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Rhizobacterial characterization for quality control of eucalyptus biogrowth promoter products

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ABSTRACT

Plant growth-promoting rhizobacteria strains from special formulations have been used to optimize eucalyptus cutting production. To undertake quality control for the formulated products, the rhizobacterial strains should be characterized to assess their purity and authentication. In the present study, we characterized nine strains of rhizobacteria, including three *Bacillus subtilis* (S1, S2 and 3918), two *Pseudomonas* sp. (MF4 and FL2), *P. putida* (MF2), *P. fulva* (Ca), *Frateuria aurantia* (R1), and *Stenotrophomonas maltophilia* (CIIb). The strains were differentiated by colony morphology after 24 h of incubation in three different solid state culture media (glucose-nutritive agar, 523 medium and yeast extract-mannitol agar), sensitivity to a panel of 28 antibiotics (expressed according to the formation of inhibition halos of bacterial growth in the presence of antibiotics), and PCR-RFLP profiles of the 16S rDNA gene produced using nine restriction enzymes. It was possible to differentiate all nine strains of rhizobacteria using their morphological characteristics and sensitivity to antibiotics. The molecular analysis allowed us to separate the strains CIIb, FL2 and R1 from the strains belonging to the genera *Bacillus* and *Pseudomonas*. By using these three methods concomitantly, we were able to determine strain purity and perform the authentication.

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Introduction

Free-living bacteria or bacteria associated with root tissues prevail in the plant rhizosphere.[1] *Plant growth-promoting rhizobacteria* (PGPR), the benefic group of these microorganisms, are a class of non-pathogenic soil microorganisms.[2–4]

Rhizobacteria are natural inhabitants of soil that are able to colonize the root systems of plants, thereby contributing several important characteristics. For example, enhanced growth can occur directly through the production of growth promoters, or it can be inhibited by the action of pathogenic microorganisms.[5]

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PGPR bacteria may directly influence plant growth by either synthesizing plant hormones, such as indol-3-acetic acid (IAA),[6,7] or favoring the uptake of nutrients from the soil through different mechanisms, such as nitrogen fixation,[8] phosphorus and potassium solubilization[9] and the synthesis of siderophores for iron sequestration.[10] PGPR can also indirectly affect plants through antagonism between the bacteria and soil-borne pathogens[11] and by inducing systemic resistance in plants against both root and foliar pathogens.

Many studies have explored the biocontrol capacity of these organisms. Additionally, their ability to produce antibiotics makes them a target for the biological control of plant diseases. Strains of rhizobacteria isolated from *Eucalyptus* spp. have been shown to promote rooting through an increase in root biomass and growth of eucalyptus cuttings[12,13] and the reduction of *Cylindrocladium* cutting rot, rust infection (*Puccinia psidii* Winter) in the nursery,[14,15] and bacterial wilt (*Ralstonia solanacearum*).[16] Based on the results, a bioproduct named Rizolyptus®[17] formulated with selected rhizobacterial strains has been used in eucalyptus cutting nurseries. The Rizolyptus® is an inoculant based on only one rhizobacteria strain in a liquid formulation. However, it is essential to know the intrinsic characteristics of each selected growth-promoting rhizobacteria strain prior to mass propagation to ensure the product's quality.

Bacterial characterization is currently based on biochemical tests, antibiotic sensitivity, microscopic observations and molecular analysis. Restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA region (rDNA) associated with polymerase chain reaction (PCR) is an appropriate and inexpensive molecular method.[1,2,18–20] Thus, in the present work, we characterized the Rizolyptus® production from nine rhizobacterial strains based on their morphology, antibiotic sensitivity, and PCR-RFLP profiles. The findings and methods presented in this study represent important tools to ensure the purity, quality and authentication of strains in the final product Rizolyptus® for commercialization.

Material and methods

Rhizobacterium strains

Nine strains of rhizobacteria isolated from eucalyptus that were previously selected for their capacity to promote rooting and the growth of eucalyptus cuttings[14] were characterized. The strains were identified and coded as follows: S1, S2 and 3918 (*Bacillus subtilis* Cobn); Ca (*Pseudomonas fulva* Lizuga & Komagata); CIIb (*Stenotrophomonas maltophilia* Hugh, Palleroni & Bradbury); R1 (*Frateuria aurantia* Swings et al.); MF2 (*P. putida* Migula); and FL2 and MF4 (*Pseudomonas* sp. Migula). The molecular identification, based on homology (>98%) of the 16S rDNA was performed as previously described.[21] The cultures of rhizobacteria are stored in the Forest Pathology Laboratory/Bioagro of the Universidade Federal de Viçosa, Minas Gerais, Brazil.

Morphological characterization

The strains were grown on 523 medium,[22] yeast extract-mannitol agar (YMA)[23] and glucose-nutritive agar (ANG)[24] for 24 h at 28 °C and were characterized according to their colony shape, elevation, edge type (Fig. 1), consistency (i.e., mucosus, fluid or mycelial), aspect of the colony surface (i.e., smooth or rough), brightness (i.e., bright, translucent or opaque), color, size (i.e., <1 mm, 1–2 mm, 2–3 mm or >3 mm), and growth speed (i.e., very fast: visible to the naked eye after less than 24 h of incubation; fast: visible within 24–48 h; intermediate: visible within 24–48 h; slow: visible within 36–96 h; or very slow: visible only after 96 h).

Antibiotic sensitivity

Strain sensitivity to 28 antibiotics was assessed using the standard antibiogram method.[25] An inoculum sample of 0.1 mL was evenly spread in a Petri dish (9 cm diameter) containing 523 medium, and four Whatman® No.1 filter paper disks (Ø = 0.7 cm) that were previously soaked in the antibiotics to be tested were distributed over the medium. A completely random design containing three replicates per antibiotic was used. After a 48-h incubation, the presence or absence of an inhibition halo was observed.

Molecular characterization

Genomic DNA from the rhizobacterial strains[26] was amplified by a PCR reaction consisting of 10–20 ng DNA, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.1 mM of each deoxynucleotide (dATP, dTTP, dCTP and dGTP) (Invitrogen), 0.1 μM of

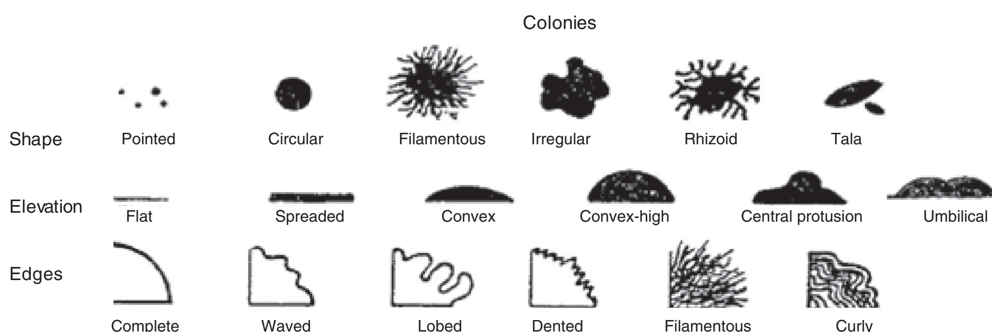

Fig. 1 – Patterns used for the morphological characterization of rhizobacterial strains based on their colony shape, elevation and edge type.
Source: Adapted from Coon et al.[36]

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