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ORIGINAL PAPER

Potential seed germination-enhancing plant growth-promoting rhizobacteria for restoration of *Pinus chiapensis* ecosystems

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Abstract Rhizosphere soil samples of three *Pinus chiapensis* sites were analyzed for their physicochemical properties, soil bacteria isolated and screened in vitro for growth-promoting abilities. Nine isolates that showed promise were identified to five genera *Dyella*, *Luteimonas, Enterobacter*, *Paraburkholderia* and *Bacillus* based on the sequences of 16S rRNA gene. All the strains were isolated from non-disturbed stands. These bacteria significantly decreased germination time and increased sprout sizes. Indole acetic acid and gibberellin production and phosphate solubilisation were detected. Results indicate that these biochemicals could be essential for *P. chiapensis* distribution and suggest the possibility that PGPR inoculation on *P. chiapensis* seeds prior to planting could improve germination and possibly seedling development.

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Keywords *Pinus chiapensis* · Plant growth-promoting rhizobacteria · *Dyella* · *Luteimonas* · *Enterobacter*

Introduction

Efforts to modify the effects of climate change and preserve biodiversity, it is apparent that trees play an important role. The loss or degradation of forest ecosystems due to climate change is of considerable concern. Mexico is rich in temperate and tropical forests, with one-quarter of the total land area classified as forests, and is one of the countries with the world's largest number of pine species.

Pinus chiapensis (Martinez) Andresen is a Neotropical pine endemic to southern Mexico and southwestern Guatemala. In Mexico, it is most abundant in Oaxaca and Chiapas states as well as small populations in Puebla, Guerrero, and Veracruz (Del Castillo et al. 2009). Compared to other Neotropical pine species, *P. chiapensis* requires humid, warm localities (Donahue et al. 1991; Alba et al. 2003; Del Castillo et al. 2009) and grows on acidic soils at 600–2200 m a.s.l. on steep slopes (Dvorak et al. 1996). *P. chiapensis* is a species of economic importance, however, some populations have been severely reduced or disappeared in recent years due to land use changes and over-exploitation for its wood quality (Zamora and Velasco 1977; Donahue et al. 1991; Del Castillo and Acosta 2002). This species is classified as 'vulnerable' according to the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List criteria (Thomas and Farjon 2013), and needs 'special protection' by the environmental agency of the Mexican Government (Secretaría del Medio Ambiente y Recursos Naturales 2001).

There are a number of methods employed to restore and expand native forests and to conserve wildlife habitats. The

application of these methods may be limited by climate conditions, soil quality, and/or characteristics of the species (Smithers 2017). Although chemical methods have proved to be efficient in increasing germination rates, they often have harmful environmental side effects, hence the need to develop effective biotechnological methods to improve forest restoration. A common approach that has been adopted by many countries is pre-farming treatment (Hoseini et al. 2013).

Plant growth promoting rhizobacteria (PGPR) have been used to promote the establishment of *Pinus* species. The mechanisms of growth promotion by rhizospheric bacteria include the production of phytohormones (indole-3-acetic acid, IAA) and siderophores, enzymatic activities that indirectly help plant growth and antagonism against pathogens (Probanza et al. 2001; Barriuso et al. 2008). The bacteria of many genera have been described as having plant growth promoting activities. For example, bacteria belonging to the genus *Paraburkholderia* are capable of producing IAA (indole acetic acid), siderophores, ACC (amino-cyclopropane carboxylic acid) deaminase and antibiotic compounds. These bacteria have shown biotechnological potential to control pathogens and to reduce biotic and abiotic stresses (Naveed et al. 2014; Eberl and Vandamme 2016; Esmaeel et al. 2018). Bacteria belonging to the genus *Dyella,* isolated from forest soils (Ou et al. 2019; Zhou et al. 2019), produced phytohormones, solubilized phosphates and synthetized ACC deaminase (Palaniappan et al. 2010; Contreras et al. 2016). Some species of this genus have the potential for biotechnological application for the degradation of phenolic compounds and biocontrol (Zhou et al. 2012; Iasur-Kruh et al. 2018). The ability of species of the genus *Bacillus* to produce compounds associated with an increase in plant size and weight, for example, IAA, is also well documented (Kumar et al. 2012). This genus has also been capable of removing toxic metals such as chrome by efflux pumps by neutralizing their negative effect, reducing Cr (VI) to Cr (III), and by activating enzymes involved in the detoxifying process of reactive oxygen species (ROS) (Sethuraman and Balasubramanian 2010). Some species of the *Enterobacter* genus were reported as PGPR because they were confirmed to be IAA producers and also able to solubilize phosphates, fix nitrogen, produce siderophores (Pramanik et al. 2018; Sarkar et al. 2018; Chakraborty et al. 2019; Shahid et al. 2019), protect plants against metal toxicity by producing antioxidants (Das and Osborne 2018) and have an ACC deaminase activity that plays a significant role in sustaining plant growth and development by reducing senescence (Upadhyay et al. 2011; Habib et al. 2016). The genus *Luteimonas* was described for the first time by Finkmann et al. (2000) and has been isolated from different contaminated environments and rhizospheric soils (Zhang et al. 2010; Sun et al. 2012; Xin et al. 2014; Cheng et al. 2016; Mu et al. 2016; Ngo and Yin 2016). These bacteria are able to eliminate fuel contaminants from soil, participating actively in soil restoration (Mu et al. 2016). All of the bacteria described, although they have been attributed to enhance plant growth, have not been reported as being a growth promoter of *P. chiapensis*.

The disappearance of many species is increasing and *P. chiapensis* is endangered due to human impact. Thus, information on plant growth promoting rhizobacteria (PGPR) could be used to improve or restore the population of this pine by seed inoculation techniques. Bacteria are likely to adapt or change their diversity and population structure as a response to changing habitats, for example, those disturbed by human activities. The analysis of PGPR in different regions with distinct of *P. chiapensis* populations could possibly provide valuable information on native PGPR populations contributing to plant restoration. This study investigates the ability of bacteria isolated from the rhizosphere of *P. chiapensis* to improve the germination of seeds and the development of the species. This would help pre-farming treatment of the seeds and produce healthy seedlings in *P. chiapensis* conservation programs, permitting the acceleration of the restoration of disturbed forests.

Material and methods

Sampled sites

Samples were collected from three pine locations, one of which was strongly affected by human activities. The three sites were located at 19° 52′02″ N and 97° 19′42″ W, Hueyapan; 19° 51′00″ N and 97° 43′42″ W, Cuautempan (highly disturbed); and 19° 47′46″ N and 97° 05′40″ W, Teziutlán, México (Fig. 1).

Soil sampling

In each of the three regions, eight soil samples were taken for analysis, with each sample a mixture of 10 subsamples. Thus, a total 80 subsampling sites were distributed across each region. The sampling system was done in a zigzag pattern, with a random starting point and at a depth of approximately 30 cm. A 1.5 kg sample was taken from each location and taken to the laboratory for analysis.

Physicochemical of soils

The physicochemical characteristics of the soil samples were measured, and included total nitrogen (N), phosphorus (P), iron (Fe), zinc (Zn), sodium (Na), potassium (K) and calcium (Ca), pH, conductivity, water content and texture, according to standard protocols (Reeuwijk 2002).

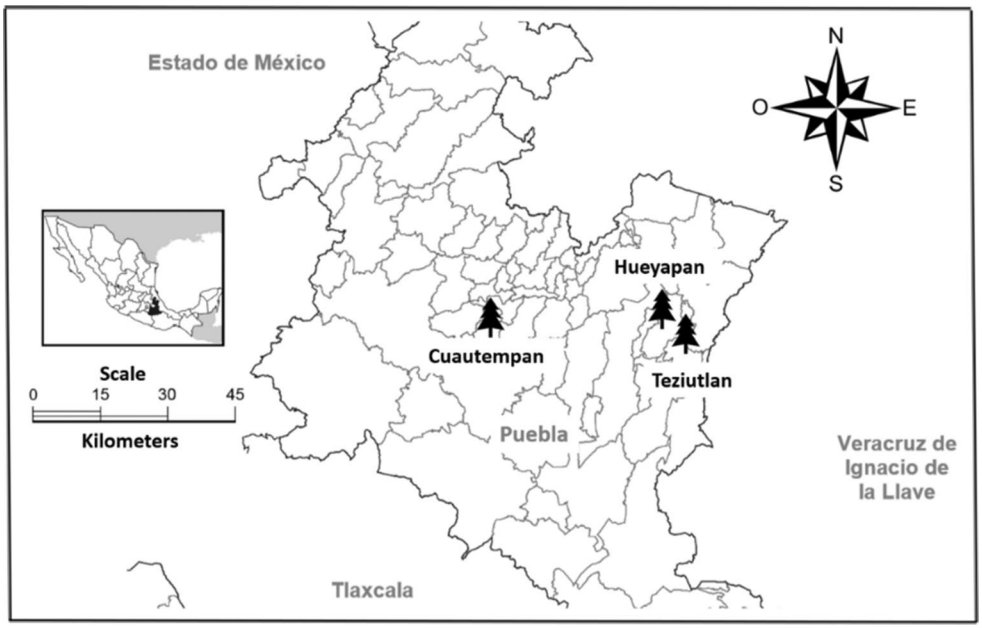

Fig. 1 Location of the *Pinus chiapensis* stands in Cuatempan, Hueyapan and Teziutlán regions, México

These characteristics were analyzed through a variance test (ANOVA) with a post-hoc Tukey-HSD with a confidence level of 95% using the Statgraphics Centurion XVI program and canonical correspondence analysis. The classification, composition, and nutrient contents from the three regions were separated based on the presence or absence of PGPR bacteria along axis 1 and axis 2, respectively.

Strains and culture conditions

To isolate the bacterial strains, one gram of soil was suspended in 99 mL sterile water, serially diluted up to 10^{-10} and cultured at 30 °C and 37 °C on six different media: TESMES, PY, LB, LGI, Congo Red and M9 (Muñoz-Rojas et al. 2005). They were further isolated by repeated streaking and by microscopic examination. The purified strains were kept in 20% (v/v) glycerol at − 80 °C. Five µL of the glycerol stock were taken to inoculate on a LB medium for experimentation.

Germination

Seeds were washed, disinfected with 0.1% Tween 20 for 10 min, 0.8% sodium hypochlorite for 10 min and 50% ethanol for 10 min, and germination carried out on water agar (0.8% w/v) in Petri dishes. The samples were incubated at 27 °C and a 12/12 h light/dark cycle at 70% relative humidity. Before the seeds germinated, they were inoculated with 10 µL of bacterial culture containing 10^8 colony-forming unit (CFU) in the exponential growth phase, because, in this growth phase, adequate interaction between bacteria and seeds has been observed (Angulo et al. 2014). Non-inoculated seeds were used as controls. Germination was calculated as the number of sprouted seeds divided by the number of total seeds, multiplied by 100. Variance tests (ANOVA) were carried out with a post-hoc Tukey test with a confidence level of 95% using the Statgraphics Centurion XVI program. The dynamics of the germination process were analyzed through a multiple regression analysis with the STATA version 12 statistical estimation application software for multilevel models.

In this regression analysis, two effects can be determined. The "intensity" effect refers to a proportion of germinated seeds during a period as compared to the control, while time is not considered a variable. The "calendar" effect refers to the germination rate at certain times. For this effect, cases of decreased germination times caused by bacteria could be analyzed, reaching the same proportion of germinated seeds but in a shorter time than the control without inoculation. Both effects are observed by plotting the ratio of seed germination against time (days), where:

$$\text{Seed germination proportion} = (\text{Number of germinated seeds} / \text{Total germinated seeds})$$

Amplification of the 16S rRNA gene

Total DNA was extracted from overnight cultures at 28 °C in 15 mL of *LB* broth harvested during the mid-log phase (OD_{620} of 0.5–1), using the *Wizard Genomic DNA Purification Kit* (Promega Corporation, Madison, WI, USA), and

stored at 4 °C. PCR was performed in a 2400 GeneAmp PCR Systems® Perkin Elmer thermocycler. The 16S rRNA amplicon was obtained from each sample using *UN27F and UN1392R primers* (Biodiversa Inc., Colima, Mexico). The PCR cycling program comprised an initial denaturation step at 95 °C for 10 min, 35 subsequent cycles including denaturation at 94 °C for 30 s, annealing at various specific temperatures for 30 s, an extension step at 72 °C for 90 s, and a final extension at 72 °C for 10 min. The reaction was carried out in a volume of 25 µL using Phusion® High-Fidelity DNA Polymerase. PCR products were purified with a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany), and visualized under ultraviolet light after staining with ethidium bromide. In all cases, the amplification product had a length of approximately 1500 bp using the GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc. Waltham, MA. USA. NYSE:TMO) as a molecular weight marker.

Sequence alignment and phylogenetic analyses

The PCR products of 16S rDNA were sent to the Instituto de Biotecnología UNAM, Cuernavaca, Mexico, for sequencing. The 16S rDNA sequences obtained were compared with those retrieved from the GenBank/EMBL/DDBJ databases. Nucleotide sequence alignments were made using CLUSTAL_X (Thompson et al. 1997), and corrected manually using GeneDoc (Nicholas and Nicholas 1997). The evolutionary history was inferred using a Maximum Likelihood (ML) analyses performed with MEGA version 7 (Kumar et al. 2016) based on Jukes and Cantor (1969) with 1000 bootstrap replications. Phylogenetic trees were visualized using MEGA version 7.

Measurement of auxins and gibberellins

For auxin determination, King's medium B (Himedia) supplemented with tryptophan (1 $\mu g \cdot mL^{-1}$) was used. Indole-3-acetic acid (IAA) was quantified using a modified *Salkowski* colorimetric method (Glickmann and Deessaux 1995), with sodium salt indole-3-acetic acid (Sigma-Aldrich 15,148) as a control. For the quantification of auxins, a milliliter of the cultures was taken at 24, 48, and 72 h of incubation and centrifuged for 5 min at 12,000 rpm. Five hundred microliters of the supernatant were removed and 500 µl of PC reagent added. The samples were incubated for 30 min and the absorbance read at 540 nm.

Gibberellins were identified using a qualitative fluorescence method (Reyes et al. 1991). Their concentration was calculated by preparing standard curve using gibberellin A3 (GA_3) (Sigma-Aldrich G7645) as reference. Bacterial cultures were incubated in 5 ml of ICI medium (a medium with a low concentration of nitrogen to allow the production of gibberellins) for 24, 48, and 72 h at 30 °C. After incubation, a 0.2-mL aliquot of the culture was mixed with 0.2 mL of ethanol (96%, v/v) and 2 mL of a cooled mixture of equal volumes of sulfuric acid and 96% ethanol. After the mixture was incubated at 48 °C for 30 min by shaking, the fluorescence emission at 464 nm was measured with a Cary Eclipse Fluorescence Spectrophotometer (excitation at 406 nm) (Candau et al. 1992).

Phosphate solubilization

The capability for solubilizing phosphate was assessed using the *Mo-blue* colorimetric method (Murphy and Riley 1962) in the National Botanical Institute's phosphate (NBRIP) medium with bromothymol blue to follow pH changes.

To determine phosphates, 1-mL of culture was incubated for 24, 48, and 72 h and centrifuged for 5 min at 12,000 rpm. Five hundred microliters of the supernatant were removed and 500 µL of reagent C (1 volume of 6 N sulfuric acid with 2 volumes of distilled water, and 1 volume of 2.5% ammonium molybdate with 1 volume of 10% ascorbic acid) and 1 mL of water were mixed, incubated for 2 h at 37 °C and the absorbance at 880 nm read. The quantification of dissolved phosphorus was performed with a KH_2PO_4 standard curve.

Results

Evaluation of *P. chiapensis* stands

A transition from humid temperate to a humid semi-warm climate is dominant in the three regions. The soils from these regions are classified as Andisols (strongly weathered soils with a high capacity to hold both nutrients and water, making these soils very productive and fertile). Soil pH values ranged between 6.4 and 7.8; total N ranged between 0.1% and 1.0% and organic matter between 2.5% and 7.5%, with a wide range of clay 18% to 50% that confer characteristic andic properties (Table 1). Silt and sand fractions range between 22 to 66% and 18% to 50%, respectively (Table 1). In the Hueyapan and Teziutlán stands, *P. chiapensis* populations are well-established with trees at different growth stages. The Cuautempan site is an anthropogenically disturbed habitat with only mature trees. No seedlings or saplings were observed. This site is commonly used as a waste dump.

Physicochemical characteristics of soils were analyzed with a correspondence analysis model and showed that data from the disturbed site formed a cluster separate from the undisturbed sites (Fig. 2). The first axis had a Cronbach's alpha greater than 0.97, explaining 71.8% of the variance. The variables principally contributed to the clustering are Zn, K, silt, P, Fe, Na, sand, organic material and Ca, while the second axis had a Cronbach's alpha 0.78, explaining

Table 1 Physicochemical characteristics of soils via ANOVA analysis for different localities

Localities	Climate	pH	Organic matter (%)	Conductivity (dS)	Texture (%)			Soil type	P (mg/kg)	Total N (%)	Fe (mg/kg)	Zn (mg/kg)	Na (mg/kg)	K (mg/kg)	Ca (mg/kg)
					Silt	Clay	Sand								
Cuatempan	Humid temperate/humid semi-warm	7.0±0.5 [a]	7.5±0.47 [b]	3.4±0.5 [a]	22.0±0.81 [a]	49.9±0.82 [b]	28.1±0.9 [b]	Andisol	12.2±0.5 [c]	0.2±0.1 [a]	111.0±1.2 [c]	170.0±4.8 [c]	<0.05±0.02 [a]	1024.0±3.09 [c]	402.0±2.86 [c]
Hueyapan	Humid temperate/humid semi-warm	7.8±1.39 [b]	7.7±2.84 [b]	4.9±0.5 [b]	38.2±8.8 [b]	18.3±0.5 [a]	43.5±4.0 [c]	Andisol	4.3±0.3 [b]	1.0±0.2 [b]	28.0±0.6 [b]	36.0±2.4 [b]	13.3±0.7 [b]	1128.2±2.7 [b]	265.0±2.52 [b]
Teziutlán	Humid temperate/humid semi-warm	6.4±0.6 [c]	2.5±1.2 [a]	3.6±0.2 [a]	66.6±2.4 [c]	22.0±0.7 [b]	11.4±0.5 [a]	Andisol	0.2±0.08 [a]	0.1±0.01 [a]	7.7±0.65 [a]	0.02±0.002 [a]	1146.4±1.1 [c]	40.1±1.4 [a]	209.4±2.7 [a]

Results of the one-way ANOVA analysis for the post hoc Tukey- HSD test among groups;
Different letters in the same row (by main effect) indicate significant difference ($P < 0.05$)

25.5% of the variance, only clay and sampled site were representative in this axis.

The more closely related variables, forming a cluster at the disturbed site, were clay, Zn, Ca, Fe, and P. Sodium was grouped at the non-disturbed cluster and at the other secluded cluster, while the other variables were related to sample sites or grouped in another cluster unrelated to the sample sites (Fig. 2).

Seed germination in response to bacterial inoculation

Four hundred and ninety-eight bacterial isolates were obtained from the rhizosphere of *P. chiapensis* stands in three localities. One hundred and seventy-seven isolates were obtained from the Teziutlán site, 241 from the Hueyapan site and 80 from Cuautempan. All isolates were analyzed for their potential to stimulate seed germination and were monitored daily for 25 days. The most effective nine isolates belonged to the *Enterobacter*, *Dyella*, *Luteimonas*, *Paraburkholderia* and *Bacillus* genera and identified by 16S rRNA gene sequence analysis (Figs. S1, S2, S3, S4, and S5). The *Bacillus* species were the most representative (GenBank accession numbers are shown in Table 2). These nine isolates increased germination rates, improved sprout sizes, and reduced germination times (Table 2).

All nine isolates were obtained from the non-disturbed sites (three from Hueyapan and six from Teziutlán) where *P. chiapensis* populations are developing under good conditions, in contrast with the disturbed Cuautempan site where no bacterial isolates were obtained.

In addition, a multilevel logistic regression analysis of time related to the event was carried out, and provided information on the dynamics of the germination process and helped determine if the bacteria improved or delayed germination compared to the controls. In this analysis, the dependent variable was the presence or absence of germination (radicle appearance), and the independent variables were germination time in days and strain inoculated to the seeds, versus the non-inoculated control (Fig. 3).

There are three distinct dynamic phases of the germination process. In the first phase (days 1 to 5), the beginning of germination (calendar effect) with the nine inoculated cultures was observed relative to the non-inoculated controls. According to the statistical test, none of the bacteria had a significant intensity effect compared to the control. In the second phase (days 6 to 10), the inoculated cultures resulted in an exponential increase in seed germination compared to the controls, of which *Dyella* sp. SD2037TM was the most effective for inducing germination. The "calendar type" positive-effect was also observed with the *Dyella* SD2037TM, *Enterobacter* SD330TM, *Luteimonas* SD2430LB, *Paraburkholderia* H130LGI and *Bacillus*

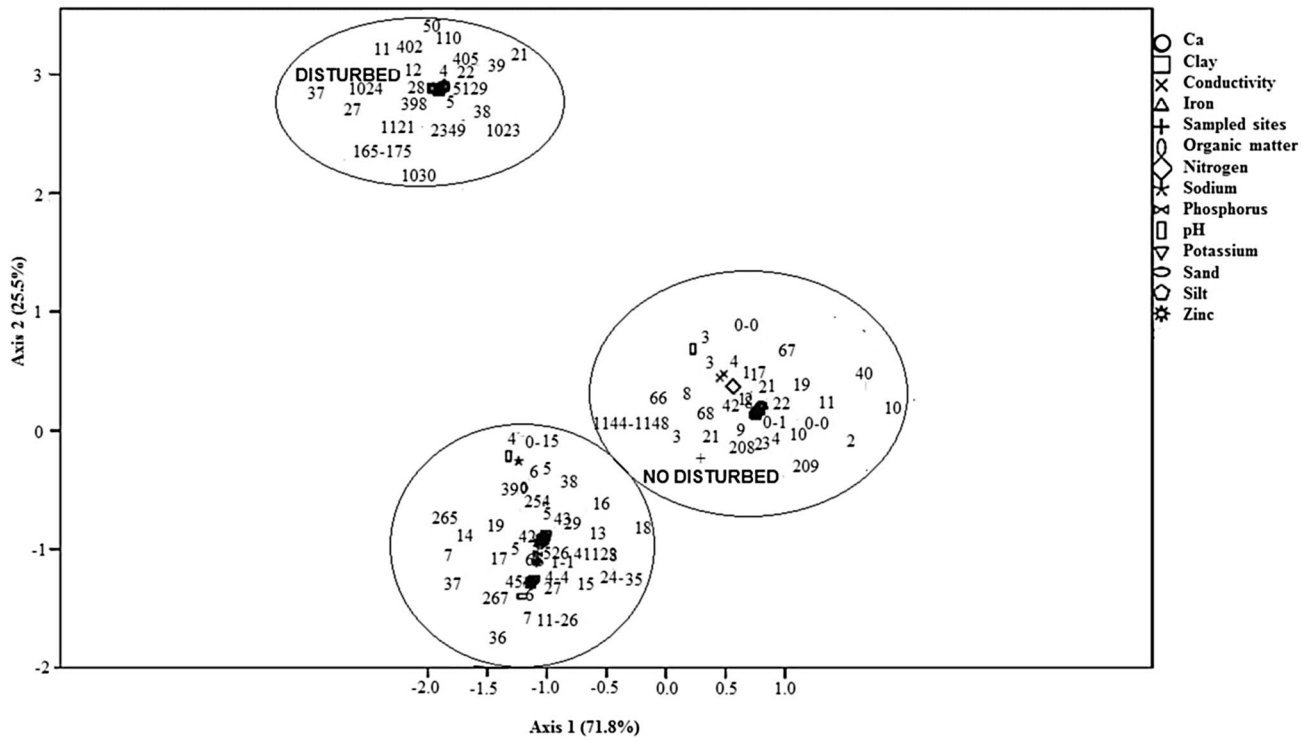

Fig. 2 Bio-spatial dispersion diagram of physicochemical characteristics on disturbed and none-disturbed sites

H3430LGI inoculates. In the third phase (days 11 to 14), the germination proportion did not change significantly. All nine cultures showed the "intensity" effect and five the "calendar" effect (Fig. 3).

Mechanisms of germination stimulation by bacterial inoculation

In this study, two phytohormones (IAA, gibberellin) production and phosphate solubilizing capacity were tested. All nine bacteria produced IAA at concentrations from 0.5 to 1.38 µg IAA·mL^{-1}, which could induce a direct effect on seeds. The *Enterobacter* sp. SD330TM inoculate attained maximum production after 24 h. The nine bacterial cultures also produced gibberellins, with *Enterobacter* sp. SD330TM and *Bacillus* sp. SD1730LB showing higher gibberellin production than the other inoculates: 0.6 ± 0.08 µg·mg^{-1} and 0.47 ± 0.03 µg·mg^{-1} respectively (Table 3). All nine cultures also had the capacity to solubilize phosphate. *Bacillus* sp. SD1330LB and *Bacillus* sp. H1830TM were the most effective, solubilizing 33.8 ± 0.4 µg phosphate·mg^{-1} and 31.8 ± 0.7 µg phosphate·mg^{-1} respectively.

Three isolates from the two undisturbed stands (*Bacillus* sp. SD1537RC, *Bacillus* sp. SD237RC, and *Bacillus* sp. H537RC) had no beneficial effect on germination, as they produced low amounts of IAA and gibberellins, much less than the nine cultures, and slightly higher than the *E. coli* DH5α control. Phosphate solubilization from these isolates was also tested, and the result was similar to the IAA and gibberellin results, implying that inoculates that have a positive effect on the germination of *P. chiapensis* are related to the production of IAA and gibberellin, and also to phosphate solubilization.

Discussion

P. chiapensis is a typical species found on Andisol and Acrisol soils (Martínez et al. 2018) where it forms well-established populations in Mexico. The soils in the three sites are Andisols, however, pine populations are decreasing and the causes are not clear. A plantation established near Hueyapan (a non-disturbed site) showed a survival rate higher than 96%, and only minor damage by animals was observed in 13% of the trees, unlikely to affect population development (Muñoz-Flores et al. 2015). These data suggested that other factors must be involved in the decline of *P. chiapensis* populations in Mexico.

Rhizospheric microorganisms possess growth promoting substances that benefit plant establishment. Bacteria of the species *Burkholderia vietnamiensis*, *Paenibacillus*

Table 2 Effect of plant growth-promoting rhizobacteria on *Pinus chiapensis* seeds

Strain (GenBank Accession number)	Germination rate (%)	Germination time (days) **	Sprout size (mm) **	Wet weight (g) **	Dry weight (g) **
Luteimonas sp. SD2430LB (MH724924)	70	6.6 ± 1.90^{a}	98 ± 23.30^{g}	0.09 ± 0.030^{bcd}	0.02 ± 0.000^{abc}
Dyella sp. SD2037TM (MH725241)	72	5.6 ± 1.84^{a}	98 ± 24.28^{g}	0.10 ± 0.018^{d}	0.02 ± 0.003^{bc}
Enterobacter sp. SD330TM (MH725240)	72	6.4 ± 2.45^{a}	95 ± 25.14^{fg}	0.09 ± 0.014^{cd}	0.02 ± 0.004^{c}
Paraburkholderia sp. H130LGI (MH725263)	60	6.6 ± 2.31^{a}	83 ± 31.63^{def}	0.07 ± 0.004^{abcd}	0.02 ± 0.001^{abc}
Bacillus sp. SD1330LB (MH725242)	66	6.4 ± 1.77^{a}	90 ± 25.78^{efg}	0.11 ± 0.025^{d}	0.02 ± 0.000^{abc}
Bacillus sp. SD730RC (MH725267)	72	6.5 ± 1.90^{a}	87 ± 23.05^{defg}	0.07 ± 0.004^{bcd}	0.02 ± 0.000^{abc}
Bacillus sp. SD1730LB (MH725243)	64	6.5 ± 1.76^{a}	85 ± 18.04^{defg}	0.10 ± 0.020^{d}	0.02 ± 0.003^{abc}
Bacillus sp. H3430LGI (MH725262)	68	6.7 ± 2.40^{a}	78 ± 28.04^{cde}	0.08 ± 0.003^{abcd}	0.02 ± 0.000^{abc}
Bacillus sp. H1830TM (MH725268)	60	6.5 ± 2.22^{a}	77 ± 20.56^{cd}	0.07 ± 0.003^{abcd}	0.02 ± 0.000^{abc}
Bacillus sp. SD1537RC (MN558947)	44	8.2 ± 1.76^{bc}	50 ± 24.20^{a}	0.04 ± 0.013^{a}	0.01 ± 0.004^{abc}
Bacillus sp. SD237RC (MN558949)	42	9.1 ± 1.89^{cd}	61 ± 21.00^{ab}	0.05 ± 0.009^{ab}	0.01 ± 0.003^{a}
Bacillus sp. H537RC (MN558948)	42	9.6 ± 1.56^{d}	59 ± 32.00^{ab}	0.05 ± 0.021^{ab}	0.01 ± 0.001^{a}
E.coli DH5 α	40	8.1 ± 1.44^{b}	65 ± 17.54^{bc}	0.07 ± 0.210^{abcd}	0.02 ± 0.006^{abc}
Non-inoculated Control	56	7.9 ± 1.78^{b}	71 ± 22.80^{bc}	0.07 ± 0.022^{abcd}	0.01 ± 0.005^{abc}

Results of the one-way ANOVA analysis for different sites $p < 0.01$ significance for the post hoc Tukey-HSD test among groups

Different letters in the same column (by main effect) indicate significant differences ($P < 0.05$)

**A significant interaction exists between simple effects

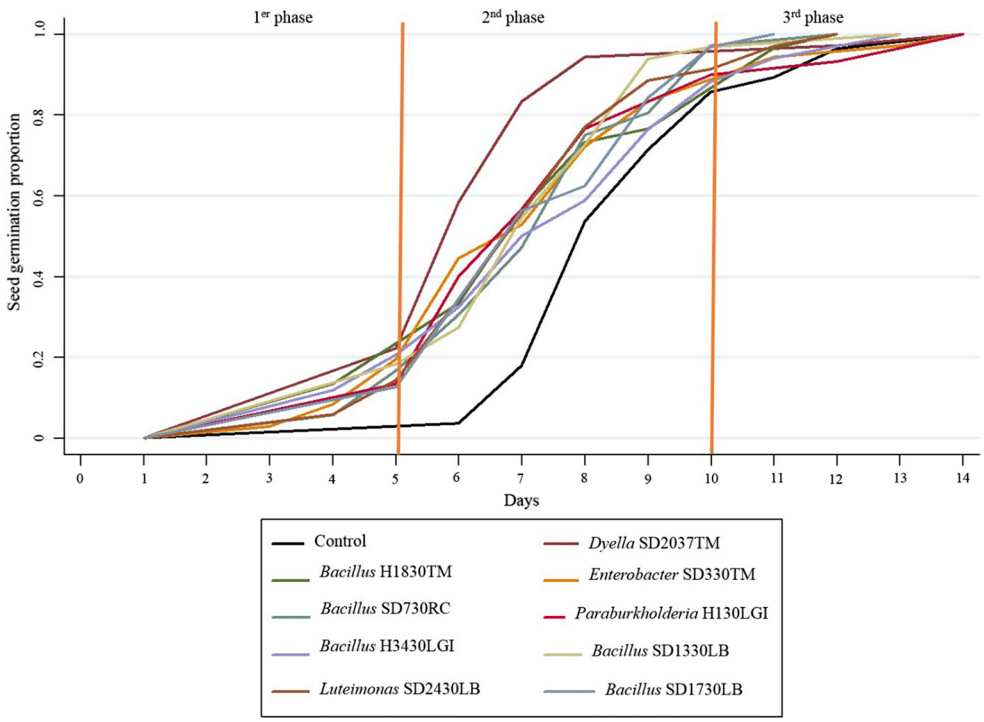

Fig. 3 Dynamics of seed germination proportion multilevel logistic regression analysis of time to the event plots ($P < 0.05$)

polymyxa, *Enterobacter cloacae*, *B. fungorum*, *Gluconacetobacter* and *Bacillus subtilis* and *Rhabdits* spp. have been associated with pine species (Madmony et al. 2005; Xin et al. 2009; Anand et al. 2013; Andreolli et al. 2013). In this study, we have identified *the rhizospheric bacteria associated with P. chiapensis.* A total of 498 isolates were tested for germination enhancement and nine isolates, belonging to the five genera, *Bacillus, Paraburkholderia, Dyella, Luteimonas and Enterobacter*, stimulated germination. All of the nine isolates were obtained from non-disturbed stands.

The mechanisms utilized by bacteria to promote plant growth could be the production of phytohormones such as

auxins and gibberellins and phosphate solubilization. Seeds accumulate high amounts of IAA during dormancy and subsequent germination (Birgit et al. 2005). In studies of breaking dormancy and germination in *Pinus sylvestris* L. seeds, IAA accumulated before germination and during the early stage of elongation. It was suggested that pines need to synthesize this hormone for successful development within the first 12 days (Ljung et al. 2001). Zhao and Jiang (2014) showed that exogenous gibberellin promoted germination and seedling vigor in *P. massoniana Lamb*. By improving respiration or decreasing levels of endogenous abscisic acid. In *P. sylvestris*, both gibberellins and IAA are required for both shoot elongation and cambial growth, and gibberellins act directly in the control of shoot growth, rather than indirectly through affecting the IAA concentration (Wang et al. 1997). All nine inoculates in this study were analyzed for IAA and gibberellin production, as well as for phosphate solubilization. *Dyella* sp. SD2037TM, the most effective bacteria for accelerating germination and increasing germination rate, is an IAA producing species. *Enterobacter* sp. SD330TM, which showed the highest gibberellin production, not only stimulated the rate of germination but also in a shorter time than the control without inoculation. These results imply that these bacterial derived hormones could help pine seed germination in the early stages.

Phosphorus is an essential nutrient element for plants, and makes up about 0.2% of a plant's dry weight (Tak et al. 2012). However, the concentration of soluble phosphorus in soil is usually very low, and is generally most available in soils with a pH around 6.5. A pH range of approximate 6.0 to 7.0 promotes availability of plant nutrients (USDA-NRCS 2020). Soils from the Teziutlán and Cuautempan sites showed pH values within the ideal range; the Hueyapan site had a pH of 7.8 that would limit phosphorous availability due to fixation by calcium. The growth of some phosphorus- solubilizing bacteria from the Teziutlán and Hueyapan sites were identified in agar media with bromothymol blue indicating acidification. Therefore, soil pH is also a factor in plant distribution by affecting phosphate availability contributed by rhizobacteria. In addition, phosphorus- solubilizing bacteria may aid the growth of plants by enhancing rhizobial activity; this is achieved by stimulating nitrogen fixation, synthesizing phytohormones and enhancing the bioavailability of elements such as zinc and iron (Wani et al. 2007), which are weakly available in soils. Considering the human-disturbed region Cuautempan where soil pH value is generally adequate for phosphorous availability, but also the Hueyapan region where pH was much higher, it suggests that pH is not an important factor for pine distribution, at least for seed germination in *P. chiapensis* in the range of pH 6.0–7.8.

The correspondence analysis showed that soil physicochemical variables contribute principally (71.8%) to the human-disturbed cluster, separating it distinctly from the undisturbed clusters. This indicates that these parameters have an important role in pine distribution. It is well- known that physicochemical soil characteristics are associated with growth development in plants. In the present study, some associations were found among certain soil characteristics (Table 1; Fig. 3). For example, clay is associated with the presence of various minerals, and cation exchange properties

Table 3 Phytohormones production and phosphate solubilizing capacity of plant-growth promoting rhizobacteria

Strain	IAA (μg·mg^{-1} protein)**	Gibberellins (μg·mg^{-1} protein)**	Phosphate solubilization (μg phosphate·mg^{-1} protein)**
	24 h	72 h	24 h
Bacillus H1830TM	0.54 ± 0.01^{bc}	0.06 ± 0.005^{ab}	31.8 ± 0.73^{e}
Bacillus H3430LGI	0.57 ± 0.14^{bc}	0.06 ± 0.004^{ab}	8.9 ± 0.51^{b}
Bacillus SD1330LB	0.72 ± 0.02^{bc}	0.07 ± 0.005^{b}	33.8 ± 0.36^{e}
Bacillus SD1730LB	0.72 ± 0.02^{bc}	0.47 ± 0.035^{e}	7.4 ± 0.14^{b}
Bacillus SD730RC	0.90 ± 0.14^{c}	0.25 ± 0.004^{d}	23.3 ± 1.93^{d}
Dyella SD2037TM	0.50 ± 0.11^{b}	$0.12 \pm .009^{bc}$	7.8 ± 0.57^{b}
Paraburkholderia H130LGI	0.82 ± 0.25^{bc}	0.05 ± 0.002^{a}	9.4 ± 0.01^{b}
Luteimonas SD2430LB	0.82 ± 0.12^{bc}	0.18 ± 0.006^{c}	8.3 ± 0.57^{b}
Enterobacter SD330TM	1.38 ± 0.14^{d}	0.60 ± 0.078^{f}	14.5 ± 1.94^{c}
Bacillus SD1537RC	0.02 ± 0.06^{a}	0.00^{a}	0.0 ± 0.002^{a}
Bacillus SD237RC	0.02 ± 0.07^{a}	0.00^{a}	0.0 ± 0.003^{a}
Bacillus H537RC	0.02 ± 0.09^{a}	0.00^{a}	0.0 ± 0.002^{a}
E.coli DH5 α	0.01 ± 0.004^{a}	0.00 ± 0.000^{a}	0.0 ± 0.000^{a}

Results of the one-way ANOVA analysis for different localities $p < 0.01$ significance for the post-hoc Tukey-HSD test among the groups

Different letters in the same column (by main effect) indicate significant differences ($P < 0.05$)

**As significant interaction exists between simple effects

of them are important for the retention of nutrient ions in soils (Dixon 1991), allowing plants to develop properly.

Another association in this study was the presence of PGPR bacteria and sodium in the soil of non-disturbed sites, suggesting that the association could be beneficial to the distribution of this species. In hypersaline soils, halotolerant PGPR bacteria have enabled plant resistance as well as provided inorganic compounds such as indole acetic acid (IAA), ethylene, 1-amino cyclopropane-1-carboxylate (ACC) deaminase, volatile organic compounds (VOC), and antioxidants for their proper growth (Abbas et al. 2019). Microbial communities also helped triggering response genes involved in plant growth, yield, and development under stress conditions (Abbas et al. 2019).

Conclusions

Microorganisms play an important role in plant development and bacteria that showed plant growth- promoting activities, isolated from the rhizosphere of *Pinus chiapensis*, belong to the genera *Dyella*, *Luteimonas*, *Enterobacter*, *Paraburkholderia* and *Bacillus*. They have stimulated seed germination and interestingly, all these bacteria were isolated from non-disturbed stands. These bacteria can be used for restoration of degraded soils for the establishment of *P. chiapensis.* Bacterial isolates *Dyella* sp. SD2037TM and *Enterobacter* sp. SD330TM were the most promising candidates, increasing germination rates and sprout sizes. The mechanisms used by these microorganisms can be related to bacterial derived auxin and gibberellin production as well as to phosphorous solubilization. Additional research is required to utilize these isolates for forest soil recovery.

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