDR, J.; WIESHUBER, A.; AMANN, R.I.; LUDWIG, W.; BACKHAUS, H. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. **Journal of Bacteriology**, v.178, p.5636-5643, 1996.

PABEL, C.T.; VATER, J.; WILDE, C.; FRANKE, P.; HOFEMEISTER, J.; ADLER, B.; BRINGMANN, G.; HACKER, J.; HENTSCHEL, U. Antimicrobial activities and matrix-assisted laser desorption/ionization mass spectrometry of *Bacillus* isolates from the marine sponge *Aplysina aerophoba*. **Marine Biotechnology**, v.5, p.424-434, 2003.

PLEBAN, S.; CHERNIN, L.; CHET, I. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. **Letters in Applied Microbiology**, v.25, p.284-288, 1997.

SAITOU, N.; NEI, N. The neighbor-joining method: a new method for reconstructing phylogenetic trees. **Molecular Biology and Evolution**, v.4, p.406-25, 1987.

STIERLE, A.; STROBEL, G.; STIERLE, D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. **Science**, v.260, p.214-216, 1993.

STURZ, A.V.; CHRISTIE, B.R.; MATHESON, B.G.; NOWAK, J. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. **Biology and Fertility of Soils**, v.25, p.13-19, 1997.

TEIXEIRA, M.A.; Diversidade de Bactérias endofíticas de mandioca (Manihotis esculenta Crantz) coletada de diferentes regiões do Brasil. São Paulo: USP/ICB, 2004. 102p. Tese (Doutorado).

TEIXEIRA, M.A.; MELO, I.S.; VIEIRA, R.F.; COSTA, F.E.C.; HARAKAVA, R. Microrganismos endofíticos de mandioca de áreas comerciais e etnovariedades em três Estados brasileiros. Pesquisa Agropecuária Brasileira, v.42, p.42-49, 2007. Available in http://www.scielo.br/pdf/pab/v42n1/06.pdf. Accessed 1 Jul. 2008.

THOMPSON, J.D.; HIGINS, D.G.; GIBSON, T.J. Clustal-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Research**, v.22, p.4673-4680, 1994.

TRISCHMAN, J.A.; TAPIOLAS, D.M.; JENSEN, P.R.; DWIGHT, R.; FENICAL, W.; McKEE, T.C.; IRELAND, C.M.; STOUT, T.J.; CLARDY, J.C. Salinamide-A and salinamide-B: anti-inflammatory depsipeptides from a marine streptomycete. **American Chemical Society**, v.116, p.757-758, 1994.

Received December 06, 2007
Accepted April 16, 2009