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Bacteriosomes in axenic plants: endophytes as stable endosymbionts

Cristina Vieira de Almeida · Fernando Dini Andreote · Ricardo Yara · Francisco André Ossamu Tanaka · João Lúcio Azevedo · Marcílio de Almeida

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Abstract Observations of cells of axenic peach palm (*Bactris gasipaes*) microplants by light microscopy revealed movements of small particles within the cells. The phenomenon was characterized initially as Brownian movement, but electron microscopy revealed the presence of an intracellular bacterial community in these plants. Microscopy observations revealed the particular shapes of bacterial cells colonizing inner tissues of analyzed plants. Applying a molecular characterization by polymerase chain reaction and denaturing gradient gel electrophoresis, it was revealed the existence of bacterial rRNA within the plants. Sequencing of the rRNA identified three different phylogenetic groups; two bands had a high degree of similarity to sequences from *Moraxella* sp. and *Brevibacillus* sp., and a third sequence was similar to a non-cultivated cyanobacterium. The presence of those endosymbionts, called bacteriosomes, in axenic peach palm microplants raises the question of whether these stable endosymbionts were acquired in the process of evolution and how could they benefit the process of plants micropropagation.

Keywords Axenic microplants · *Bactris gasipaes* · Endosymbionts · Organelles · Peach palm · 16S rRNA

C. V. de Almeida
In Vitro Palm Consultoria, Estudo e Desenvolvimento Biológico Ltda, Rua Itajobi, 421, CEP 13432-000 Piracicaba, São Paulo, Brazil

F. D. Andreote
Laboratório de Microbiologia Ambiental, Embrapa Meio Ambiente, Rod. SP340, km 127.5, Cx. Postal 69, 13820-000 Jaguariúna, São Paulo, Brazil
e-mail: fdandreo@gmail.com

R. Yara
Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPE), Rua Prof. Arthur de Sá, S/N-Cidade Universitária, CEP 50670-091 Recife, Pernambuco, Brazil

F. A. O. Tanaka
Núcleo de Apoio a Pesquisa em Microscopia Eletrônica (NAP/MEPA), Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ/USP), Av. Pádua Dias, 11, Cx. Postal 09, 13418-900 Piracicaba Piracicaba, São Paulo, Brazil

J. L. Azevedo
Departamento de Genética, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ/USP), Av. Pádua Dias, 11, Cx. Postal 83, 13418-900 Piracicaba, São Paulo, Brazil

M. de Almeida (✉)
Departamento de Ciências Biológicas, Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ/USP), Av. Pádua Dias, 11, Cx. Postal 83, 13418-900 Piracicaba, São Paulo, Brazil
e-mail: malmeida@esalq.usp.br

Introduction

Endophytic bacteria are characterized by the colonization of inner tissues of plants. There is an ongoing discussion toward a better definition of these microorganisms; but a commonly used definition of endophytes is those whose isolates form on surface-disinfected plant tissues (Hallmann et al. 1997). However, the separation of endophytes according to their essentiality in niche occupations is explored. In that case, the endophytic community is divided into "passenger" endophytes, i.e. bacteria that eventually invade internal plant tissues by stochastic events and "true" endophytes, those with adaptive traits enabling them to strictly live in association with the plant (Hardoim et al. 2008).

Concerning the endophytic bacteria associated with plants cultivated in vitro, one can considered them as a more intimate relationship plant-bacteria. Dias et al. (2009) has shown the potentiality of these bacteria in promoting

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plant growth during acclimatization of micropropagated strawberry. Also, these microorganisms have been described as compulsory endosymbionts, due to the essentiality of the host to their survival (Bodyt et al. 2007). It is widely expanded the knowledge on the on evolution and diversity of the host and the endosymbiont interactions. Bodyt et al. (2007) showed by a molecular approach the presence of endosymbiontic cyanobacteria in amoeba (*Paulinella chromatophora*).

Here we describe a microbiological affiliation of particles, with constant movement, observed within parenchymatic cells of the leaf, root and rhizome of axenic peach palm (*Bactris gasipaes* Kunth.-Arecaceae) microplants. We used light microscopy, electron microscopy and molecular analysis to investigate this microbial community in the intracellular space of axenic peach palm microplants.

Materials and methods

Plant material

All plants used in this work were cultivated under the same conditions: MS medium (Murashige and Skoog 1962), under controlled conditions of temperature and light (26(±1)°C and 16 h photoperiod with 42 μmol m^{-2} s^{-1}). The analyses used a total of 50 axenic peach palm (*B. gasipaes* Kunth-Arecaceae) plants originating from embryos excised from seeds.

Tentative isolation of bacteria

In a laminar flow hoods samples of roots and leaves of axenic peach palms were ground in a sterilized mortar with 5 ml of PBS (NaCl, 8 g L^{-1}; KCl, 0.2 g L^{-1}; Na_2HPO_4, 1.44 g L^{-1}; KH_2PO_4, 0.24 g L^{-1}; pH 7.4). Five plants were selected for isolation of bacteria. Samples of the suspensions were submitted to serial dilutions, which were inoculated into liquid or solid (1.5% agar amended) of three different culture media: TSA (Tryptic Soy Agar, OXOID), LB (Luria-Bertani: tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 5 g L^{-1}) and BG-11 (Allen 1968). Flasks and plates were incubated at 28°C. The colonies and bacterial growth in liquid media were monitored weekly during 6 months. As a control, portions of the three media were inoculated with the PBS used in the grinding and in the dilutions.

Light microscopy (LM) analysis

Samples of roots and leaves from axenic peach palm plants were used for examination by microscopy. Fresh histological sections were obtained by tissue slicing with sheet steel, suspended in deionized, sterilized water, and assembled in non-permanent histological blades for observation by light microscopy (Olympus CH2).

Fresh plant sections were submitted to specific reagents: Sudan III and IV (Jensen 1962) to characterize lipid compounds; Lugol's solution to identify starch grains (Langeron 1949); and ferrous sulfate and formalin (Johansen 1940) to visualize phenolic compounds.

In permanent histological blades, root and leaf segments, approximately 1 × 0.2 cm, were obtained from axenic plants and fixed for 48 h in Karnovsky solution (Karnovsky 1965). The samples were then dehydrated in an ethanol series, and infiltrated with resin (glycol-methacrylate; Leica historesin embedding kit). The blocks were sectioned with a manual rotary microtome. The 0.5 μm thick slices were further adhered in histological blades, stained with 0.05% toluidine blue and mounted in the synthetic resin Entellan®. The histological blades were examined by light microscopy (Zeiss Jenemed 2), and all of the images were captured at the same magnification with a Samsung (SDC-313) camera.

Scanning electronic microscopy (SEM) analysis

Samples of roots and leaves (4 × 4 mm) were fixed for 24 h in Karnovsky solution, rinsed three times (10 min each) in 0.05% sodium cacodylate buffer and post-fixed in 1% osmium tetroxide for 1 h. The three-dimensional structure of the mesophyll was visible in samples that had been immersed in 30% glycerol for 1 h then fractured in liquid nitrogen (which results in a better definition of sample faces, avoiding interference by the cutting blade), then dehydrated by passage through an acetone series of increasing concentration. After the wash in 100% acetone, samples were dried to the critical point by dehydration with CO_2 (Balzers CPD 030). The dehydrated fragments were placed on onto metallic stubs and coated with gold using a Bal-Tec SCD 050 metallizer. Samples were examined with an LEO 435 VP scanning electronic microscope.

Transmission electronic microscopy (TEM) analysis

Similar samples to those examined by SEM were also subjected for TEM. These samples were approximately 2 × 4 mm. Samples were fixed in Karnovsky solution for 24 h, washed three times (10 min each) with 0.05% cacodylate buffer and post-fixed with 1% osmium tetroxide for 1 h. After that, samples were overnight submitted to contrasting and dehydrated in an acetone series of increasing concentration. After washing with 100% acetone, samples were submitted to pre-infiltration with 1:1 Spurr resin/100% acetone for 5 h with agitation. The subsequent infiltration of the samples was done with pure resin for

12 h. The samples were then shaped and polymerized at 70°C for 3 days. The blocks were cut on an ultramicrotome (Leica Ultracut UCT) with a diamond blade (450) into 70 nm thick sections and mounted on uncoated 200-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds 1963). TEM observations were made with a Zeiss EM 900 microscope operating at 50 kV. Preparation of samples for examining the microorganisms was similar to that described above for the plant material. However, at each change of solution during the infiltration process, the samples were centrifuged for 10 min at 5,000*g*, yielding better concentration of the cells.

DNA extraction from plants and cultures

Roots and leaves were separated from the seedling with a scalpel, and fragments of these tissues (4 fragments of approximately 5 mm) were incubated in sterile 15 mL tubes (3 samples per tube) each containing 2 mL of sterilized 0.8% (w/v) NaCl. The tubes were shaken for 30 min at 120 rpm. The solutions were then transferred to new sterile tubes and centrifuged for 5 min at 12,000*g*. The resulting pellets were suspended in 500 µL of TE buffer and DNA was extracted with the Wizard bacterial DNA isolation kit (Promega, USA). The DNA was analyzed by electrophoresis in a 1.0% (w/v) agarose gel and staining with ethidium bromide for visualization under UV light. The DNA was extracted from bacterial cultures by similar methodology, as the start material was 2 mL of cultures submitted to centrifugation and treatments similar to those used for the plant samples.

PCR-DGGE analyses of microplants cultivated in vitro

Amplification of 16S ribosomal RNA (rRNA) gene fragments from genomic DNA was performed in 50 µL reactions with 400 nM universal primers U968/GC and R1387 (Heuer et al. 1997), using 30 cycles with an annealing temperature of 56°C. The PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel and staining with ethidium bromide for visualization under UV light. DGGE was performed as described (Muyzer et al. 1993) with the Ingeny phorU2 apparatus (Ingeny, The Netherlands). PCR samples were loaded onto 6% (w/v) polyacrylamide gels in 0.5 × TAE buffer. The polyacrylamide gels were made with gradients ranging from 45 to 65% denaturant, where the 100% denaturant was 7 M urea and 40% (v/v) formamide. The gels were run for 16 h at 100 V and 60°C, then soaked for 1 h in SYBR GreenI nucleic acid stain (1:10,000 dilution; Molecular Probes, Leiden, The Netherlands) and immediately photographed under UV light. From DGGE analyses, bands were selected for identification, cut from the gel, mashed in sterile water, and submitted to a new PCR amplification. The resulting amplicons were loaded onto a new DGGE gel to check band purity and co-migration with the selected band. Amplicons were then purified and sequenced in an ABI Prism 377 automated sequencer (PE Applied Biosystems, USA).

Phylogenetic affiliation of selected bands

The sequences generated in this study were submitted to a BLASTn analysis at GenBank database (nr/nt), considering the three most similar sequences for band phylogeny association (Table 1). Also, the bands similatities were evaluated at Ribosomal Data Project (RDP–http://rdp.cme.msu.edu/) database for affiliation with complete database and also type strains. The sequences obtained of tree bands have been submitted to GenBank under subscription codes DQ494142, DQ494143 and DQ494144.

Results and discussion

The examination of the axenic microplants of peach palm (*B. gasipaes*) by light microscopy revealed the movement of small "particles" inside parenchymatic cells (Fig. 1a), which were initially considered as Brownian movement. However, these movements are driven by motility patterns, with quick, wavy and constant trajectory, which seems to respond to light incidence, resembling bacterial phototaxis. Particles with such movements were observed inside the parenchymatic cells of leaves, roots and rhizomes and were present in all examined plants. Elaioplasts, amiloplasts and phenolic compounds were distinguished from the supposed endosymbionts by histological tests, which revealed a size discrepancy between the particles and the putative endosymbionts (Fig. 1b).

Scanning electronic microscopy revealed the presence of the endosymbionts inside the plant cells (Fig. 1c, d). These observations were reinforced by analysis of the ultrastructure of the roots, which showed the presence of a bacterial community colonizing the intracellular space of plant cells (Fig. 1e). Such community is constituted by microorganisms which are able to migrate from the hyaloplasm to the vacuole (Fig. 1f). The thylakoid structure, unique to cyanobacteria, was also observed in the TEM analysis. The migration of the microorganisms to the vacuole is illustrated by Fig. 2a–c, which show the presence of bacilli and cocci morphology of bacterial cells. It is remarkable that much of the scientific literature refers to these structural vesicles as "Multivesicular bodies", not addressing the characterization its contents. Here we show that these particles should not be characterized as microbodies or osmophiles, which are composed of a single membrane and granular or crystalline protein. The Fig. 2d

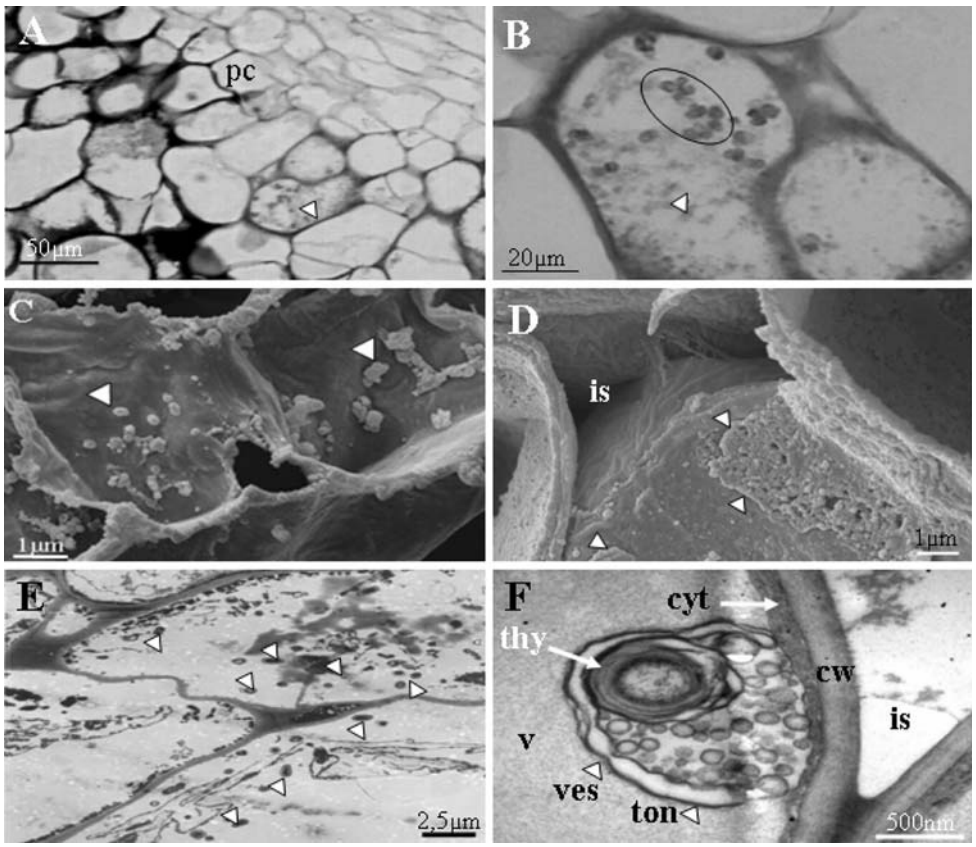

Fig. 1 Microscopy of peach palm microplants cultivated in vitro. **a** Transverse section of *Bactris gasipaes* leaf showing, by light microscopy, the presence of the particles considered as bacteriosome (*arrow*) in the parenchymatous cells (pc). **b** Detail of the figure **a** showing the discrepancy between the dimensions of bacteriosomes and organic compounds (*circle*). **c** Scanning electronmicrograph showing bacterial cells inside parenchymatous cells of the root (*arrow*). **d** Evidence of the microorganism colony present within the plant cell (*arrow*); intercellular spaces (is), cell wall (cw). **e** Transmission electronmicrograph showing the microorganisms (*arrows*) within rhizome cells of the axenic microplants; **f** Transmission electron micrographs emphasizing migration of the microorganism colony from the cytoplasm (cyt) to the inside of the vacuole (v). The vesicle formation (ves) grouping the colony. The thylakoids (thy) and coccoids present within the vesicle; cell wall (cw), intercellular space (is), tonoplast (ton)

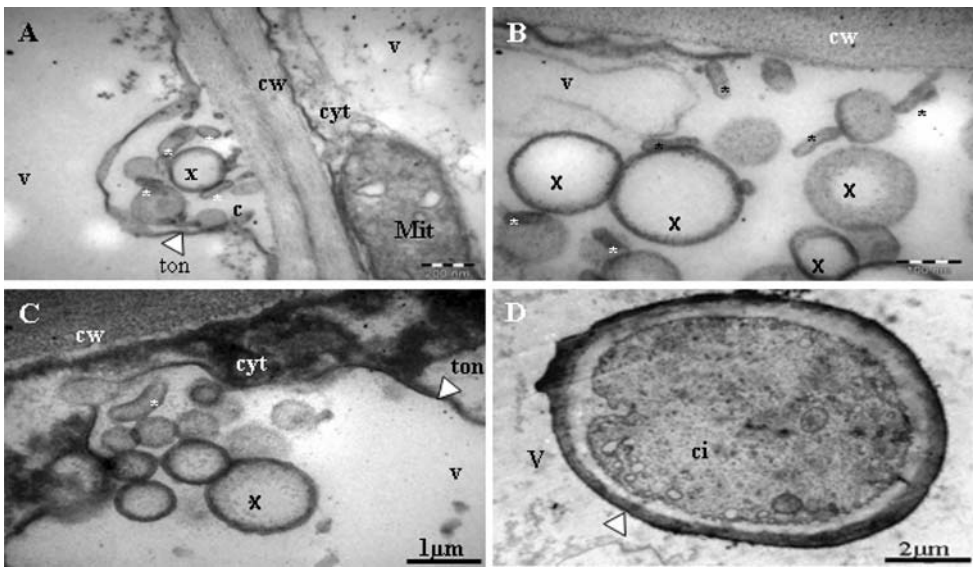

Fig. 2 Microscopy showing the translocation and the characterization of bacteriosomes. **a** Transmission electronmicrograph of axenic plant root demonstrating the migration of microorganisms to the vacuole (v), the presence of bacilli (*) and coccoids (x) structures. The tonoplast shaping (ton), cell walls (cw), cytoplasm (c) and mitochondria (Mit) can be seen. **b, c** Detail of endosymbiont morphology. **d** Coccoids morphology of endosymbionts inside the vacuole (v), showing the presence of the cell membrane (*arrow*), and cytoplasmic inclusions (ci)

shows the coccoid shape of the endosymbiont and the presence of cytoplasmic inclusions. Then, we have an important support to characterize such particles as microorganisms, which inhabit and interact with the host plant.

Concerning the isolation of these possible endosymbionts, the developments of bacteria in the used media were very slow or absent. The Isolation was not possible in the TSA and LB media commonly used for bacteria isolation. In the BG11 medium, specific for cyanobacteria, the growth was observed after 5 months of incubation. At this time, the development of cells was observed near the surface, reflecting the microaerophilic behavior of the endosymbiontic microbial community (Fig. 3). At this time, the development of cells was not observed in the control tubes.

Observations of the isolated cells by light microscopy showed the presence of cocci, bacilli and filamentous morphology (Fig. 4a–c). Among these structures, only the filamentous shapes, which resemble cyanobacteria, were not observed inside the plant cells. This might indicate the differential development of this microbial group in culture media and inside the plant. Concerning the cyanobacterium morphology, it was observed in plant tissues only in a unicellular shape, evidenced by the presence of thylakoids (Fig. 1f). This possible dimorphism may be an advantage for cyanobacteria adapting to the different ecological niches where these species live, varying from unicellular to multicellular organization, or even with or without heterocyst (Mays-Figueira 2004; Gorelova 2006). Scanning electronic microscopy of isolated bacteria showed the coccoid, filamentous and bacillus shapes, without flagella or fimbriae (Fig. 4).

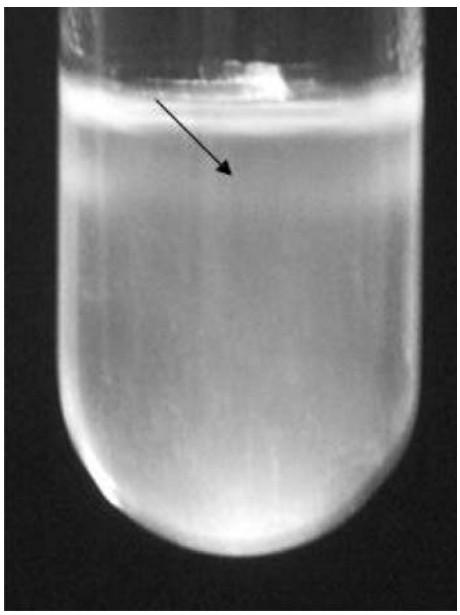

Fig. 3 Cultivation of endosymbionts in BG-11 medium after incubation for 5 months. The development of the cells is shown close to the surface of the medium (*arrow*), characterizing the microaerophilic behavior of bacteriosomes

Similarities between isolated bacteria and those found in the plant cytoplasm were revealed by transmission electronic microscopy (Fig. 4g–i), which showed the presence of a double membrane, typically found in groups of bacteria. The presence of multiple membranes, characteristic for thylakoids, inside parenchymatous cells is compatible with the presence of endosymbiont cyanobacteria in plants.

The culture-independent molecular analysis confirmed the presence of microorganisms in analyzed samples, detecting the microbial community associated with axenic peach palm plants. It was made by the direct access of the DNA of these endosymbionts. The DGGE patterns show the presence of extra bands, as well as the strong band in the lower part of the gel that is typical of chloroplastidial DNA (Fig. 5). Comparison of the migration profile of the bands from total DNA with those observed for the bacterial cultures shows correlation of three bands. It corroborates with the results of isolation and microscopy, showing that the endosymbionts colonize the plant tissues, and can be isolated on BG-11 medium. Sequence analysis of the three bands present in cultivations and plants showed the possible phylogenic affiliation of the endosymbionts (Table 1). Band sequences were distributed in three phylogenetic branches within the bacteria domain. The band B1 (Bacteriosome 1) is highly similar to *Moraxella* sp. (Gammaproteobacteria) showing its inter-relation with plant species. The sequence B2 (Bacteriosome 2) has a similarity to *Brevibacillus* sp. The presence of *Moraxella* sp. and *Brevibacillus* sp. is in line with data from the literature describing the isolation of these species from microplants (Vivas et al. 2003a, b; Vivas et al. 2005; Thomas 2006). Araújo et al. (2001) described the colonization of *Citrus jambhiri* root xylem by endophytes, and remarked the establishment of bacterial cells in the apoplastic or inside of the vessels cells.

The sequence generated from band 3 in the DGGE gel shows similarity with the 16S chloroplastidial DNA from different species, but not with peach palms or related plants. In addition, matches were obtained with non-cultivatable bacteria and cyanobacteria. Combining the DGGE results, the analysis of G + C content of the sequence, and the localization of the bands in the gel, it suggests that this sequence is from a non-described cyanobacterium. These data are in agreement with the SEM and TEM analysis, suggesting that one of the endosymbionts is related phylogenetically to cyanobacteria, which is here described colonizing inner tissues of in vitro cultivated plants. However, cyanobacteria have been observed inside cells of several groups of organisms in different taxonomic kingdoms (Monera, Protist, Plantae and Metazoa), where it is described as elected syncyanoses, cyanels or cyanobionts

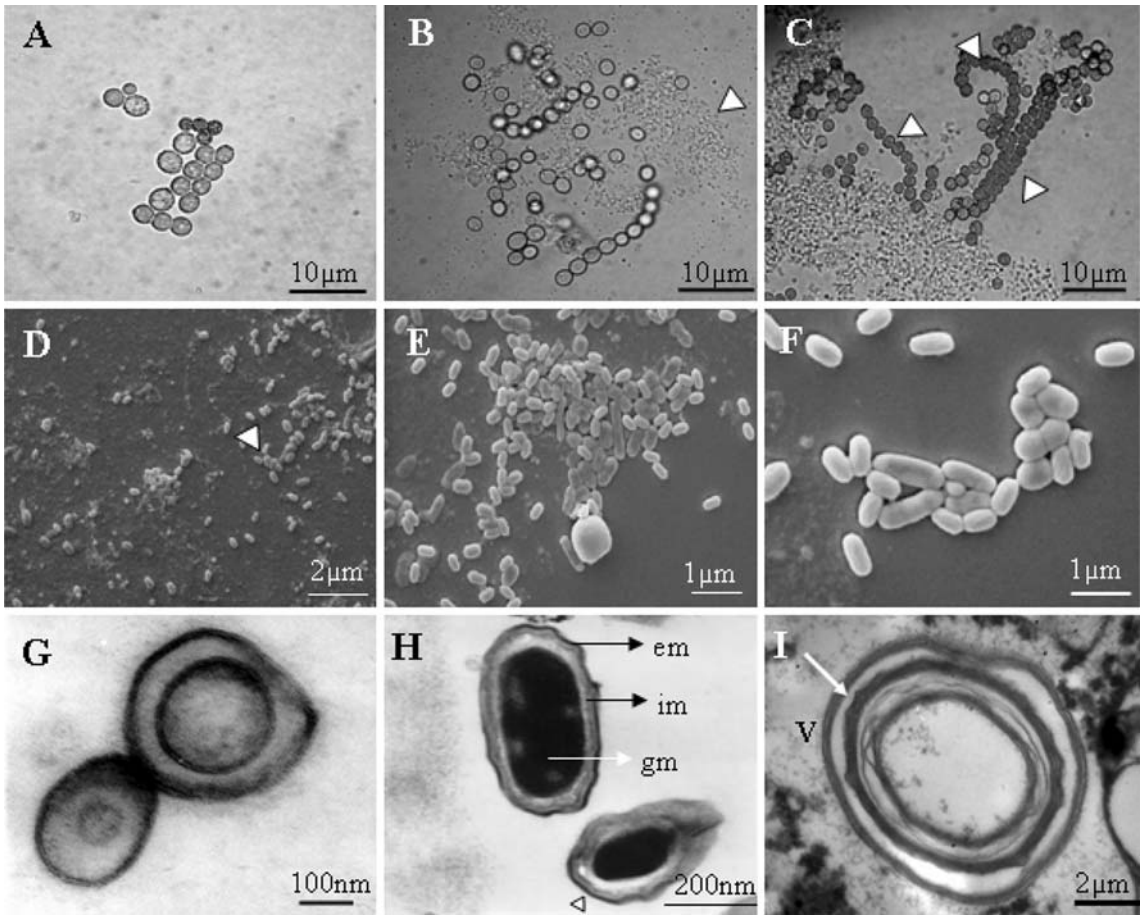

Fig. 4 Light microscopy of endosymbionts isolated from palm peach plants in BG-11 liquid medium. **a** Coccoids morphology of isolated species; **b** Formation of filamentous and bacilli structures (arrows); **c** Formation of filamentous colony structures (*arrows*); **d**, **e** and **f** SEM analysis showing the development of bacilli in the culture medium; **g**, **h** and **i** TEM images revealing the morphology of microbial isolates, where *arrows* indicate the thylakoids typical of cyanobacteria (*v* vacuole; *em* external membrane; *im* internal membrane; *gm* genetic material)

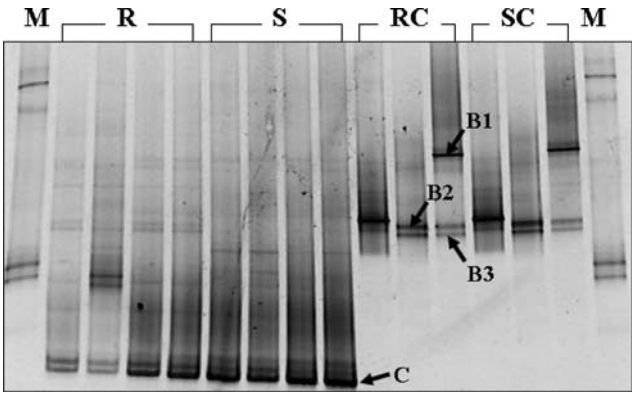

Fig. 5 PCR-DGGE fingerprints of peach palm microplant (R and L) and culture samples (RC and LC) based on amplification of universal primers. Markers (M) were loaded onto the gels to verify the gradient and electrophoresis efficiency. Seedling samples were divided into root plus rhizome (R) and shoot (S). Cultivations from the same tissues were obtained to compose the cultivation samples of bacteriosomes from root (RC) and shoot (SC). Only three replications are presented for cultivation due to the contamination of one sample. *Arrows* indicate the sequenced bands B1, B2 and B3 found in both plants and cultures. The *arrow* labeled C indicates the chloroplastidial band on the DGGE gel

(Pascher 1929; Geitler 1959; Reisser 1984; Schenk 1990; Burey et al. 2005; Bodyt et al. 2007). Concerning the interactions with plants, cyanobionts are affiliated to the *Nostoc* genus, found within cells of diatomaceous plants and the pulvinus on the petiole base of *Gunnera magellanica*, the only angiosperm described in the literature to harbor such association (Bonnett 1990; Osborn et al. 1991; Benson and Margulis 2002; Gusev et al. 2002; Janson 2002; Gorelova 2006).

The presence of bacteria inside axenic plant cells is very intriguing if we consider that the explants of peach palm used for micropropagation were from embryos excised from seeds, where the microorganisms are less abundant. The maintenance of these bacteria over the micropropagation process indicates that they are very stable and important for the plant development and metabolism. Hence we can define the observed bacteriosomes as endophytes with a behavior of stable endosymbionts. Further studies are needed to better understand the methods of infection and transmission (cell to cell, organism to

Table 1 Phylogeny of endosymbionts detected in peach palm axenic microplants, based on the partial 16S rRNA sequence from DGGE bands

DGGE band	Accession number	GC content (%)	Best match (BLASTN) (%)	Accession number	Best match (RDP) (%)	Accession number
B1	DQ494142	51.7	*Moraxella* sp. (97)	AY162144	*Moraxella* sp. (97)	AY162144
			Moraxella sp. (97)	DQ199213	*Moraxella* sp. (97)	FJ357597
			Uncultured bacterium (97)	AB193927	*Moraxella* sp. (97)	FJ006859
B2	DQ494143	53.0	*Brevibacillus* sp. (98)	DQ116777	*Brevibacillus* sp. (98)	AJ313027
			Brevibacillus centrosporus (98)	AB112719	*Brevibacillus* sp. (98)	DQ116777
			Brevibacillus sp. (98)	AJ313027	*Brevibacillus* sp. (98)	EU497965
B3	DQ494144	54.1	Unidentified cyanobacterium (95)	AJ428862	Unclassified *Cyanobacteria* (95)	–
			Arabidopsis thaliana chloroplast (95)	AP000423	Unclassified *Cyanobacteria* (95)	–
			Uncultured bacterium (95)	AB200661	Unclassified *Cyanobacteria* (95)	–

organism) of this microbial community. Also, another issue still to be addressed it the role of such bacteria in the host plant. Endophytes are known to have a sort of functions when interacting with plants; protection against pathogens, supplying of nutrients and modulation of genetic and metabolic functions (Hallmann et al. 1997; Hardoim et al. 2008; Dias et al. 2009). The techniques used in this work only proved the presence of microbes colonizing peach palm microplants cultured in vitro.

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