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SHORT COMMUNICATION

Endophytic bacteria in long-term in vitro cultivated "axenic" pineapple microplants revealed by PCR–DGGE

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Abstract In vitro propagated plants are believed to be free of microbes. However, after 5 years of in vitro culture of pineapple plants, without evidence of microbial contamination, the use of culture-independent molecular approach [classifying heterogeneous nucleic acids amplified via universal and specific 16S rRNA gene by polymerase chain reaction (PCR)], and further analysis by denaturing gradient gel electrophoresis (DGGE) revealed endophytic bacteria in roots, young and mature leaves of such plants. The amplification of 16S rRNA gene (*Bacteria* domain) with the exclusion of the plant chloroplast DNA interference, confirmed the presence of bacterial DNA, from endophytic microorganisms within microplant tissues. PCR–DGGE analysis revealed clear differences on bacterial communities depending on plant organ. Group-specific DGGE analyses also indicated differences in the structures of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* communities in each part of plants. The results suggest the occurrence of a succession of bacterial communities colonizing actively the microplants organs. This study is the first report that brings together evidences that pineapple microplants, previously considered axenic, harbor an endophytic bacterial community encompassing members of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* group which is responsive to differences in organs due to plant development.

Keywords Micropropagation · *Ananas comosus* · Endophyte · PCR–DGGE

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Introduction

Although endophytic bacteria are ubiquitously inhabiting most plant species and have been isolated from a variety of plants (Lodewyckx et al. 2002), the shoot meristems regions have been considered virtually free-of-microbial cells (Pirttilä et al. 2000). Nevertheless, the presence of bacteria in micropropagated plants is commonly mentioned as microbial contamination, which must be prevented and eliminated (George et al. 2008). Only a few scientists consider these microorganisms endophytes present in the plant tissues. Recently, the presence of such bacteria was reported in peach palm plants (Almeida et al. 2009). Moreover, studies that confirm and characterize the presence of beneficial endophytic microorganisms in "axenic" plant cultures are even rarer (Almeida et al. 2005, 2009; Dias et al. 2009; Pirttilä et al. 2000). The association of beneficial endophytic bacteria and micropropagated plants may be more frequent than it is reported, and can lead to positive effects on micropropagation and cell culture studies (Dias et al. 2009; Pirttilä et al. 2000).

Another important gap about the endophytic colonization of micropropagated plants is the role of these bacteria

in plant development. It is already known that in field-cultivated plants, endophytes can help on plant development by promoting phosphate solubilization activity, indole acetic acid production, osmotic adjustment, stomatal regulation; uptake of minerals and alteration of nitrogen accumulation and metabolism (reviewed by Ryan et al. 2008). However, although the potentiality of these bacteria is extremely high to improve the micropropagation and acclimatization of micropropagated plants, only a few attributions are made for endophytic bacteria in such plants. Dias et al. (2009) has shown that these bacteria can promote the growth of strawberry plants during the acclimatization process in greenhouse.

With regard to pineapple, that represents an important tropical crop, isolation and characterization of diazotrophic bacteria have been reported. Bacteria related to the groups of *Azospirillum amazonense*, *Azospirillum lipoferum*, *Burkholderia* sp. could be detected in roots, stems, leaves and fruits of different genotypes (Weber et al. 1999). Additionally, studies have demonstrated positive agronomic effects due to the inoculation of diazotrophic bacteria in in vitro propagated plants (Weber et al. 2009).

In order to address the endophytic bacterial community structures and diversity in micropropagated healthy-looking pineapple plants, we used culture-independent approaches, based on the analysis of heterogeneous nucleic acids via PCR amplification targeting universal and specific 16S rRNA gene and further DGGE fingerprinting method. It allowed us to answer the following questions: (1) are endophytic bacteria present in micropropagated pineapple plants, maintained in vitro for long periods of cultivation? (2) are these bacteria equally distributed throughout the plant organs?

Materials and methods

Samples were collected from pineapple microplants (*Ananas comosus* (L.) Merrill, cv. IAC Gomo-de-mel) from the Laboratório de Morfogênese e Biologia Reprodutiva de Plantas (Piracicaba, São Paulo, Brazil). Such plants, originally derived from meristem culture, had been maintained in vitro for over 5 years (25 ± 2°C, 16 h photoperiod, 42 µmol m^{-2} s^{-1} photosynthetic photon flux density). The microplants were cultured singly in glass culture tubes (150 × 25 mm) containing 15 ml of half strength MS (Murashige and Skoog 1962) salt medium containing 3% (w/v) sucrose, and sub-cultured at intervals of 35 days. Each sample consisted of fragment of roots, young leaves (unexpanded) and mature leaves (fully expanded, length > 4 cm, lamina width ≥ 0.5 mm), without any evidence of microbial contamination. Still, they were surface sterilized according to a methodology previously described for endophytic bacterial isolation (Araújo et al. 2002). Root and leaf tissues were immersed in 70% ethanol for 1 min, washed with sodium hypochlorite solution (2.5% available Cl^-) for 20 min, rinsed with 70% ethanol for 30 s, and washed three times with sterile distilled water. To confirm that the sterilization process was successful, the aliquots of the sterile distilled water used in the final rinse were set on tryptic soy agar (TSA) medium plates. The plates were examined for bacterial growth after incubation at 28°C for 7 days. Plant samples that were not contaminated as detected by culture-dependent sterility test were used for further analysis.

Approximately 200 mg of each plant sample was ground in liquid nitrogen, and the whole genomic DNA was extracted using the CTAB procedure (Araújo et al. 2002). Four replicates were performed for the roots, young and mature leaves, resulting in 12 samples. DNA extraction and integrity were assessed in a 1% (w/v) agarose gel. For the analysis of total endophytic bacterial community, and to avoid the interference of plant chloroplast DNA, the amplification of 16S rRNA gene (*Bacteria* domain) was carried out with primers f799 (Chelius and Triplett 2001) and r1492 (Heuer et al. 1997), performing 35 cycles of 94°C for 20 s, 53°C for 40 s, and 72°C for 40 s.

For amplification of alpha and betaproteobacterial or actinobacterial 16S rRNA gene, initial PCRs were performed with group-specific primers: f203α, f948β and f243, respectively, and combined with primer r1387 (Heuer et al. 1997; Gomes et al. 2001). The amplified products were used as templates in (separate) nested PCRs with primers U968⊥GC and r1387 (Heuer et al. 1997). DGGE was performed as described previously (Muyzer et al. 1993) with the Ingeny phorU2 apparatus (Ingeny, Goes, The Netherlands). The amplicons obtained by PCR were loaded onto 6% (*m/v)* polyacrylamide gels in 0.5× TAE (Tris–acetate-EDTA) buffer. The polyacrylamide gels were made with denaturing gradients ranging from 45 to 65% (where the 100% denaturant contained 7 mol l^{-1} urea and 40% formamide). The gels were run for 4 h at 200 V and 60°C, after gels were washed for 30 min in a fixing solution (10% v/v ethanol and 0.5% v/v acetic acid), stained for 20 min in a staining solution (0.2% w/v $AgNO_3$), washed in developing solution for 10 min (1.5% w/v sodium hydroxide and 0.8% v/v formaldehyde), and washed in fixing solution for 5 min.

Denaturing gradient gel electrophoresis gels images were normalized and analyzed using the BioNumerics software platform (Applied Maths, Belgium). The unweighted-pair group method using arithmetic average (UPGMA) cluster analysis was performed based on the Pearson correlation coefficient. The position tolerance was set at 1% and background subtraction was applied. Both strong and weak bands were included in the analysis, thus taking into account the

presence and absence of bands at specific positions. The species richness was expressed as the total number of detectable 16S rRNA gene amplicons on the DGGE profiles.

Selected DGGE bands were excised, macerated in sterile water and submitted to a further PCR amplification using primers U968 and r1387 (Heuer et al. 1997). The new PCR products were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, USA) and cloned into a pGEM®-T Easy Vector (Promega, USA) in accordance with the manufacturer's instructions. Plasmids were isolated from *Escherichia coli* DH5α by using standard protocols. The purified plasmids with the correct insert (proper lengths were evaluated on agarose gels) were then sequenced in both directions with universal M13 primers. Analyses of sequences were performed with the basic sequence alignment BLASTn program run against the BLAST database (National Center for Biotechnology Information website [http://www.ncbi.nlm.nih.gov]).

Results and discussion

PCR amplifications with primers f799 and r1492 resulted in two products: one band with approximately 700 bp (bacterial 16S rRNA gene amplicons), and another between 900 and 1,100 bp (cloroplastidial 16S rRNA gene amplicons). Thus, it confirmed the presence of bacterial DNA in association with plant material. The PCR–DGGE analysis revealed that endophytic bacterial communities were colonizing all plant organs (roots, young and mature leaves) in all the studied microplants (Fig. 1). According to Andreote et al. (2006), the presence of bands in the DGGE fingerprints in supposedly bacterium-free plants, grown under sterile conditions, indicated that these endophytes were hidden into plant tissues. Similar observations were reported for in vivo plants, in studies with endophytic bacteria in citrus (Araújo et al. 2002; Andreote et al. 2006), eucalyptus (Andreote et al. 2009) and peach palm (Almeida et al. 2009), where non-culturable bacterial communities could only be fully assessed by culture-independent approaches.

Concerning the selectivity of these endophytic bacteria, the cluster analysis based on DGGE profiles, derived from specific bacterial groups, revealed differences between the community structures of *Actinobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* for the examined plant tissues (Fig. 1). The dendrograms showed common and specific bands in each group of samples. The specificity and the sharing of bands (species) were more evident in Venn diagrams (Fig. 1) and suggest that different bacterial groups perform diverse colonization in the microplants. These variations can be attributed to plant physiological patterns, which may influence differentially each tissue, and can lead to a differential selection of root-associated (Andreote et al. 2009) or leaf-associated microbes.

Regardless the bacterial group analyzed, numerous bands in DGGE profiles could be clearly distinguished exhibiting a complex pattern of varying composition, especially in leaves samples. Furthermore, the richness was always higher within leaves (young and mature) than within root samples. Although leaves DGGE patterns were more complex than root patterns, young leaves patterns were visually distinguishable from those for mature leaves. Besides that, the DGGE profiles of total bacterial community, *Alphaproteobacteria* and *Actionobacteria* communities, of the mature leaves samples revealed the closest resemblance to those of the young leaves than to root samples. When focus was put on the *Betaproteobacteria* community a higher similarity between roots and young leaves was clear, yielding slight differences from the other two group profile.

Such similarities between endophytic communities might be related to the ecology of these bacterial groups, which have a populational dynamics directly related to plant metabolism factors and also with the availability of nutrients that modulate the niche occupation and the structure of bacterial communities (Baudoin et al. 2003). Normally, in the rhizosphere, organic carbon is considered as the driving force for microbial density and activity (Baudoin et al. 2003). And once inside the plant tissue, the different mechanisms of distribution might be due to interactions with other bacteria or to the different abilities of each microorganism that allows them to exploit different niches (Lodewyckx et al. 2002). However, this dynamic nutrition in micropropagation process is altered. In vitro plants are grown under exogenous supply of carbon source in the medium, leading to a heterotrophic or mixotrophic metabolism (Pospíšilová et al. 1987). Roots from in vitro plants are considered non-functional and may not be important storage organs in plant tissue culture, due to the strong sink force from leaves, which have limited photosynthetic capacity (Pospíšilová et al. 1987).

In mixotrophic plants, the leaves, young or mature, are not the major source of energy and photoassimilates. On the contrary, in vitro leaves may become the principal carbon reserve owning to it sink behavior. This suggests that this higher carbon reserve could support a higher group of bacteria in that niches, resulting in a higher number of bands in the DGGE profile. Additionally, mature leaves, as sink organs, are capable of non-specific trafficking of molecules through simple plasmodesmata (Roberts et al. 2001), and can provide as a nutrient-rich environment for endophyte growth as that which occurs in the young leaves, justifying the resemblance in bands patterns from these two organs. Indeed, source-sink relationships in the host appear

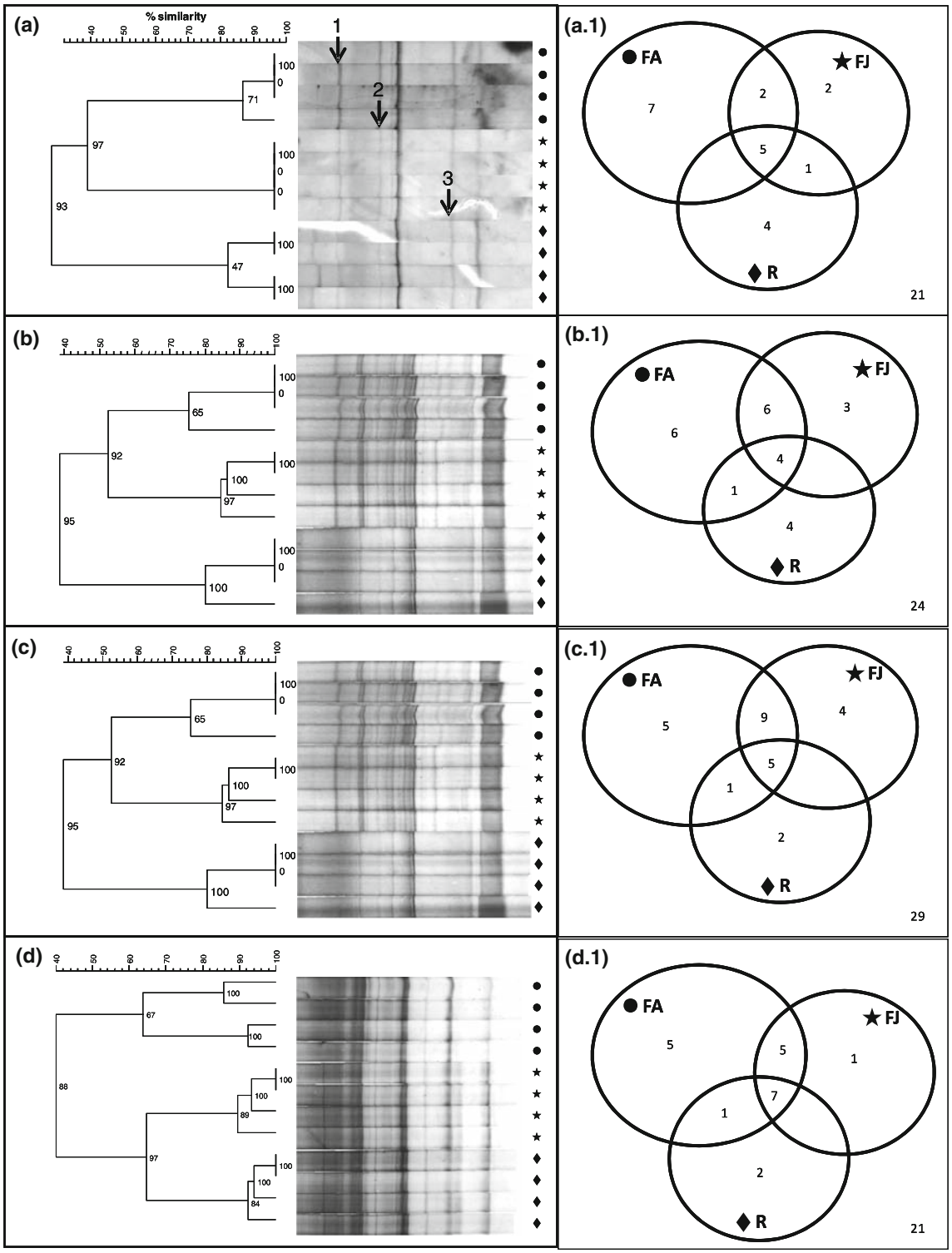

Fig. 1 Cluster analysis of DGGE fingerprintings of total bacterial community (**a**), *Actinobacteria* (**b**), *Alphaproteobacteria* (**c**) and *Betaproteobacteria* (**d**) with UPGMA algorithm and Pearson correlation coefficient. *Numeric* values at branch nodes indicate the cophenetic correlation coefficient associated with each cluster. The indications *a.1*, *b.1*, *c.1* and *d.1* show Venn diagrams with common amplicons 16S rDNA richness detected for DGGE fingerprintings in different plant organs and stages of development (mature leaves *filled circle*, young leaves *filled star* and roots *filled diamond*). *Number* indicate the amount of common or unique bands found in samples. *Arrows* show excised bands (no. 1, 2 and 3)

to be a determining factor in the growth of endophytes (Bethlenfalvay et al. 1982).

Sequence analyses retrieved from the bands 1–3 (Fig. 1) were similar to some culturable and unculturable strains. Band (1) showed highest similarity to uncultured *Gammaproteobacteria* (*Pseudoxanthomonas* sp.), band (2) to cultured *Gammaproteobacteria* (*Stenotrophomonas maltophilia*), and band (3) to cultured *Betaproteobacteria* (*Burkholderia cenocepacia*). Sequence matches from the GenBank nucleotide database ranged from 96 to 100%. These results are related to those revealed by other studies, in which the *Proteobacteria* group presents itself as a predominant group of endophytic bacteria that shows beneficial effects on host plants, such as growth promotion and plant pathogen antagonism (Andreote et al. 2006, 2009; Chelius and Triplett 2001; Dias et al. 2009). These benefits should also be present in in vitro propagated pineapple plants, which was not the aim of this study. However, this merits further investigation.

A high similarity was found among the replicates. This was expected once the microplants were clones (keeping a genetic fidelity) and cultivated under the same controlled conditions. So, the environmental variations that are known to modulate the structure of bacterial communities (Araújo et al. 2002) were limited. Then, the only differences between the samples were restricted to the microclimate of each test tube and the subcultures history of each microplant, differences that were almost negligible.

This study concluded that even in long-term in vitro pineapple plants, which were considered axenic, endophytes remains into plant tissues, without causing any visible external symptoms, but constitute a complex and diverse bacterial community, influenced by the different physiological conditions found in each plant organ.

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