Research Letter

Diversity of endophytic bacteria from *Eucalyptus* species seeds and colonization of seedlings by *Pantoea agglomerans*

Anderson Ferreira¹, Maria Carolina Quecine¹, Paulo Teixeira Lacava¹, Shintiro Oda², João Lúcio Azevedo¹ & Welington Luiz Araújo¹,³

¹Departamento de Genética, Escola Superior de Agricultura ‘Luiz de Queiroz’, Universidade de São Paulo, Piracicaba, SP, Brazil; ²Suzano Bahia Sul Papel e Celulose S/A, Suzano, SP, Brazil; and ³Núcleo Integrado de Biotecnologia, Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brazil

Correspondence: Welington Luiz Araújo, Departamento de Genética, Escola Superior de Agricultura ‘Luiz de Queiroz’, Universidade de São Paulo, PO Box 83, 13400-970 Piracicaba, Brazil. Tel.: +55 19 34294251; fax: +55 19 34336706; e-mail: wilaraujo@esalq.usp.br

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Abstract

The diversity and beneficial characteristics of endophytic microorganisms have been studied in several host plants. However, information regarding naturally occurring seed-associated endophytes and vertical transmission among different life-history stages of hosts is limited. Endophytic bacteria were isolated from seeds and seedlings of 10 *Eucalyptus* species and two hybrids. The results showed that endophytic bacteria, such as *Bacillus*, *Enterococcus*, *Paenibacillus* and *Methylbacterium*, are vertically transferred from seeds to seedlings. In addition, the endophytic bacterium *Pantoea agglomerans* was tagged with the *gfp* gene, inoculated into seeds and further reisolated from seedlings. These results suggested a novel approach to change the profile of the plants, where the bacterium is a delivery vehicle for desired traits. This is the first report of an endophytic bacterial community residing in *Eucalyptus* seeds and the transmission of these bacteria from seeds to seedlings. The bacterial species reported in this work have been described as providing benefits to host plants. Therefore, we suggest that endophytic bacteria can be transmitted vertically from seeds to seedlings, assuring the support of the bacterial community in the host plant.

Introduction

The genus *Eucalyptus* (*Myrtaceae*) is composed of over 700 species, distributed primarily in the tropics and subtropics. Regions under *Eucalyptus* cultivation have been expanding in the last few decades and the members of the genus now occupy an important position in the world economy. The countries delivering the most *Eucalyptus* to the world markets include Brazil, India, South Africa, Portugal, Angola, Spain and China (Poke et al., 2005). Worldwide, Brazil occupies the 11th position in cellulose production and the seventh in paper production (BRACELPA, 2006). Over 3 million hectares of *Eucalyptus* are cultivated in Brazil, which represents c. 50% of the area covered by exotic forests (Embrapa, 2006). These data demonstrate the importance of this crop for the Brazilian economy.

Endophytic bacteria are defined as bacteria that live within a plant for at least a part of its life, without causing apparent harm to the host. These bacteria can be isolated from surface-disinfected plant tissues or extracted from these host plants (Hallmann et al., 1997). The most comprehensive definition was proposed by Azevedo & Araújo (2007), which described an endophyte as all microorganisms that may or may not be successfully cultured, that either internally colonize the host plant and do not cause apparent damage and/or visible external structures. The potential for practical applications of endophytes has led to studies addressing the bacteria’s ability to control both disease and insect infestations, as well as promoting plant growth (Azevedo et al., 2000; Kozdrój et al., 2004; Kavino et al., 2007).

A variety of microorganisms have been isolated from surface-disinfected seeds, including endophytes and pathogens (Granér et al., 2003; Majewska-Sawka & Nakashima, 2004; Rudgers et al., 2004; Jordaan et al., 2006; Rudgers & Clay, 2007). However, information regarding naturally occurring seed-specific endophytes and interactions is limited (Mundt & Hinkle, 1976; Cankar et al., 2005; Vega et al., 2005). Seeds are one of two methods used in *Eucalyptus* cultivation; the second is the generation of...
micro-propagated plants. In some species, seed sowing and subsequent germination allows for vertical transfer of endophytic microorganisms (Majewska-Sawka & Nakashima, 2004; Gao & Mendgen, 2006). In fact, endophytic bacteria with beneficial characteristics are frequently applied artificially to seeds. However, bacterial strategies for colonizing different plant tissues are still unknown (Hallmann et al., 1997).

_Pantoea agglomerans_ has been described as an effective bacterium to control plant disease (Costa et al., 2002; Hsieh et al., 2005), generate antibiotics (Wright et al., 2001) and promote plant growth (Procópio, 2004; Feng et al., 2006). In South Africa, _Pantoea_ species were described previously as a phytopathogen of _Eucalyptus_ (Coutinho et al., 2002; Medrano & Bell, 2007). However, _P. agglomerans_ strain 33.1 was isolated from _Eucalyptus_ and caused no harm to the plants (Procópio, 2004). In addition, this author showed that strain 33.1 possessed other beneficial characteristics, including plant growth promotion.

A relationship between endophytes and seeds that results in seedling protection against various threats, such as disease or herbivory, is an interesting strategy to increase plant fitness. Therefore, the aims of this study were twofold: (1) detect and identify the endophytic bacterial community in seeds and seedlings of different _Eucalyptus_ species via isolation and (2) study the colonization of _Eucalyptus_ seedlings by genetically modified _P. agglomerans_, an endophytic bacterium, and evaluate the characteristics of the bacterium by isolation and fluorescence microscopy.

**Materials and methods**

**Bacteria, plants and plasmids**

The following _Eucalyptus_ species were chosen for the study: _Eucalyptus grandis_ (W. Hill.), _Eucalyptus dumii_ (Maiden), _Eucalyptus robusta_ (Sm.), _Eucalyptus camaldulensis_ (Dehn.), _Eucalyptus citriodora_ (Hook.), _Eucalyptus urophylla_ (S.T. Blake), _Eucalyptus brassiana_ (S.T. Blake), _Eucalyptus saligna_ (Sm.) and also the hybrids _E. grandis_ × _Eucalyptus globulus_ (Labill.) and _E. robusta_ × _E. grandis_. Seeds were kindly provided by Cia. Suzano Papel e Celulose (Itapenininga, SP, Brazil).

The bacterium _P. agglomerans_ strain 33.1 was isolated previously from healthy _E. grandis_ (Procópio, 2004) and belongs to the collection of Laboratório de Genética de Microorganismos, Genetics Department, ESALQ/USP. Also, _Escherichia coli_ DH5α pir, a DH5α derivative strain expressing the R6K _π_ protein, was used as the host for cloning experiments. The plasmid pNKBOR carrying a mini-Tn10 derivative with a kanamycin (kn) resistance gene (Rossignol et al., 2001) was used as a scaffold for subcloning the gfp gene from commercial plasmid pMUT-GFP (Clontech Laboratories).

**Seed germination**

Seed germination was induced following seed surface disinfection [70% ethanol for 1 min, sodium hypochlorite solution (2% available Cl) for 2 min, 70% ethanol for 1 min and two washes in sterilized distilled water]. Seeds were germinated on MS medium (Murashige & Skoog, 1962) at 25°C with 12 h of dark/light. Thirty-day-old seedlings were used for bacterial isolation.

**Isolation of endophytic bacteria from seeds and seedlings**

Total endophytic bacteria were isolated from surface-disinfected seeds and seedlings using a serial washing as described above. After surface disinfection, the seeds were triturated in sterile phosphate-buffered saline [PBS, containing (g L⁻¹) Na₂HPO₄, 1.44; KH₂PO₄, 0.24; KCl, 0.20; NaCl, 8.00; pH 7.4] and maintained at 28°C under 150 r.p.m. agitation. Appropriate dilutions were subsequently plated onto 10% trypticase soy agar (TSA-Merck) supplemented with 50 μg mL⁻¹ of benomyl fungicide to prevent fungal growth. Plates were incubated at 28°C for 20 days, and the number of CFU was determined to estimate population density. The disinfection process was verified by plating aliquots of the final sterile-distilled water wash onto 10% TSA and incubated under the same conditions as described above. Following purification, single colonies were suspended in 20% glycerol solution and stored at −70°C.

**Amplified rDNA restriction analysis (ARDRA) and molecular identification of endophytic bacteria**

The 16S rDNA gene was amplified using colony-PCR. Isolates were grown in TSA and transferred to a tube containing 200 μL of sterilized ultra pure water. The bacterial suspension was used as the source of DNA to conduct PCR. The following primer sequences were chosen for 16S rDNA gene amplification: PO27F (5′-GAGGTTTTGA TCCGGGCTCAG-3′) and 1387R (5′-CGGTGTGTACAA GGCAGGAGACG-3′). The PCR reaction mixture included 1 μL of bacterial suspension, 10 mM Tris-HCl (pH 8.3), 3.75 mM MgCl₂, 0.2 mM of each dNTP, 200 mM of each primer and 2.5 U of Taq DNA polymerase in a 50 μL final volume. A negative control (PCR mixture without DNA) was included in all PCR amplifications. Amplifications were performed in a thermal cycler with the following PCR parameters: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, primer annealing at 62.5°C for 1 min and primer extension at 72°C for 1 min.
followed by a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide in 0.5 × TBE buffer.

Following PCR amplification, ARDRA was performed as follows: 1 µg amplified 16S rRNA partial gene sequence was digested with 2 units of HhaI restriction enzyme (Fermentas Life Sciences, Brazil) according to the manufacturer’s recommendations. The products were resolved in 2.5% (w/v) agarose gel and stained with ethidium bromide.

16S rRNA gene PCR products were purified with polyethylene glycol (PEG) (20% PEG 8000; 2.5 mM NaCl) and sequenced in the Instituto do Genoma Humano (USP, São Paulo, Brazil). The sequences were evaluated in BLASTN (National Center for Biotechnology Information website) against the database of the GenBank and further deposited in GenBank.

**Development of GFP-tagged P. agglomerans (33.1:gfp strain)**

The gfp gene was amplified from pMUT-GFP using the primers PGFP (forward – 5'-TACGCCGAAATTCAGCT TGCATGCGTACGG-3') and PGFPII (reverse – 5'-GG CCCGAGTCTACGGCCATGATTGTC-3'), purified and cloned in the BamHI-digested site of the integrative pNKBOR plasmid (Rossignol et al., 2001), generating the pNKGFP integrative plasmid. All molecular procedure was carried out according to a standard protocol (Sambrook et al., 1989), using E. coli DH5α pir as a host.

The endophytic P. agglomerans 33.1 strain was grown (OD560nm 1.0) for 1 day at 28 °C in 5 mL Luria–Bertani (LB) liquid medium, harvested by centrifugation at 4 °C, resuspended with 1 mL of cold ultrapure water and electrophoresed (2.5 kV; 25 mA; 25 µM and 400 Ω) with 0.1 µg of pNKGFP plasmid. After transformation, 1 mL of LB medium was added, incubated for 1 h at 28 °C and plated on LB medium supplemented with kanamycin (50 µg mL⁻¹). The identification of clones carrying the gfp gene was carried out on UV light.

**Seed inoculation and P. agglomerans strain 33.1:gfp reisolation**

The P. agglomerans 33.1:gfp strain was inoculated onto surface-disinfected seeds (process as described previously) of E. grandis, E. urophylla and the hybrid E. grandis × E. globulus. The seeds were maintained for an hour in a bacterial suspension (10⁶ CFU mL⁻¹) of strain 33.1:gfp and planted in the PlantMax® (Eucatex) substrate and maintained under greenhouse conditions.

Genetically modified P. agglomerans was isolated from surface-disinfected seedlings (germinated from inoculated seeds) after 15 and 30 days of germination. Five seedlings from each species were harvested and endophytic bacterial community was evaluated from the rhizoplane and plant tissues (roots, stems and leaves). Bacteria from the rhizoplane were isolated using the following procedure: roots were placed in a 500-mL Erlenmeyer flask containing 25 g of 0.1-cm-diameter glass beads and 50 mL of PBS; flasks were agitated at 150 r.p.m. at 28 °C for 1 h and after agitation, appropriate dilutions of bacterial suspension were plated onto LB medium supplemented with 50 µg mL⁻¹ of kanamycin antibiotic. The endophytic bacteria in the leaves, stems and roots were isolated by surface disinfection, as described previously. Tissues were triturated in sterile PBS and maintained at 28 °C with 150 r.p.m. agitation for 1 h. Appropriate dilutions of the bacterial suspension were then plated onto LB medium supplemented with 50 µg mL⁻¹ of kanamycin antibiotic. Following 3 days of plate incubation at 28 °C, CFUs were determined to estimate the population density.

**Fluorescence microscopy**

Seedlings were removed from pots 15 days after germination and washed in running tap water. Leaves, stems and roots were cut into small pieces (c. 0.5 cm²) and mounted on a bridged slide with 10% (v/v) glycerol (microscope grade). Fluorescence microscopy was carried out on a Zeiss Axio-phot-2 epifluorescence microscope with the recommended filter for GFP analysis. GFP-tagged bacterial cells were excited using a 490 nm filter and the images were captured with a video camera, using the ISIS software (Meta Systems, Germany).

**Statistical analysis**

Data analysis was carried out using the SAS software package (SAS Institute, Cary, NC). Bacterial counts were transformed using log₁₀ of X + 1 before implementing an ANOVA. An a priori 0.05 level of significance was established for data analysis. In addition, Tukey’s least significant difference test was used for paired comparison of means.

**Results**

**Isolation and identification of indigenous endophytic bacteria**

We successfully isolated endophytic bacteria from six out of 10 Eucalyptus species and studied hybrids (Table 1). Plating of triturated seed samples showed that total cultured bacterial densities were significantly different (P ≤ 0.05) among seeds of Eucalyptus species and hybrids. The density of endophytic bacteria ranged from 0.33 to 1.83 × 10⁶ CFU g⁻¹, for E. camaldulensis and E. urophylla seeds, respectively.

Bacteria were consistently isolated from seeds and seedlings of E. brassiana, E. citriodora, E. globulus × E. grandis,
E. robusta × E. grandis and E. urophylla (Table 1). However, bacteria were not obtained from seeds and seedlings of E. dunnii, E. grandis, E. robusta and E. saligna. Although bacteria were isolated from seeds of E. camaldulensis, bacteria were not successfully obtained from seedlings. The total bacterial culture densities were significantly different (P ≤ 0.05) among Eucalyptus seedlings (Table 1), ranging from 0.27 to 0.87 × 10^2 CFU g⁻¹ in E. citriodora and the hybrid E. robusta × E. grandis, respectively.

We successfully amplified the 16S rRNA gene directly from bacterial colonies without prior DNA extraction. Eight ribotypes were obtained from 50 endophytic bacteria characterized using ARDRA (Table 2). Of these, four ribotypes were common to both seed and seedling tissues and the other four were observed exclusively in one of the tissue types (Table 2). The isolates representative of these ribotype groups were identified by 16S rRNA gene sequence data (Table 2). The molecular identification of the cultured endophytic bacteria associated with Eucalyptus seeds belonged mainly to the classes Alphaproteobacteria, Firmicutes and Actinobacteria. These bacteria remained in the seedling following germination, except Actinobacteria. The 16S rRNA gene sequence similarities allowed us to verify that the bacterial community associated with the Eucalyptus seeds is constituted by Bacillus sp., Bacillus megaterium, Enterococcus mundtii, Methylobacterium sp., Methylobacterium variabile, Methylobacterium gregens, Paracoccus sp., Paenibacillus sp, Paenibacillus humicus, Sphingomonas phyllosphaerae and bacteria from the Frankiaceae (Actinobacteria) family (Table 2).

### Seedling colonization by P. agglomerans

The total bacterial population expressing the gfp gene was determined by examining plant tissues. Pantoea agglomerans continued to colonize the seeds after planting and were subsequently isolated from Eucalyptus seedlings. The 33.1:gfp strain was identified from the rhizoplane and from plant tissue dissections (roots and stems) of E. grandis and the hybrid E. grandis × E. globulus. The bacterial densities from the hybrid E. grandis × E. globulus rhizoplane were significantly (P ≤ 0.05) higher than that of E. grandis. Differences in bacterial densities were not observed in the roots and stems.

The Eucalyptus tissues auto-fluorescence hindered but did not disable the visualization of gfp expression; however, the fluorescence emitted by 33.1:gfp reduced the image contrast. Fluorescence microscopy confirmed a 33.1:gfp strain endophytic colonization in roots, mainly in intercellular spaces (Fig. 1a and b), in stem and xylem vessels (Fig. 1c and d). Eucalyptus urophylla seedlings were not colonized by inoculated bacterium. In all Eucalyptus species included in this...
The presence of seed microorganisms has been reported for many plant species (Granér et al., 2003; Majewska-Sawka & Nakashima, 2004; Rudgers et al., 2004; Jordaan et al., 2006; Rudgers & Clay, 2007) and endophytic microorganisms have even been isolated from flowers (Majewska-Sawka & Nakashima, 2004). Most studies have focused on fungal endophytic microorganisms in seeds, but few reports regarding the presence or persistence of endophytes after germination and seedling growth are available (Gao & Mendgen, 2006; Jordaan et al., 2006; Bennett & Whipps, 2007). In this study, we detected endophytic bacteria in the seeds of *Eucalyptus* species and bacterial presence following germination. In some cases, bacteria were isolated from seeds but not from seedlings, for example, *E. camaldulensis*, suggesting that the plants and not the seeds were axenic. Also, this result could be observed whether the bacteria become unculturable at some point of plant development.

Several studies have shown that microorganism density in seeds of the same species decreased during seed dormancy (Mundt & Hinkle, 1976; Bacon & Hinton, 1996; Pirritila et al., 2000; Granér et al., 2003; Mano et al., 2007). The 16S rRNA gene sequence identification indicated that the bacterial species associated with *Eucalyptus* are not described as pathogenic. Moreover, some of these genera have been reported as beneficial, such as *Bacillus* with the potential of biological control (Ryu et al., 2006; Senthilkumar et al., 2007), *Paracoccus* and *Frankia* as nitrogen fixers (Stroh et al., 2004; Gaulke et al., 2006), *Bacillus* and *Paracoccus* in growth hormone production (Tsavelkova et al., 2007) and *Methylobacterium* in systemic resistance induction (Madhaiyan et al., 2004). *Methylobacterium* may also stimulate seed germination and promote root growth (Freyermuth et al., 1996; Holland, 1997).

The presence of seed microorganisms may facilitate future studies to investigate beneficial seed microorganisms that later colonize plants (Bennett & Whipps, 2007). We showed that 33.1:gfp inoculated on *Eucalyptus* seeds had the capability to colonize seedlings following germination and growth. The endophytic 33.1:gfp strain could be carried initially into the embryo through the break in the seed husk. Inside the seedlings, the colonization profile of 33.1:gfp was similar to field observations, i.e. larger colonization density in the rhizoplane, followed by roots and stems (Fisher et al., 1992; Elvira-Recuenco & van Vuurde, 2000; Kuklinsky-Sobral et al., 2004; Mendes et al., 2007).

This is the first report to describe the detection, identification and persistence of bacterial communities in *Eucalyptus* seeds, and the subsequent colonization of the same bacteria in *Eucalyptus* seedlings. The results of this study suggested that vertical transfer of endophytic microorganisms by seeds might maintain the stability of bacterial communities in *Eucalyptus* plants. A plant that already supports an established endophytic bacterial community has the potential to pass the microorganisms and the possible intrinsic benefits to their offspring.

However, the present study may not explain how the bacterial community is transmitted by seeds in terms of whether seed infection occurs directly by gametic cells or by maternal host plant tissues. Majewska-Sawka & Nakashima (2004) observed that the hyphae of the endophytic fungus *Neotyphodium loli* spread through specific flower tissues and developing seeds, suggesting that the plant colonization follows a precisely coordinated and unknown mechanism that defines the flower part that could be colonized by the endophyte. In *Eucalyptus*, the seed infection could occur after or during seed development, but the presence of the same genotypes, such as *B. megaterium*, *P. humicus* and *M. variabile*, inside plants, seeds and seedlings suggests that the host plant could be the source of the bacterial endophytic community.

Also, in this study, we reported the inoculation of *Eucalyptus* seeds and later seedlings, with the endophytic bacterium *P. agglomerans*, isolated from *Eucalyptus*. This
may serve as a point of departure for the use of genetic engineering to manipulate endophytic microorganisms with beneficial characteristics. The engineered endophytes could be used to inoculate seeds, and the beneficial characteristics, derived from the microorganisms, be expressed in host plants.

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