J. For. Res. (2019) 30(5):1727–1734
https://doi.org/10.1007/s11676-018-0723-5

ORIGINAL PAPER

Isolation, characterization and effect of plant-growth-promoting rhizobacteria on pine seedlings (*Pinus pseudostrobus* Lindl.)

Cristina Heredia-Acuña[1] · Juan J. Almaraz-Suarez[1] · Ramón Arteaga-Garibay[2] · Ronald Ferrera-Cerrato[1] · Deisy Y. Pineda-Mendoza[1]

Received: 26 May 2017 / Accepted: 19 September 2017 / Published online: 18 June 2018

Abstract In this study, 10 bacterial strains were isolated from the rhizosphere of coniferous trees on Mount Tláloc in Mexico. The strains were characterized by their capacity to produce auxins, solubilize phosphates and stimulate mycelial growth of the ectomycorrhizal fungus *Suillus* sp. All isolates were identified at the molecular level. Moreover, an experiment was established to evaluate the response of *Pinus pseudostrobus* seedlings to inoculation with the rhizobacteria strains. The isolated strains belonged to the species *Cupriavidus basilensis*, *Rhodococcus qingshengii*, *R. erythropolis*, *Pseudomonas* spp., *P. gessardii*, *Stenotrophomonas rhizophila* and *Cohnella* sp. All of the strains produced auxins; the best producer was *R. erythropolis* CPT9 (76.4 μg mL^{-1}). *P. gessardii* CPT6 solubilized phosphate at a significant level (443 μg mL^{-1}). The strain *S. rhizophila* CPT8 significantly increased the radial growth of the ectomycorrhizal fungus *Suillus* sp. by 18.8%. Five strains increased the dry mass of the shoots; *R. qingshengii* CPT4 and *R. erythropolis* CPT9 increased growth the most, by more than 20%. Inoculation with plant-growth-promoting rhizobacteria can be a very useful practice in a forest nursery to produce healthy, vigorous plants.

Keywords Biofertilizers · Forest species · Rhizobacteria · Indole acetic acid · Phosphate solubilization

Introduction

Plant growth-promoting rhizobacteria (PGPR) in the rhizosphere can have beneficial effects on plants (Vessey 2003) by directly affecting growth through the production of plant hormones and increasing nutrient availability or indirectly by inhibiting pathogens (Richardson et al. 2009; Ahangarar et al. 2012; Zhang et al. 2014; Pii et al. 2015). Microorganisms that can promote growth of plants of agricultural interest have been widely tested. Species such as maize, tomato and cereals (Matiru and Dakora 2004; Pii et al. 2016) have shown positive responses to inoculation with PGPR, such as increased biomass, improvement of fruit quality and resistance to pathogens such as *Fusarium, Phythium* and *Phytophthora* (Whipps 2001; Zehnder et al. 2001). However, in tree species, particularly in coniferous species, studies related to inoculation with PGPR are limited (Enebak et al. 1998; Bent et al. 2001; Barriuso et al. 2005; Brunetta et al. 2007; de Vasconcellos and Cardoso 2009; Singh et al. 2010). The benefits provided by the PGPR to the host include greater nitrogen and phosphorous

Project funding: The work was supported by the project "Impact of Climatic Change and Agricultural Activity on the Emission of Greenhouse Gases and on the Microbial Resources of the Sierra Nevada, Mexico" ["Impacto del Cambio Climáticos y actividad agrícola en la emisión de gases de efecto invernadero y en los recursos microbianos de la Sierra Nevada, México"] No. 213059 funded by the National Council of Science and Technology [Consejo Nacional de Ciencia y Tecnología (CONACyT)].

The online version is available at http://www.springerlink.com

Corresponding editor: Tao Xu.

✉ Juan J. Almaraz-Suarez
jalmaraz@colpos.mx

[1] Área de Microbiología, Postgrado de Edafología, Colegio de Postgraduados, Carretera México-Texcoco km 36.5, CP 56230 Montecillo, Estado de México, Mexico

[2] Laboratorio de Recursos Genéticos Microbianos, Centro Nacional de Recursos Genéticos, INIFAP, Boulevard de la Biodiversidad No. 400, CP 47600 Tepatitlán de Morelos, Jalisco, Mexico

contents (Rojas et al. 2001; Bashan and Holguin 2002; Holguin et al. 2003; Anand et al. 2013) and an increase in biomass (Chanway and Holl 1992) and the production of organic acids, phenolic compounds and siderophores (Calvaruso et al. 2006) that help in the release of nutrients. In forest ecosystems, the PGPR affect processes leading to the release of low-availability nutrients, such as phosphorus (Calvaruso et al. 2006; Ouahmane et al. 2009). They also induce growth of plants by releasing growth factors (Probanza et al. 2002) and promoting resistance to environmental stress (Rincón et al. 2008; Sharma and Rai 2015).

In Mexico, deforestation and fires have generated losses in forest areas (Cuevas-Guzmán et al. 2011). The regeneration of forest resources is gradual, mainly because of the slow growth rate of forest species. Additionally, the degradation of the soil as a consequence of deforestation hinders the establishment of new plants. Reforestation programs using nursery-produced seedlings have been implemented as a strategy for the recovery of forest areas. The production of such seedlings requires time and high production costs, and despite all care measures, the mortality rate of plants in Mexican reforestation programs is approximately 60–90% (Gómez-Romero et al. 2012). One of the species most used in Mexican reforestation and forest plantation programs is *Pinus pseudostrobus*, which is widely distributed in Mexico and Central America and is highly prized for its wood quality and productivity. It has also been successfully introduced in countries such as Brazil, New Zealand and South Africa (Mitchell et al. 2012; Cambrón-Sandoval et al. 2013; Zenni and Simberloff 2013). The use of PGPR, alone or in combination with ectomycorrhizal fungi, to produce high-quality seedlings are expected to decrease costs, nursery time and the use of fertilizers and pesticides. The aim of this study was to evaluate the growth promoter characteristics of rhizobacteria strains isolated from coniferous trees and to determine their effect on pine seedlings (*P. pseudostrobus*) in a nursery.

Materials and methods

Isolation of bacterial strains

Samples of rhizosphere soil were collected at a depth of 0–15 cm on Mount Tláloc, North of the Sierra Nevada in Central México, at an altitude between 3000 and 3800 m above sea level. Samples were sourced from the mycorhizosphere of oyamel (*Abies religiosa*), cedar (*Cupressus lusitanica*), and pine (*Pinus hartwegii*) trees. Additionally, samples were collected from the rhizosphere of 1-year-old *Pinus montezumae* trees grown in a nursery. The samples were transported to the laboratory in a cooler on ice. Bacterial strains were isolated using the dilution technique and plated on nutrient agar. Colonies showing abundant growth and with different morphologies were selected and purified by streaking onto nutrient agar. Pure bacterial cultures were stored at 4 °C until further use.

Auxin production

The bacterial strains were incubated in Luria–Bertani broth at 28 °C on a rotatory shaker (Model 2–16, Sigma) at $4\times g$ for 180 rpm. Next, 3 mL of the bacterial cultures was transferred to 1.5 mL microcentrifuge tubes and centrifuged at $2655\times g$ for 10 min. Then, 100 µL samples of the obtained supernatant were transferred to 96-well plates (microplates Costar 3591, Corning, NY); 200 µL of Salkowski reagent was added (2% v/v 0.5 M $FeCl_3$ in 35% v/v perchloric acid), and the plates were incubated in darkness for 30 min for the development of color. Absorbance of the samples at 530 nm was measured with a spectrophotometer (Synergy 2 microplate reader, BioteK Instruments, Vermont, USA). The concentration of auxins was determined from a standard curve generated using 0–60 µg mL^{-1} of indole acetic acid.

Phosphate solubilization

The strains were cultured in tubes with Pikovskaya medium (10 g glucose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.2 g KCl, 0.5 g yeast extract, 0.002 g $MnSO_4$, 0.002 g $FeS0_4{\cdot}7H_2O$, 1 L distilled water, pH 7)., incubated at 28 °C under agitation at $4\times g$ for 180 rpm. After incubation, the bacterial cultures were centrifuged in Falcon tubes at $2655\times g$ for 10 min. The supernatant was filtered through Whatman No. 1 filter paper, then 200 µL samples of each filtrate were transferred to 1.5 mL microcentrifuge tubes. Next, 100 µL of a solution of vanadate (0.25% w/v NH_4VO_3 in 35% v/v HNO_3) and 100 µL of molybdate solution [5% w/v $(NH_4)_6MO_7O_{24}$ in distilled water] were added to each tube, which were mixed and incubated for 5 min. After this time, the samples were read at 420 nm. The amount of solubilized phosphorus was determined from a standard curve from 0 to 300 µg KH_2PO_4 mL^{-1} water.

Stimulation of ectomycorrhizal fungus growth

The ability of bacterial strains to stimulate the growth of ectomycorrhizal fungi was evaluated using strain CPEc1 of *Suillus* sp. from the collection of the Laboratory of Microbiology, Colegio de Postgraduados, Montecillo, Mexico. The strain was cultured in potato-dextrose agar (PDA) and incubated for 20 days, then 9-mm-diameter

disks from the periphery of the colony was placed on PDA (pH 6.5) (Kataoka and Futai 2009). The bacterial strain was streaked in a straight line 2 cm from the disk of *Suillus* sp. The bacterial strains were cultured previously in nutrient broth for 7 days at 28 °C under constant agitation. As a control, a small sample of the medium without bacteria was streaked as above on a plate with a disk of the fungus. Three replicates per treatment were used. Petri dishes were incubated at 25 °C for 2 weeks, then the diameter of the fungal colony was measured.

Molecular identification

DNA was extracted from biomass equivalent to seven 24-h cultures of each bacterial strain with an EZ-10 Spin Column Bacterial DNA mini-prep kit (Bio Basic, Canada). The integrity of the DNA was evaluated by electrophoresis in a 1% agarose gel stained with Green-DNA Dye (Bio Basic) and visualized with a transilluminator.

A ~ 1500-bp fragment of the 16S rDNA was amplied for reliable identification to the level of genus and species using primers 27f (forward) 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 1492r (reverse) 5′-CGG TTA CCT TGT TAC GAC TT-3′ and amplification reaction mixture containing 1× PCR Buffer, 2 mM dNTPs, 0.2 mM $MgCl_2$, 10 µM of each primer and 0.05 U/µL DNA polymerase in a final volume of 25 µL. The thermocycler program included an initial denaturation for 5 min at 95 °C; 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 1.5 min; with a final extension at 72 °C for 7 min. The amplicons were electrophoresed (100 V) in a 1.5% agarose gel and visualized with a transilluminator after staining with Green-DNA Dye (Bio Basic). The PCR products were then purified with an Agencourt AMPure XP kit (Beckman Coulter, USA) using the manufacturer's instructions. The purified amplicons were sequenced at MACROGEN, Korea.

The sequences were edited with BioEdit software version 7.0.9.0 (Hall 1999) and Seaview version 4.0 (Galtier et al. 1996), and the phylogenetic trees were constructed with MEGA version 6.0 (Tamura et al. 2013). The sequences were used as queries in a search for homologous genes using the BLAST platform (http://blast.ncbi.nlm.nih.gov/Blast.cgi); the criteria reported by Rosselló-Mora and Amann (2001) were used to identify species (identity > 97%) and genus (identity of 95–96%), with coverage values higher than 85%. A phylogenetic analysis was performed in MEGA version 6 (Tamura et al. 2013) by mining sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank) obtained from a MegaBLAST analysis (Morgulis et al. 2008) to confirm the identity through the agreement in topology and the BLAST identity for each sequence of the strains. A phylogenetic tree was then constructed with using sequences of the strains and the mined sequences of microorganisms associated with plant growth promotion and a multiple alignment was done in ClustalX version 2.0 (Larkin et al. 2007) in MEGA6 (Tamura et al. 2013).

Nursery experiment

Seeds of *P. pseudostrobus* were submerged in water for 24 h; the water was changed every 6 h. The seeds were scarified with 30% H_2O_2 for 20 min under constant agitation, then rinsed with sterile distilled water to remove the H_2O_2. The seeds were then planted in germination trays using peat moss as substrate (previously sterilized for 6 h at 120 °C); two seeds were planted per well. The trays were covered with black plastic to accelerate germination and were maintained in a growth chamber at 28 °C for 28 days. Watering was performed with sterile distilled water, and every third day, a Captan solution was added (2 g L^{-1}) to prevent the growth of pathogenic fungi. Two weeks after emergence, seedlings were transplanted to 140-mL tubes that were previously washed and disinfected with commercial chlorine and alcohol. The tubes were then filled with the substrate to 80% of capacity. The substrate consisted of a mixture of peat moss and sand (2:1), sterilized for 6 h at 120 °C. After the transplant, the seedlings were maintained in a nursery.

The bacterial strains were grown in nutrient broth (Merck) at 28 °C for 96 h in constant agitation, reaching 10^9 CFU mL^{-1}. The culture was centrifuged at 2655×*g* for 10 min; the supernatant was removed, and the bacterial pellet was resuspended in sterile distilled water. The seedlings were inoculated with 2 mL of this suspension at the base of the seedling stem. Inoculum was added twice to ensure the persistence of the bacterium in each treatment: the first, 2 weeks after the transplant; and the second, 3 months after the first inoculum. The plants were watered with distilled water.

The treatments consisted of 10 rhizobacterial strains and an unfertilized and noninoculated control, which were established in a randomized block experimental design with 10 replicates. The plants were harvested 150 days after the establishment of the experiment. The variables evaluated were the height, diameter and dry mass of the shoots and roots. The plant material was oven-dried (Jouan Model EUSS ELTS SN) at 70 °C until reaching constant mass. Total nitrogen and phosphorus in the plant was determined by the micro-Kjeldahl and molybdo-vanadate methods, respectively. The data were analyzed using SAS version 9 (SAS Institute Inc 1999) for Windows (Microsoft, Redmond, WA, USA) to obtain the analysis of

variance (ANOVA) and means comparison testing using the least significant difference (LSD) with $\alpha = 0.05$.

Results

Isolation and characterization

Ten strains were isolated from the mycorrhizosphere of *C. lusitanica*, *A. religiosa*, *P. hartwegii*, and *P. montezumae* (Table 1). All strains produced 1.6 μg to 76.4 μg mL^{-1} indole acetic acid. Strains CPT5, CPT6, CPT7, CPT8, and CPT9 produced more than 33 μg mL^{-1} IAA. Seven strains solubilized phosphorous; strain CPT6 solubilized 433 μg mL^{-1} phosphorous.

Induction of mycelial growth of *Suillus* sp. in vitro

Bacterial strain CPT8 induced mycelial growth of *Suillus* sp. in vitro by 18.8% compared with the control mycelia. Strains CPT5 and CPT6 inhibited growth of *Suillus* sp. by 6 and 4%, respectively. The remaining strains did not significantly affect fungal growth.

Bioassay of bacterial inoculation in *P. pseudostrobus*

Some of the strains had significant beneficial effects on the variables evaluated (Table 2). Strain CPT2 increased the height of *P. pseudostrobus* by 68.9% compared to that of the control. Strains CPT4, CPT6 and CPT9 increased the dry mass of the shoots by 20.4, 15.2 and 24.2%, respectively (Fig. 1). Other strains inhibited plant growth. For example, plants treated with strain COT7 had the lowest values for stem diameter (1.38 mm), shoot dry mass (0.24 g), and total biomass (0.3437 g).

Identification of strains by molecular biology

The 10 strains were identified as belonging to the following species: *C. basilensis* (CPT1, CPT2, CPT3), *R. qingshengii* CPT4, *Pseudomonas* sp. CPT5, *Pseudomonas gessardii* CPT6, *P. gessardii* CPT7, *S. rhizophila* CPT8, *Rhodococcus erythropolis* CPT9 and *Cohnella* sp. CPT10. Of the group of strains isolated, those belonging to the genus *Pseudomonas* came from the ectomycorrhizosphere of *P. hartwegii* and *A. religiosa*. The nucleotide sequences for the strains were submitted to GenBank database and assigned accession numbers KU195255 (CPT1), KU195256 (CPT2), KU195257 (CPT3), KU195258 (CPT4), KU195259 (CPT5), KU195260 (CPT6), KU195261 (CPT7), KU195262 (CPT8), KU195263 (CPT9), and KU195264 (CPT10).

In the phylogenetic analysis of the genetically identified strains using sequences from bacterial strains related to these species and the ectomycorrhizosphere from which they were isolated (Fig. 2), the sequences clustered in the taxonomic groups Beta-proteobacteria, Gamma-proteobacteria and Actinobacteria.

Discussion

The results obtained in this study showed that bacteria of the genera *Rhodococcus* and *Pseudomonas* act as growth promoters in *P. pseudostrobus*. However, the increase in the production of biomass did not always depend on the production of IAA or on phosphorous solubilization by the

Table 1 Molecular identification and concentrations of IAA produced and phosphorous solubilized (Sol.) by the isolated rhizobacterial strains

Strain	Species	IAA (μg mL^{-1})	Sol. PO_4^{-3} (μg mL^{-1})	pH	Growth of *Suillus* sp.	
					(cm)	(%)
Control		–	–	7.0	1.30 ± 0.11 b	–
CPT1	*Cupriavidus basilensis*	7.8 ± 1.7 c	–	7.2	1.35 ± 0.03 b	3.7
CPT2	*Cupriavidus basilensis*	12.2 ± 0.9 c	–	7.5	1.38 ± 0.08 b	5.5
CPT3	*Cupriavidus basilensis*	7.0 ± 0.5 c	–	7.3	1.33 ± 0.07 b	1.9
CPT4	*Rhodococcus qingshengii*	1.7 ± 0.2 c	–	7.1	1.35 ± 0.05 b	3.7
CPT5	*Pseudomonas* spp.	34.0 ± 4.2 b	–	7.3	1.23 ± 0.08 b	− 5.7
CPT6	*Pseudomonas gessardii*	45.3 ± 3.9 b	443.1 ± 1.6 a	6.1	1.25 ± 0.09 b	− 4.0
CPT7	*Pseudomonas gessardii*	33.5 ± 4.4 b	5.0 ± 0.8 b	7.8	1.30 ± 0.04 b	0
CPT8	*Stenotrophomonas rhizophila*	43.4 ± 2.5 b	2.2 ± 0.2 b	7.5	1.60 ± 0.04 a	18.8
CPT9	*Rhodococcus erythropolis*	76.4 ± 2.7 a	2.0 ± 0.3 b	6.9	1.30 ± 0.09 b	0
CPT10	*Cohnella* sp.	5.5 ± 2.2 c	5.0 ± 0.2 b	8.1	1.35 ± 0.13 b	3.7

Different letters within a column indicate a significant difference among strains for that variable in a Tukey's test ($\alpha = 0.05$, $n = 8$)

Table 2 Height, diameter and P and N content for *P. pseudostrobus* seedlings 150 days after inoculation with different rhizobacterial strains

Strain	Height (cm)	Diameter (mm)	P (μg g^{-1})	N (mg $plant^{-1}$)
Control	4.7 ± 0.2 c	1.60 ± 0.05 ab	1.4 ± 0.4 a	6.3 ± 0.2 ab
CPT1	5.7 ± 0.4 abc	1.70 ± 0.04 a	1.4 ± 0.2 a	6.4 ± 0.4 ab
CPT2	6.6 ± 0.3 a	1.68 ± 0.05 a	1.3 ± 0.2 a	6.4 ± 0.2 ab
CPT3	5.6 ± 0.4 abc	1.71 ± 0.05 a	1.4 ± 0.5 a	7.2 ± 0.4 a
CPT4	4.6 ± 0.6 c	1.71 ± 0.08 a	1.2 ± 0.3 a	6.1 ± 0.5 b
CPT5	5.3 ± 0.4 abc	1.65 ± 0.05 ab	1.4 ± 0.2 a	6.1 ± 0.4 b
CPT6	5.1 ± 0.3 c	1.70 ± 0.07 a	1.2 ± 0.4 a	7.3 ± 0.3 a
CPT7	5.2 ± 0.5 cb	1.54 ± 0.08 b	1.0 ± 0.5 a	6.6 ± 0.3 ab
CPT8	6.4 ± 0.3 ab	1.55 ± 0.09 b	1.1 ± 0.3 a	6.2 ± 0.1 b
CPT9	5.2 ± 0.5 cb	1.69 ± 0.03 a	1.2 ± 0.4 a	7.0 ± 0.3 a
CPT10	5.9 ± 0.4 abc	1.66 ± 0.05 ab	1.3 ± 0.1 a	4.8 ± 0.6 c

The same letters after values within a column indicate that no significant differences were found using Tukey's test ($\alpha = 0.05$, $n = 8$)

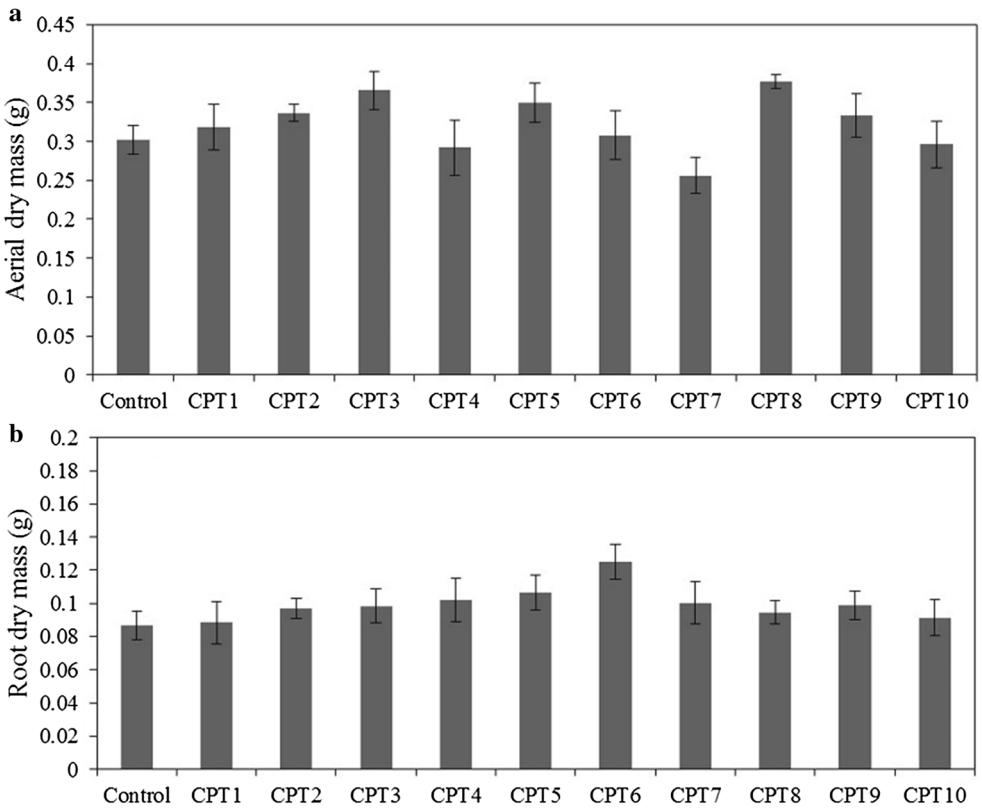

Fig. 1 Effect of different rhizobacteria strains in *P. pseudostrobus* on dry mass of **a** shoots and **b** roots 150 days after inoculation

bacteria. The effect of the secondary metabolites produced by the bacteria might depends on the affinity between organisms. *Pseudomonas aeruginosa* showed strong chemotaxis in the presence of the root exudates of *Pinus roxburghii* (Singh et al. 2010). *Pseudomonas fluorescens* is capable of producing auxins and dissolving calcium phosphate, which allows it to properly establish itself in the rhizosphere of *Pinus pinea* (García et al. 2004; Karnwal 2009). *P. fluorescens* has been widely studied because of its growth-promoting capacity and has been found in the mycosphere of ectomycorrhizal fungi (ECMF) such as *Lactarius* sp. and *Rhizopogon* sp. (Preston 2004; Aspray et al. 2006; Frey-Klett et al. 2007). The three strains of *Pseudomonas* tested in the experiment increased the shoot and root biomass of *P. pseudostrobus*; however, they did not enhance growth of the ectomycorrhizal fungi. *R. qingshengii* and *R. erythropolis* also had a positive effect on biomass production. However, *R. qingshengii* promoted growth of the ECMF; therefore, it can promote mycorrhizal colonization. Few studies have been performed with

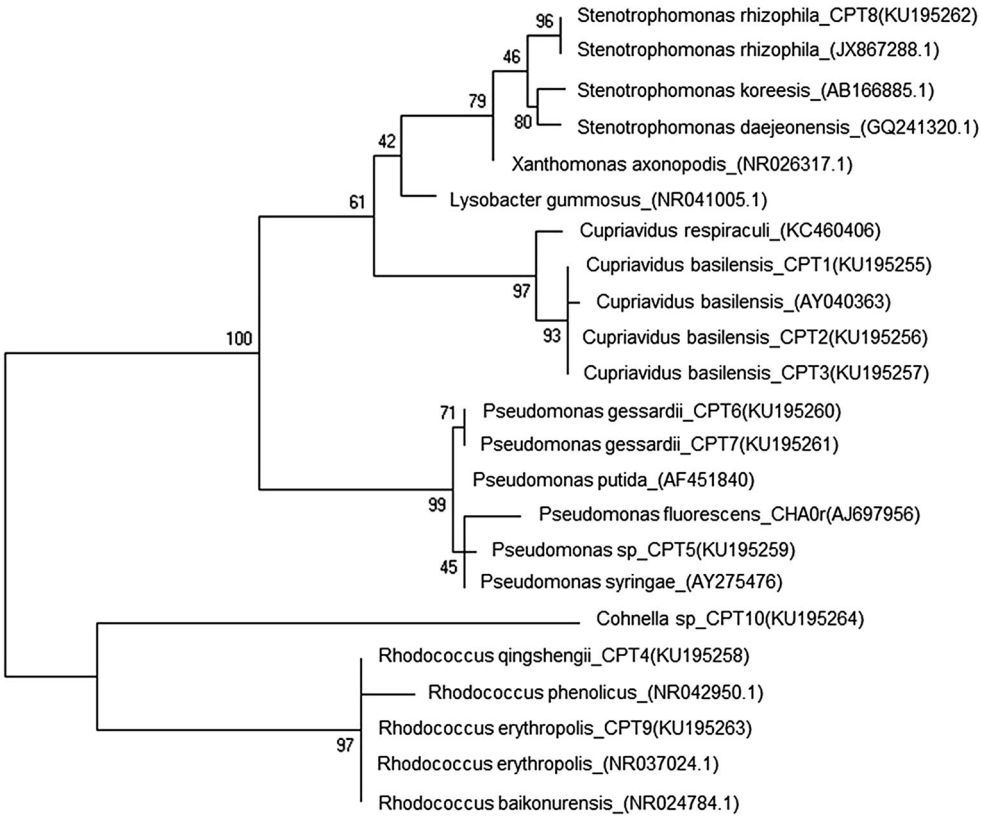

Fig. 2 Phylogeny of the identified strains

bacteria from the genus *Rhodococcus*, although found in the mycorrhizosphere of ECMF such as *Lactarius rufus* (Poole et al. 2001). Although *R. erythropolis* has not been reported as a species that promotes mycorrhizal colonization, it has potential in areas such as bioremediation as a sulfur-removing agent (Naito et al. 2001; Gogotov and Khodakov 2008; Cumming et al. 2015). It is also capable of using phenol and pyrocatechol, among other substrates, for its growth (Čejková et al. 2005) and can degrade paraffin (Zhukov et al. 2007) and diesel (Zhang et al. 2007). Although *C. basilensis* has not been reported as PGPR, some species of *Cupriavidus* form associations with legumes and are resistant to heavy metals (Estrada de los Santos et al. 2012). *C. basilensis* has been reported for its capacity to degrade furanic compounds (Wierckx et al. 2011). *S. rhizophila* also enhanced biomass and root growth. So dual inoculation with these two strains could induce mycorrhizal formation in plant species of interest for forestry, such as *P. pseudostrobus*. Dual inoculation (bacterial-ECMF) has been widely studied with positive, neutral or negative responses depending on the affinity between microorganisms (Bonfante and Anca 2009; Hrynkiewicz et al. 2010; Wu et al. 2012).

The PGPR promote plant growth by increasing nutrient availability (Pii et al. 2015). Nutrient solubilization and mineralization can be biological or biochemical through the production and release of organic acids, such as citric, butyric, acetic and fumaric acids, phenolic compounds, and siderophores (Ouahmane et al. 2009). Organic acids produced by rhizobacteria acidifies the medium (pH 7 to pH 4.2), allowing the solubilization of phosphorous (Song et al. 2008). Similarly, strain CPT6 in the present study acidified the pH of the medium compared to the control (pH 8 to 6). *Paenibacillus polymyxa* decreases the mortality of *Pinus contorta* var. *Latifolia* seedlings and increases the concentration of N in seedlings (Anand et al. 2013). Strains of the genus *Pseudomonas* increase the height and dry mass of *Pinus taeda* seedlings (Brunetta et al. 2010) There are reports in which *C. basilensis* is capable of solubilizing phosphorous (Qian et al. 2010), but the strains in this experiment did not show this capacity. Accordingly, the isolated strains are capable of solubilizing considerable amounts of phosphorous, which remains available for the plant. In soils in which phosphorous is immobilized, this mechanism is a viable solution for decreasing fertilizer use (White and Metcalf 2007).

The use of PGPR to produce seedlings of forest species for the reforestation of altered areas still requires more research because little information is available. The present studies showed favorable increases in biomass. The use of dual inoculants is a strategy to decrease the application of fertilizers and pesticides. This strategy would allow the establishment of endophyte communities in the rhizosphere that promote positive effects on plant physiology, such as

stimulation of photosynthesis, release of root exudates, and production of growth factors (Fuentes-Ramírez and Cabellero-Mellado 2005; Barka et al. 2006).

Despite the benefits PGPR offer, potential consequences to human health need to be assessed (Fuentes-Ramírez and Cabellero-Mellado 2005). For example, *R. erythropolis* has been reported in six cases of infection in immunocompromised patients (Park et al. 2011). Therefore, bacterial inoculants should be applied in the field with great care and with appropriate quality control.

Conclusions

The bacterial strain that best stimulated the growth of *P. pseudostrobus* increased shoot dry mass by 24.2% over the control and by dry mass of roots by 33.9%. A more profuse root system leads to better nutrient capture from the soil. The strains were molecularly identified to belong to the genera *Pseudomonas*, *Rhodocccocus*, *Cupriavidus*, *Stenotrophomonas* and *Cohnella*, which have been found in association with hyphae of ectomycorrhizal fungi; thus, these strains may be more efficient growth promoter after a dual inoculation with an ectomycorrhizal fungus. However, studies evaluating the effect of mycorrhizal colonization in conjunction with plant-growth promoting bacteria are still needed.

References

Ahangarar MA, Dar GH, Bhat ZA (2012) Growth response and nutrient uptake of blue pine (*Pinus wallichiana*) seedlings inoculated with rhizosphere microorganisms under temperate nursery conditions. Ann For Res 55(2):217–227

Anand R, Grayston S, Chanway C (2013) N_2-Fixation and seedling growth promotion of lodgepole pine by endohytic *Paenibacillus Polymyxa*. Microb Ecol 66:369–374

Aspray TJ, Frey-Klett P, Jones JE, Whipps JM, Garbaye J, Bending GD (2006) Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. Mycorrhiza 16:533–541

Barka EA, Nowak J, Clément C (2006) Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. Appl Environ Microbiol 72(11):7246–7252

Barriuso J, Pereyra MT, García JL, Megías M, Manero FG, Ramos B (2005) Screening for putative PGPR to improve establishment of the symbiosis *Lactarius deliciosus- Pinus* sp. Microb Ecol 50:82–89

Bashan Y, Holguin G (2002) Plant growth-promoting bacteria: a potential tool for arid mangrove reforestation. Trees 16:159–166

Bent E, Tuzun S, Chanway CP, Enebak S (2001) Alterations in plant growth and in root hormone levels of lodgepole pines inoculated with rhizobacteria. Can J Microbiol 47:793–800

Bonfante P, Anca IA (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annu Rev Microbiol 63:363–383

Brunetta CFMJ, Cuoto AA, Goncalves MR, Gomes JM, Binoti DB, EdeP Fonseca (2007) Avaliação da especificidade de rizobactérias isoladas de diferentes espécies de *Pinus* sp. Rev Árvore 31(6):1027–1033

Brunetta CFMJ, Alfenas CA, Mafia GR, Gomes JM, Binoti DB, Fonseca NAN (2010) Isolamiento e seleçã de rizobactérias promotoras do crescimento de *Pinus taeda*. Rev Árvore 34(3):399–406

Calvaruso C, Turpault MP, Frey-Klett P (2006) Root-associated bacteria contribute to mineral weathering and to mineral nutrition in trees: a budgeting analysis. Appl Environ Microbiol 72(2):1258

Cambrón-Sandoval VH, Sánchez-Vargas NM, Sáenz-Romero C, Vargas-Henández JJ, España-Boquera ML, Herrerías-Diego Y (2013) Genetic parameters for seedling growth in *Pinus pseudostrobus* families under different competitive environments. New For 44:219–232

Čejková A, Masák J, Jirku V, Veselý M, Pátek M, Nešvera J (2005) Potential of *Rhodococcus erythopolis* as a bioremediation organism. World J Microbiol Biotechnol 21:317–324

Chanway CP, Holl FB (1992) Influence of soil biota on Douglas fir *Pseudotsuga menziesii* seedling growth: the role of rhizosphere bacteria. Can J Bot 70:1025–1031

Cuevas-Guzmán R, Cisneros-Lepe EA, Jardel-Peláez EJ, Sánchez-Rodríguez EV, Guzmán-Hernández L, Núñez-López NM, Rodríguez-Guerrero C (2011) Análisis estructural y de diversidad de *Abies* de Jalisco, México. Rev Mex Biodiv 82:1219–1233

Cumming JR, Zawaski C, Desai S, Collart FR (2015) Phosphorus disequilibrium in the tripartite plant-ectomycorrhiza-plant growth promoting rhizobacterial association. J Soil Sci Plant Nutr 15(2):464–485

de Vasconcellos RLF, Cardoso EJBN (2009) Rhizospheric streptomycetes as potential biocontrol agents of *Fusarium* and *Armillaria* pine rot and as PGPR for *Pinus taeda*. Biocontrol 54(6):807–816

Enebak SA, Wei G, Kloepper JW (1998) Effects of plant growth-Promoting rhizobacteria on loblolly and slash pine seedlings. For Sci 44(1):139–144

Estrada de los Santos P, Martínez-Aguilar L, López-Lara IM, Caballero-Mellado J (2012) *Cupriavidus alkaliphilus* sp. nov. a new species associated with agricultural plants that grow in alkaline soils. Syst Appl Microbiol 35(5):310–314

Frey-Klett P, Garbaye JA, Tarkka M (2007) The mycorrhiza helper bacteria revisited. New Phytol 176:22–36

Fuentes-Ramírez LE, Cabellero-Mellado J (2005) Bacterial biofertilizers. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Netherlands, pp 143–172

Galtier N, Gouy M, Gautier C (1996) SeaView and Phylo_win: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12(6):543–548

García JAL, Domenech J, Santamaría C, Camacho M, Daza A, Mañero FJG (2004) Growth of forest plants (pine and holm-oak) inoculated with rhizobacteria: relationship with microbial community structure and biological activity of its rhizosphere. Environ Exp Bot 52:239–251

Gogotov IN, Khodakov RS (2008) Surfactant production by the *Rhodoccocus erythropolis* SH-5 bacteria grown on various carbon sources. Appl Biochem Microbiol 44(2):186–191

Gómez-Romero M, Soto-Correa JC, Blanco-García JA, Sáenz-Romero C, Villegas J, Lindig-Cisneros R (2012) Estudio de especies de pino para restauración de sitios degradados. Agrociencia 46(8):795–807

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Holguin G, Bashan Y, Puente E, Carrillo A, Bethlenfalvay G, Rojas A, De Bashan LG (2003) Promoción del crecimiento en plantas por bacterias de la rizosfera. Agric Tec Mex 29:201–211

Hrynkiewicz K, Baum C, Leinweber P (2010) Density, metabolic activity, and identity of cultivable rhizosphere bacteria on *Salix viminalis* in disturbed arable and landfill soils. J Plant Nutr Soil Sci 173(5):747–756

Karnwal A (2009) Production of indole acetic acid by fluorescent Pseudomonas in the presence of L-tryptophan and rice root exudates. J Plant Pathol 91(1):61–63

Kataoka R, Futai K (2009) A new mycorrhizal helper bacterium, *Ralstonia* species, in the ectomycorrhizal symbiosis between *Pinus thunbergii* and *Suillus granulatus*. Biol Fertil Soils 45:315–320

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Thompson JD (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23(21):2947–2948

Matiru VN, Dakora FD (2004) Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops. Afri J Biotechnol 3(1):1–7

Mitchell RG, Wingfield M, Hodge GR, Steenkamp ET, Coutinho TA (2012) Selection of *Pinus* spp. in South Africa for tolerance to infection by the pitch canker fungus. New For 43:473–489

Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schäffer AA (2008) Database indexing for production MegaBLAST searches. Bioinformatics 24(16):1757–1764

Naito M, Kawamoto T, Fujino K, Kobayashi M, Maruhashi K, Tanaka A (2001) Long term repeated biodesulfuration by inmobilized *Rhodoccocus erthropolis* Ka2-5-1 cells. Appl Microbiol Biotechnol 55:374–378

Ouahmane L, Revel JC, Hafidi M, Thioulouse J, Prin Y, Galiana A, Duponnois R (2009) Responses of *Pinus halapensis* growth, soil microbial catabolic functions and phosphate-solubilizing bacteria after rock phosphate amendment and ectomycorrhizal inoculation. Plant Soil 320:169–179

Park SD, Uh Y, Jang IH, Yoon KJ, Kim HM, Bae YJ (2011) *Rhodococcus erythopolis* septicaemia in a patient with acute lymphocytic leukaemia. J Med Microbiol 60:252–255

Pii Y, Mimmo T, Tomasi N, Terzano R, Cesco S, Crecchio C (2015) Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. Biol Fertil Soils 51(4):403–415

Pii Y, Borruso L, Brusetti L, Crecchino C, Cesco S, Mimmo T (2016) The interaction between iron nutrition, plant species and soil type shapes the rhizosphere microbiome. Plant Physiol Biochem 99:39–48

Poole EJ, Bending GD, Whipps JM, Read DJ (2001) Bacteria associated with *Pinus sylvestris–Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro*. New Phytol 151:743–751

Preston GM (2004) Plant perceptions of plant growth-promoting *Pseudomonas*. Philos Trans R Soc Lond 359:907–918

Probanza A, Garcia JL, Paomino MR, Ramos B, Mañero FG (2002) *Pinus pinea* L. seedlings growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus* (*B. licheniformis* CECT5106 and *B. pumilus* CECT5105). Appl Soil Ecol 20:75–84

Qian YC, Shi JY, Chen YX, Lou LP, Cui XY, Cao RK, Li PF, Tang J (2010) Characterization of phosphate solubilizing bacteria in sediments from a shallow eutrophic lake and a wetland: isolation, molecular identification and phosphorus release ability determination. Molecules 15(11):8518–8533

Richardson AE, Barea JM, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorous and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321:305–339

Rincón A, Valladares F, Gimeno TE, Pueyo JJ (2008) Water stress responses of two Mediterranean tree species influenced by native soil microorganisms and inoculation with a plant growth promoting rhizobacterium. Tree Physiol 28:1693–1701

Rojas A, Holguin G, Glick BR, Bashan Y (2001) Synergism between *Phyllobacterium* sp. (N_2-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semiarid mangrove rhizosphere. FEMS Microbiol Ecol 35:181–187

Rosselló-Mora R, Amann R (2001) The species concept for prokaryotes. FEMS Microbiol Rev 25(1):39–67

SAS Institue Inc (1999) The SAS system for windows. Ver. 9.0 SAS Institute Inc., North Carolina **(EUA)**

Sharma T, Rai N (2015) Isolation of Plant Hormone (Indole-3-Acetic Acid) Producing Rhizobacteria and Study on their Effects on Tomato (*Lycopersicum esculentum*) Seedling. Int J PharmaTech Res 7:099–107

Singh N, Kumar S, Bajpai VK, Dubey RC, Maheshwari DK, Kang SC (2010) Biological control of *Macrophomina phaseolina* by chemotactic fluorescent *Pseudomonas aeruginosa* PN1 and its plant growth promontory activity in chir-pine. Crop Prot 29:1142–1147

Song OR, Lee SJ, Lee YS, Lee SC, Kim KK, Choi YL (2008) Solubilization of insoluble inorganic phosphate by *Burkholderia cepacia* DA23 isolated from cultivated soil. Braz J Microbiol 39(1):151–156

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30(12):2725–2729

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

White AK, Metcalf WW (2007) Microbial metabolism of reduced phosphorus compounds. Annu Rev Microbiol 61:379–400

Wierckx N, Koopman F, Ruijssenaars HJ, de Winde JH (2011) Microbial degradation of furanic compounds: biochemistry, genetics, and impact. Appl Microbiol Biotechnol 92(6):1095–1105

Wu XQ, Hou L, Sheng JM, Ren JH, Zheng L, Chen D, Ye JR (2012) Effects of ectomycorrhizal fungus *Boletus edulis* and mycorrhiza helper *Bacillus cereus* on the growth and nutrient uptake by *Pinus thunbergii*. Biol Fertil Soils 48(4):385–391

Zehnder GW, Murphy JF, Sikora EJ, Kloepper JW (2001) Application of rhizobacteria for induced resistance. Eur J Plant Pathol 107:39–50

Zenni RD, Simberloff D (2013) Number of source populations as a potential driver of pineinvasions in Brazil. Biol Invasions 15:1623–1639

Zhang Q, Tong MY, Li YS, Gao HJ, Fang XC (2007) Extensive desulfuration of diesel by *Rhodoccocus erythropolis*. Biotechnol Lett 29:123–127

Zhang YG, Cong J, Lu H, Yang CY, Yang YF, Zhou JZ, Li DQ (2014) An integrated study to analyze soil microbial community structure and metabolic potential in two forest types. PLoS ONE 9(4):e93773

Zhukov DV, Murygina VP, Kalyuzhnyi SV (2007) Kinetic of the degradation of aliphatichydrocarbons by the bacteria *Rhodococcus rube* y *Rhodoccocus erythropolis*. Appl Biochem Microbiol 43(6):587–592