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RESEARCH ARTICLE

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Detection of indigenous endophytic bacteria in *Eucalyptus urophylla in vitro* conditions

Abstract The presence of indigenous endophytic bacteria in aseptically grown seedlings of *Eucalyptus urophylla* germinated from surface sterilized seeds was investigated using dilution plating, microscopy, and PCR detection. No culturable endophytic bacteria could be detected in suspensions of ground plant tissue incubated on solid or in liquid cultivation media. However, a large number of endophytic bacterial cells, mostly rod-shaped and measured 2–3 μm×0.5–0.8 μm, were observed in *in vitro* cultured seedlings of *E. urophylla* using both light and electron microscopy. Using the universal bacterial 16S rDNA primers, a predicted 190-bp fragment was amplified from total DNA isolated from the seedlings of *E. urophylla*. We concluded that the endophytic bacteria originated from the seed were present in seedlings of *E. urophylla*. However, the bacterial cells observed appeared to be nonculturable.

Keywords electron microscopy, endophytic bacteria, *Eucalyptus urophylla*, PCR

1 Introduction

Eucalyptus spp. mostly originated in Australia belong to the family of Myrtaceae. They are fast-growing tree species and one of the most valuable hardwoods. Eucalypt plantations can be found in more than 90 countries with a total area of around 2.0×10^7 hm^2. The main eucalypt-growing countries are India (8.0×10^6 hm^2), Brazil (3.0×10^6 hm^2), and China (1.5×10^6 hm^2) (FAO, 2001). In south China, it is grown mainly for production of pulpwood, flakeboard, plywood, construction poles, and railroad crossties (Li and Liu, 2003).

Endophytic bacteria actively colonize host plant tissues and mainly reside in the intercellular spaces without causing obvious harm to the plant (Yang et al., 1998). These bacteria can be isolated from roots, leaves, stems, and fruits of different plants, such as cotton, potato, rice, and tomato (Mukhopadhyay et al., 1996; Reiter et al., 2002; Yan et al., 2004; Li et al., 2005). It has been suggested that some endophytic bacteria can effectively control disease (Fisher et al., 1992; Chen et al., 1995; Fu et al., 1999; Kong and Ding, 2001; Mercado-Blanco and Bakker, 2007). The presence of bacteria within seeds of various plant species has also been reported (Mundt and Hinkle, 1976; Bacilio-Jiménez et al., 2001; Cankar et al., 2005; Mano et al., 2006). Moreover, endophytes can accelerate seedling emergence and enhance plant growth and development (Qiu et al., 2004).

In a previous study, we found that some rhizosphere bacterial strains could suppress bacterial wilt in *Eucalyptus* (Ran et al., 2005a), but their colonization readily decreased with time. It was also found that the population of endophytic bacterium *Pseudomonas poae* isolated from tomatoes applied to eucalypt seedlings showed an initial increase followed by a subsequent reduction in the population size (Ran et al., unpublished). Since endophytic bacteria in seeds or plants can inhibit colonization of other bacteria (Bacilio-Jiménez et al., 2001), it was hypothesized that indigenous endophytic bacteria in eucalypt seedlings could hamper the effective colonization of exogenous biocontrol bacterial strains. In this study, we explored the presence of indigenous endophytic bacteria in *E. urophylla* seedlings using cultivation-dependent dilution plating and cultivation-independent microscopical and molecular techniques.

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2 Materials and methods

2.1 Seeds

Seeds of *E. urophylla* were kindly provided by the Forestry Bureau of Leizhou, Guangdong Province, China, and stored in a sealed plastic bottle at 4°C for 5 years.

2.2 Cultivation of plants

Seeds of *E. urophylla* were surface-sterilized by treatment with 70% ethanol for 30 s, followed by 0.1% acidic mercury chloride for 2.5 min and subsequently, three washes in sterile distilled water. To check surface contamination, seeds were aseptically transferred to Erlenmeyer flasks containing 50-mL solid agar MS medium (Murashige and Skoog, 1962) and about 15 seeds per flask. Then the germinated seedlings in the flasks without surface contamination were put in an incubator with a 12-h light and 12-h dark cycle at 25°C and 23°C, at a relative humidity of 70%. When the plants in the flasks were about 6 cm high, each of them was cut into four segments and transferred to a new flask containing MS, respectively. In this way, the identical seedlings with the same background of endophytes can be obtained.

2.3 Isolation of culturable endophytic bacteria

Each of three *E. urophylla* seedlings grown under an aseptic condition was triturated separately in 1 mL sterile water using an autoclaved pestle and mortar. One hundred microliters of this suspension was plated on King's medium B (KB) agar plates (King et al., 1954), 1/10 KB, and modified nutrient agar (NA) plates (Zheng et al., 2008), respectively, and incubated at 28°C in the dark for two weeks. Alternatively, 200 μL of the suspension was transferred to Erlenmeyer flasks containing 15 mL of either liquid KB, 1/10 KB, or modified NA, and the flasks were placed in an orbital shaker at 150 $r \cdot min^{-1}$ at 28°C in the dark for two weeks.

2.4 Light microscopy

Each of three eucalypt seedlings at a height of 5 cm was ground with an autoclaved pestle and mortar in 1 mL sterile water under aseptic conditions, and this experiment was repeated three times. The suspensions were transferred into a sterile screw-capped tube and centrifuged at 1141×g for 5 min. Thereafter, the supernatant was transferred into a new sterile tube followed by centrifugation at 10146×g for 5 min. The pellet was suspended in sterile water. A droplet of the suspension was put onto a clean glass slide, dyed with 5% acidic fuchsin sodium salt for 1–2 min, air dried, and observed using a light microscope at 1600× magnification.

2.5 Scanning electron microscopy (SEM)

Three stem segments from eucalypt seedlings (thickness 3–5 mm) were fixed with 4% (v/v) glutaraldehyde in 0.1 $mol \cdot L^{-1}$ sodium cacodylate at pH 7.4 for 12 h at 4°C and further fixed in 1% (w/v) osmium tetroxide in the same buffer for 2 h at 4°C. The specimens were dehydrated in a graded ethanol series. Then the samples were treated with CO_2 and mounted on an aluminum cylinder with silver paste, and finally covered with a steam of carbon and ionized gold. Metal-coated specimens were observed under a SEM (Hitachi S-3500N) at an accelerating voltage of 20 kV.

2.6 Transmission electron microscopy (TEM)

Concentrated suspensions of indigenous bacteria from eucalypt seedlings were prepared by centrifuge as previously described and diluted with sterile distilled water to adjust the population density at $1\times10^8 - 1\times10^9$ $cells \cdot mL^{-1}$ using the light microscope. Samples of the suspensions were mixed with equal volume of 2% sodium phosphotungstic acid, and aliquot of the mixture was mounted onto a cupreous mesh with capillary. The specimen was observed under a TEM (Hitachi-7500) at an accelerating voltage of 80 kV after air drying.

2.7 Amplification of bacterial 16S rDNA

Total DNA was extracted from seedlings of *E. urophylla* using a CTAB-based extraction protocol (Li et al., 2001; Han and Zhang, 2007). The PCR primers used to amplify 16S rDNA gene fragments were the universal bacterial primer pair (Xing et al., 2006): F357 (5′-CCTAC GGG AGG CAG CAG-3′), R518 (5′-ATT ACC GCG GCT GCT GG-3′).

The PCR reaction mixture contained 2 U of *Taq* DNA polymerase with 2.5 μL 10×PCR buffer (contained 20 $mmol \cdot L^{-1}$ Mg^{2+}), 12.5 $mmol \cdot L^{-1}$ of dNTP mixture, 12 $pmol \cdot L^{-1}$ of each primer, and 2 μL of template DNA in a 25 μL final volume. The amplification program was run by first denaturation of template DNA at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on 1.2% (w/v) agarose gels in 1×TAE buffer.

3 Results

3.1 Cultivation of indigenous endophytic bacteria in *E. urophylla*

No bacterial colony developed from the suspensions of ground tissues of *in vitro* grown eucalypt seedlings after

incubation on KB, 1/10 KB, and modified NA agar plates, or in liquid KB, 1/10 KB, and modified NA medium for two weeks.

3.2 Light microscopy to examine endophytic bacteria in eucalypt seedlings

The concentrated suspensions of eucalypt seedlings were observed under optical microscope either directly (Fig. 1 (a)) or after dyeing with acidic fuchsin (Fig. 1(b)). The indigenous endophytic bacteria in eucalypt seedlings were rod-shaped or short club-shaped with the size of 2–3 μm×0.5–0.8 μm.

3.3 Scanning electron microscopy

The stem segments from eucalypt seedlings were observed by SEM. Endophytic bacterial cells were localized on the grooves formed by the junctions among the trachea cells (Fig. 2(a)) or localized on the inner cellular walls of the trachea cells (Fig. 2(b)) with the length of 2–3 μm and short club shape (Fig. 2).

3.4 Transmission electron microscopy

The suspensions of eucalypt seedlings as described previously were treated by negative staining and examined by TEM. At least two different sizes of bacterial cells with rod shape were observed, and the typical cell of a single bacterium was showed in Fig. 3. The size of the bacterium in (a) is 2.6 μm×1.1 μm (Fig. 3), and the one in (b) is 1.1 μm×0.6 μm (Fig. 3).

3.5 Amplification of bacterial 16S rDNA

A clear fragment of 190 bp could be readily amplified from the total DNA of seedlings of *E. urophylla* using the universal bacterial 16S rDNA primers (Fig. 4), indicating that there were indigenous endophytic bacteria colonizing the tissue of seedlings in *E. urophylla*.

4 Discussion

Bacterial endophytes of trees are poorly documented, and seed-associated endophytes have only been reported for a limited number of trees (Mundt and Hinkle, 1976). Bacterial endophytes within the seeds of Norway spruce have been reported in fresh seeds from cones taken from trees immediately after cutting (Cankar et al., 2005). Recently, diversity of endophytic bacteria from seeds of different *Eucalyptus* species was reported (Ferreira et al., 2008), and culturable bacteria from both seeds and seedlings of *E. urophylla* were described.

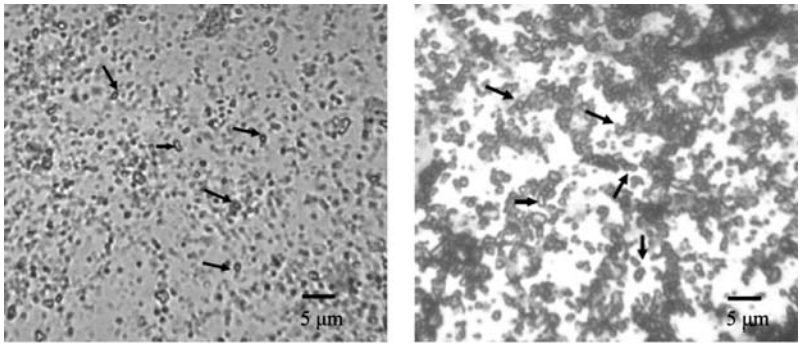


Fig. 1 Observation of endophytic bacteria in eucalypt seedlings under optical microscopy (×1600)
Note: Observed directly (a); observed after dyeing with 5% acidic fuchsin sodium salt (b).

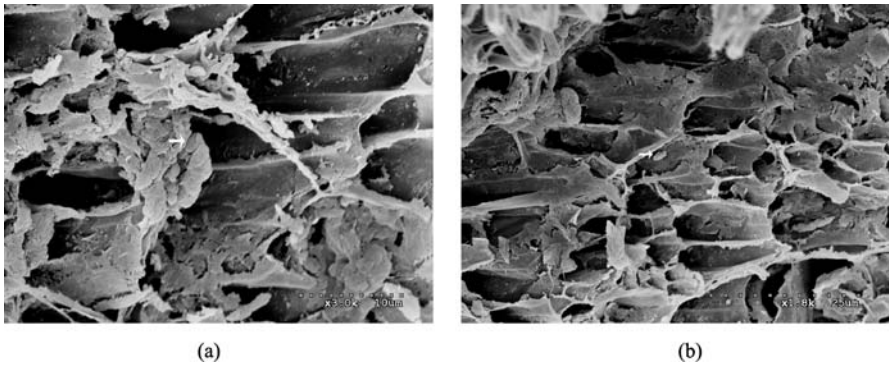
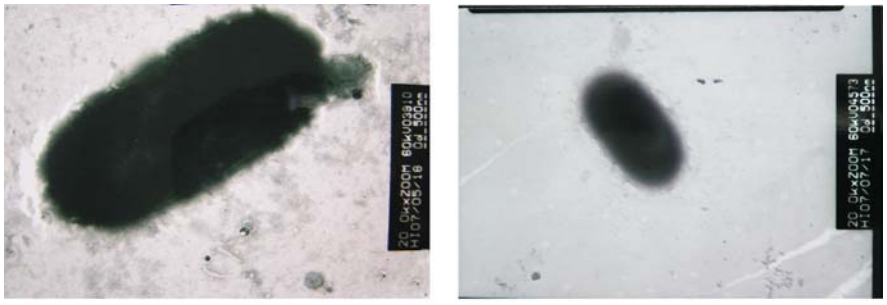


Fig. 2 Observation of endophytic bacteria in eucalypt seedlings under SEM



(a) (b)

Fig. 3 Observation of endophytic bacteria with negative staining in eucalypt seedlings under TEM

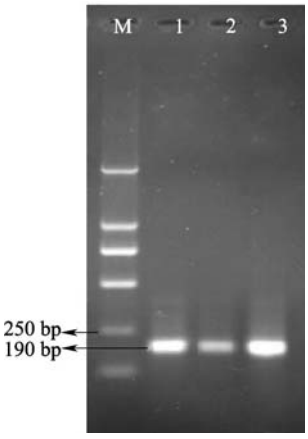


Fig. 4 PCR amplification of 16S rDNA fragment from the total DNA of eucalypt seedlings
Note: M represents Marker DL2000; Lanes 1–3 stand for the 16S rDNA amplified from three single seedlings of *E. urophylla*, respectively.

In our present study, dried seeds of *E. urophylla* stored for 5 years at 4°C were used as starting materials and geminated aseptically to obtain eucalypt seedlings. The seeds were too small to get their suspensions, so the seedlings were used. However, culturable bacteria were not isolated from eucalypt seedlings by conventional culturing methods. The results of observation with microscopy and molecular detection of eubacterial 16S rDNA showed that there were indigenous endophytic bacteria in eucalypt seedlings. It might be inferred that the indigenous endophytic bacteria in eucalypt seedlings were in a viable-but-nonculturable state (Tholozan et al., 1999; Keep et al., 2006). The results might suggest that these endophytic bacteria were originated from the *E. urophylla* seeds.

Eucalypt bacterial wilt is a soilborne, vascular disease caused by *Ralstonia solanacearum* (Smith) Yabuuchi (Coutinho et al., 2000), and it was first reported in *E. grandis* and *E. saligna* in Guangxi Autonomous Region of China in 1982 (Cao, 1982). Since then, the disease has become epidemic and caused great losses in eucalypt plantations in south China, including Guandong, Guangxi, Fujian, Yunnan, and Hainan Provinces (Xiang and Ran, 2004). Efforts to control bacterial wilt in *Eucalyptus* spp. include the use of antagonistic rhizosphere colonizing fluorescent *Pseudomonas* spp. (Xiang and Ran, 2004; Ran et al., 2005b).

The function of the presence of endophytes in host plants is not yet clear. They colonize an ecological niche similar to that of phytopathogens, which might favor them as candidates for biocontrol agents (Li et al., 2003). In some cases, they can accelerate seedling emergence and promote plant establishment under adverse conditions and enhance plant growth and development (He et al., 2004). Interactions may occur between indigenous bacterial endophytes and bacteria that colonize the plant root from the soil or that are applied as biocontrol agents (Bacilio-Jiménez et al., 2001) that result in reduced colonization by the latter. To use the exogenous endophytic bacteria as the control agents of bacterial wilt in *E. urophylla*, further work needs to be done to understand the functions of indigenous endophytic bacteria in plants.

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