

Plant growth promoting rhizobacteria from Juan Fernández archipelago improve germination rate of endangered plant *Solanum fernandezianum* Phil.

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ABSTRACT

Robinson Crusoe Island, Chile, has one of the highest densities of endemic plants in the world, however many of its native and endemic species, such as *Solanum fernandezianum* Phil., are currently declared endangered. Coating the seeds of native plants with locally sourced plant-growth-promoting bacteria can be used as a tool for conservation programs of endangered plant species. Isolation and screening of rhizosphere bacteria from Robinson Crusoe Island resulted in the selection of three bacteria: *Serratia* sp. RGM 2525, *Raoultella* sp. RGM 2526, and *Pseudomonas* sp. RGM 2607, due to their capacity of producing indole compounds (30-45 $\mu\text{L mL}^{-1}$) and indoleacetic acid (IAA) (5-10 $\mu\text{g mL}^{-1}$). The effect of these strains on the seed germination rate of *S. fernandezianum* was evaluated under five treatments: individual inoculation of each bacteria, inoculation of a mixture of the three bacteria and a treatment without bacteria (control). Inoculation of bacteria improved the seed germination rate of *S. fernandezianum* compared to the control treatment, with the bacterial mix as the best treatment with 26.9% germination ($p < 0.05$), 10.2% higher than control. Bioinoculants formulated with bacteria isolated from rhizosphere soils could improve the seed germination rate of the endangered plant *S. fernandezianum*.

Key words: Bioinoculant, germination rate, IAA production, plant-growth-promoting rhizobacteria, *Solanum fernandezianum*.

INTRODUCTION

The Robinson Crusoe Island (33°37' S, 78°51' W), Chile, exhibits one of the highest densities of endemic flora on Earth (Vargas et al., 2011). Endemic vegetation on the island represents 63% of all plants, which is equivalent to 2.1 native species per km² (Bernardello et al., 2006). However, many factors affect this unique biodiversity and therefore, the island is one of the most vulnerable places in the world with more than 70% of its endemic flora classified as threatened (Ricci, 2006). The endemic vegetation of Robinson Crusoe Island has been displaced by the introduction of foreign plants and animals which have destroyed the habitat of endemic plants and prevented their recovery (Bourne et al., 1992; Ricci, 2006; Vargas-Gaete et al., 2018). In some cases, the native and endemic vegetation is represented by a low number of individuals, probably due to their complex germination requirements and diverse germinative responses (Cuevas and Figueroa, 2007). The combination of these factors, along with erosion, fires, and habitat fragmentation,

make the regeneration of the native flora residing on Robinson Crusoe Island difficult. One particular example is *Solanum fernandezianum* Phil., an herbaceous endemic vascular plant of Robinson Crusoe Island that displays extreme resistance to *Potato leafroll virus* and *Potato viruses Y* and *A*, harbors exclusive alleles, potentially useful for breeders, but is currently categorized as endangered with less than 1000 individuals on the island. Therefore, restoration initiatives should address this problem through a multidisciplinary point of view (Ricci, 2006; Solano et al., 2011).

Plants are intimately related to the microbial community residing in the soil area that is in direct contact with their roots, known as the rhizosphere (Beneduzi et al., 2012). Studies based on the 16S rRNA gene sequences have revealed that the bacterial communities of the rhizosphere, or rhizobacteria, are diverse, with more than 10^4 species per gram of soil (Berendsen et al., 2012; Brader et al., 2017). The constant interaction between plants and rhizobacteria throughout evolution has resulted in a complex mutualistic association, where groups of these bacteria are capable of promoting plant-growth, which are known as plant growth-promoting rhizobacteria (PGPR) (Compant et al., 2019). PGPR can promote plant growth by several mechanisms, including symbiotic N_2 fixation, phosphate, mineral solubilization, production of siderophores, ammonia (NH_3) and phytohormones such as indoleacetic acid (IAA). The latter increases seed germination rate and seedling emergence in plants (Backer et al., 2018).

A Darwin Initiative project (2015-2018) was aimed at restoring the native and endemic vegetation on Robinson Crusoe Island by incorporating locally sourced PGPR to improve the germination of native plants and, the *in situ* restoration of endangered populations of plants. Different reports have demonstrated the utility of inoculation of IAA producing PGPR to increase the germination rate of plants. Thus, the isolation of rhizospheric microorganisms that are capable of producing phytohormones, such as IAA, could potentially be used as biostimulants for improving plant germination and growth rate of the endangered species, as has been shown in other reports (Galdiano Júnior et al., 2011; Gumiere et al., 2014; Liu et al., 2017). Therefore, it was hypothesized that the use of PGPR could improve the rate germination of endangered plants from Robinson Crusoe Island.

In this work, we evaluated the production of indole compounds and IAA of rhizobacteria isolated from plant-associated soils of Robinson Crusoe Island and their use as bioinoculants to improve seed germination rate of *S. fernandezianum*.

MATERIALS AND METHODS

Source of plant germplasm and collection sites

Seeds of *S. fernandezianum* were collected from plants on Robinson Crusoe Island (January and February, 2015) and stored in Petri dishes in the dark until use in the Chilean Collection of Microbial Genetic Resources (CChRGM). Samples of rhizosphere soil of endemic and invasive plants were collected from nine areas (Figure 1). Samples were collected by excavating the surroundings of the plant with a clean metal shovel; collected soils were stored in sterile plastic bags and kept inside a cooler until processed.

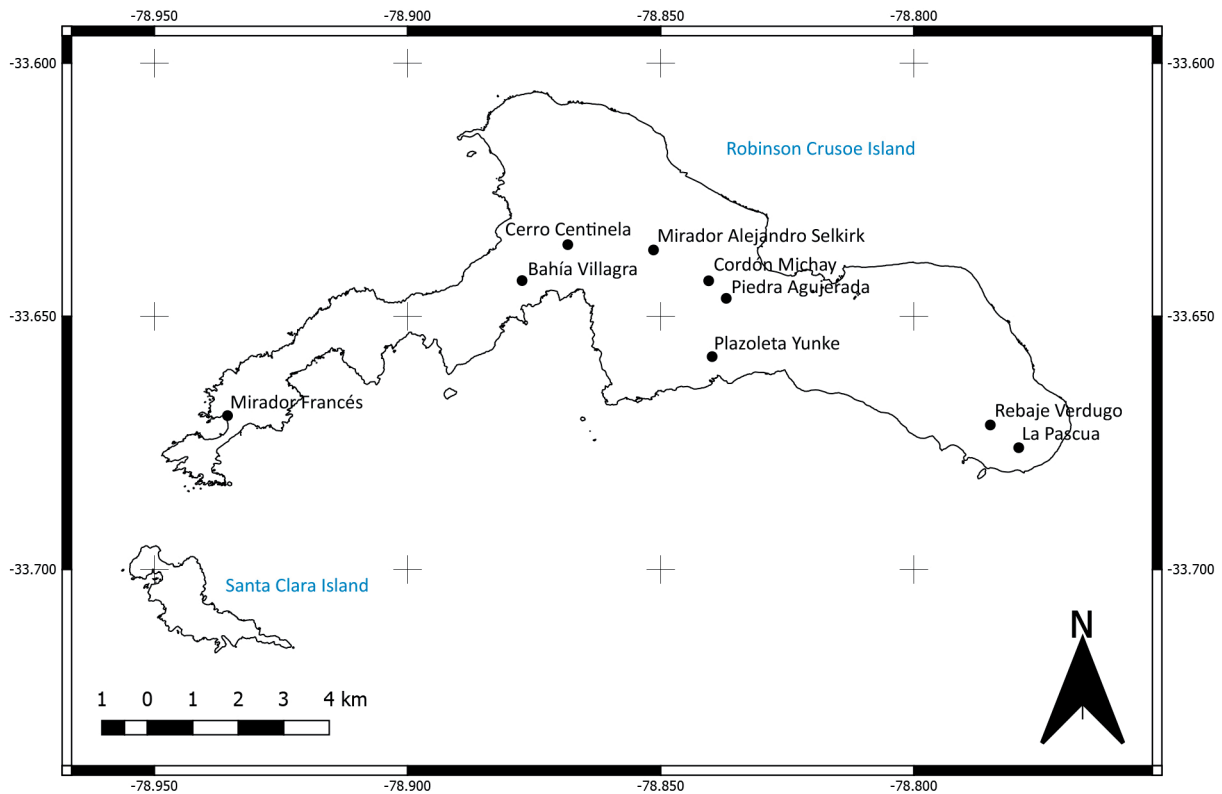
Isolation and preservation of bacteria

Isolation of bacteria from soil was carried out using the methods described by Dinesh et al. (2015) and Walker et al. (1998): 0.5 g soil were suspended in 9 mL sterile water; 80 μ L of serial dilutions were spread on nutrient agar (NA), trypticase soy agar (TSA) and King's B medium (KB). Plates were incubated for 72 h at 28 °C. The arisen colonies were streaked on fresh NA medium and incubated for 2 d at 28 °C. The isolates obtained were stored in 20% glycerol at -80 °C until use.

Assessment of indole compounds production

A screening of the 54 bacteria isolates was carried out with the Salkowski's reagent (Ehmann, 1977) after Gutierrez et al. (2009) to determine the production of indole compounds. The absorbance of the solution was measured in a spectrophotometer (Epoch, BioTek Inc., Winooski, Vermont, USA) at 535 nm. The concentration of indole compounds was calculated after interpolating the absorbance in a linear regression made with known concentrations of commercial indoleacetic acid (IAA) (Sigma-Aldrich) as standard.

Figure 1. Map of Robinson Crusoe Island showing the nine areas (black dots) selected for rhizosphere soil collection. Map generated with Google Earth Pro Geographic and processed in QGIS software version 3.0.2. (QGIS Development Team, Open Source Geospatial Foundation Project; <http://qgis.osgeo.org>).



Quantification of IAA

Three strains with the highest production of indole compounds were subject to a quantitative evaluation for IAA production by high resolution liquid chromatography (HPLC) according to Hoffman et al. (2013). Samples were analyzed in a 20A HPLC system (Shimadzu, Kyoto, Japan), equipped with an SPD-M20A diode array detector fitted with an Inertsil ODS-3 column (GL Sciences, Tokyo, Japan) with a particle size of 5 μm and dimension of 4.6 \times 250 mm with injection volumes of 10 μL .

Molecular identification of selected bacteria and bioinformatic analyses

Genomic DNA from selected strains was extracted with the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), following the manufacturer's instruction. A standard PCR was carried out to amplify the 16S rRNA gene using a temperature cycle program reported by Galkiewicz and Kellogg (2008), with primers 27F (5'-AGAGTTTGTATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). All reactions were carried out with GoTaq Green Master Mix 2X (Promega, Madison, Wisconsin, USA). Amplicons were sent for sequencing to Macrogen Inc. (Seoul, South Korea).

The nucleotide sequences were assembled and edited in Sequencher version 5.4.6 (Gene Codes Corporation, Ann Arbor, Michigan, USA). The 16S rRNA gene sequences of isolates were compared in the EzBioCloud website (www.ezbiocloud.net; Yoon et al., 2017). The sequences of the nearest type species for each isolate were retrieved from GenBank (Benson et al., 2017). Phylogenetic analyses were carried out after Castro et al. (2018); phylogenetic trees were inferred with MEGA software version 7.0 (Kumar et al., 2016). The bacterial strains were deposited in the Chilean Collection of Microbial Genetic Resources (<http://www.cchrgm.cl>) with the following access codes RGM 2525, RGM 2526 and RGM 2607.

Assessment of growth compatibility between selected bacteria

The three strains selected were simultaneously streaked on nutrient agar as follows: one strain was streaked as a line of 4 cm in the middle of the plate and the other two strains were streaked perpendicularly across this line (Prasad and Babu,

2017). Plates were incubated at 25 °C for 48 h. Positive compatibility of the strains was recorded if no inhibition zones were observed near the strains. All experiments were done in triplicate.

Bacterial inoculation on seeds of *S. fernandezianum*

Liquid cultures of strains RGM 2525, RGM 2526, and RGM 2607 were carried out in 6% liquid molasses for 72 h at 28 °C. Afterwards, bacterial cells were washed three times with phosphate buffer solution (PBS), cultures were centrifuged to 6000 g for 10 min, the supernatant was removed and the pellet resuspended in PBS. Seeds of *S. fernandezianum* were surface sterilized by sequentially soaking the seeds in solutions of 1.5% sodium hypochlorite, 70% ethanol and sterile distilled water. Surface sterilized seeds were submerged for 4 h in bacterial suspensions (10^8 CFU mL⁻¹): (i) RGM 2525, (ii) RGM 2526, and (iii) RGM 2606 strains, as well as in a suspension containing the (iv) mixture of three isolates (bacterial mix) in proportions (1:1:1), (v) PBS was used as a control. The treated seeds were sown in seedling plugs (4 × 4 × 5 cm), filled with peat, perlite and vermiculite (1:1:1) and sterilized twice at 121 °C for 1 h. The seedlings were placed in a phytotron set to 20 °C with a light:dark cycle of 14:10 h; seedlings were watered with 5 mL distilled water each day to maintain the humidity of the substrate. Germination of seeds was monitored every 4 d for 40 d. The criterion for germination was that both cotyledons were out of their seed. The germination rate was calculated as the ratio of the germinated seed and total seeds per treatment. Each treatment contained 72 seeds in a randomized complete block design with three replicates.

Statistical analysis

Germination rate, total indole compounds, and IAA concentration data were analyzed using the ‘emmeans’ package (Lenth et al., 2019) in R environment (R Core Team, 2018). Due to the nature of the data, germination rates were transformed (arcsine of the square root of the proportion) to meet statistical analysis assumptions. For each evaluation point, each treatment differences respect to the control were determined by using the Dunnett’s test (Lenth et al., 2019) at a confidence level of 95%; results were expressed using de-transformed means. Differences for total indole compounds and IAA between each strain were determined by using the Tukey’s test at a confidence level of 95%.

RESULTS

A total of 52 rhizosphere bacteria were isolated from endemic and invasive plants found in Robinson Crusoe Island from which 25 strains (48%) were capable of producing indole compounds. Three strains in particular: RGM 2525 isolated from rhizosphere soil of *Fagara mayu* (Bert. ex Savi) Engler, RGM 2526 isolated from *Aristotelia chilensis* (Molina) Stuntz and RGM 2607 isolated from *Myrceugenia fernandeziana* (Hook. & Arn.) Johow (Table 1), showed production of indole compounds above 30 µg mL⁻¹, with production by strain RGM 2526 higher ($p < 0.05$) than that of RGM 2525 and RGM 2607 (Figure 2A). In turn, HPLC quantification of IAA showed that strain RGM 2525 produced 8.6 µg mL⁻¹, RGM 2526 produced 12.4 µg mL⁻¹ and RGM2607 produced 4.4 µg mL⁻¹ (Figure 2B). The HPLC analysis confirmed that strain RGM 2526 was the highest producer of IAA compared to the other strains, although the production achieved by this strain was not significantly higher than that obtained with RGM 2525.

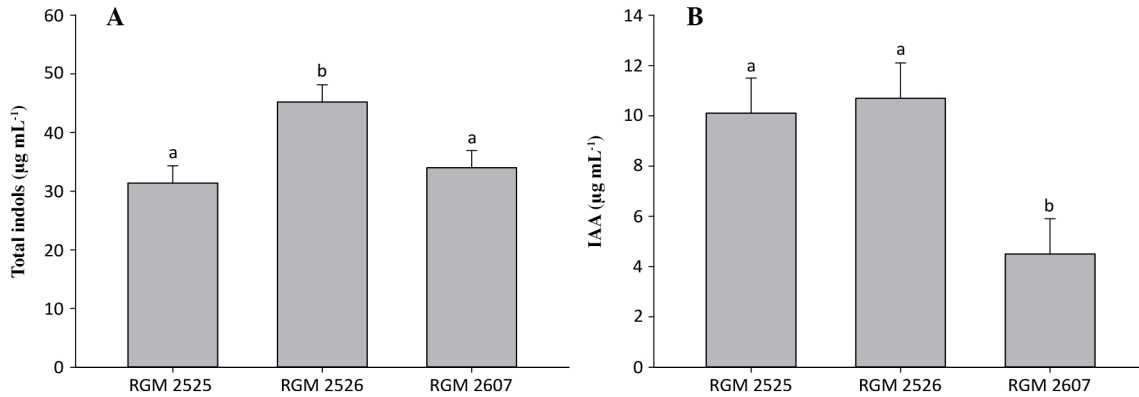
Table 1. Strains selected for inoculation of seeds of *Solanum fernandezianum*.

Strain (Genbank accession number)	Host plant rhizosphere soil	Geographic location (longitude and latitude)	Closest type-strain (GenBank accession number)
RGM 2525 (MH332648)	<i>Fagara mayu</i> (Bert. ex Colla) Engl.	33°39'01.96" S 78°50'40.72" W	<i>Serratia proteamaculans</i> DSM 4543 ^T (AJ233434)
RGM 2526 (MH332648)	<i>Aristotelia chilensis</i> (Molina) Stuntz.	33°38'13.79" S 78°51'35.53" W	<i>Raoultella terrigena</i> DSM 4687 ^T (Y17658)
RGM 2607 (MH332648)	<i>Myrceugenia fernandeziana</i> (Hook & Arn.) Kausel.	33°39'05.9" S 78°50'41.6" W	<i>Pseudomonas laurylsulfatiphila</i> DSM 105097 ^T (KY462012)

Genbank accessions correspond to 16S rRNA gene sequences.

DSM: Deutsche Sammlung von Mikroorganismen from the German Collection of Microorganisms and Cell Cultures (DSMZ); RGM: Recursos Genéticos Microbianos from the Chilean Collection of Microbial Genetic Resources (CChRGM).

Figure 2. Quantification of the production of indole compounds (A) and indoleacetic acid (IAA) (B) by isolates RGM 2525, RGM 2526, and RGM 2607.



Different letters mean significant difference according to Tukey test ($p < 0.05$).

Comparison of the 16S rRNA gene sequences of the bacterial isolates RGM 2525, RGM 2526 and RGM 2607 indicated taxonomic circumscription to the genera *Serratia*, *Raoultella*, and *Pseudomonas*, respectively (Figure 3). Isolate RGM 2525 showed a similarity value of 99.64% with the nearest type-species, *Serratia proteamaculans* DSM 4543^T; in turn, isolates RGM 2526 and RGM 2607 were 99.64% and 99.84% similar to the type species *Raoultella terrigena* DSM 2687^T and *Pseudomonas laurylsulfatiphila* DSM 105097^T.

Co-cultivation of the three isolates confirmed compatibility of the strains since no growth inhibitions were visualized when grown simultaneously on agar plates, indicating that the simultaneous use of the three microorganisms as bacterial mixture could be feasible.

The effect of the inoculation of isolates *Serratia* sp. RGM 2525, *Raoultella* sp. RGM 2526 and *Pseudomonas* sp. RGM 2607 on the seed germination rate of *S. fernandezianum* under laboratory-controlled conditions is shown in Figure 4. The first germinated seeds were observed after 16 d for treatment *Pseudomonas* sp. RGM 2607 and after 20 d for the five treatments. The greatest increase in germination rate was observed in the bacterial mix treatment (26.9% germination), which produced a significant ($p < 0.05$) difference at day 36, in comparison with that achieved in the control treatment (without bacteria) (16% germination). All bacteria, except *Raoultella* sp. RGM 2526, increased seed germination compared to the control treatment. During the time course of the experiment, the bacterial treatment did not affect the development of plants.

DISCUSSION

Efforts for *in situ* and *ex situ* conservation of endangered plant species has enabled the protection of the native and endemic phytogenetic resources from extinction, which is of utmost importance considering the current endangered state of several plant species from Robinson Crusoe Island (Bourne et al., 1992; Ricci, 2006). This work proposed the isolation of bacteria from rhizosphere soils of endemic and invasive species of Robinson Crusoe Island and the assessment of their effect on the germination rate of a model plant, *S. fernandezianum*, a native species declared as endangered due to the scarce number of individuals found on the island (Ricci, 2006). A challenging aspect of the conservation of *S. fernandezianum* is its low germination rate (14.3%) reported by Solano et al. (2011). As expected, a similar result was achieved in this work (16.7%) when seeds of this species were sown under similar laboratory conditions in the control treatment without bacteria (Figure 4). These results suggest the occurrence of a germination rate constraint, probably due to the genetics of *S. fernandezianum* that might also be influenced by environmental factors.

The production of phytohormones, such as the auxin IAA, has been reported in several bacterial genera with plant-growth promotion traits (Han and Yang, 2015). The quantities of indole compounds produced by *Serratia* sp. RGM 2525, *Raoultella* sp. RGM 2526, and *Pseudomonas* sp. RGM 2607 are similar to those reported by Gumiere et al. (2014). The quantities of IAA obtained for the three isolates represented a fraction of the total indole compounds quantified by the Salkowski's assay, which is expected due to the nature of the Salkowski's assay, which detects indole compounds.

Figure 3. Neighbor joining tree based on almost complete 16S rRNA gene sequences showing relationships between the 16S rRNA gene of isolates RGM 2525 (A), RGM 2526 (B) and RGM 2607 (C) with their respective closely related type-strains. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (ML) and maximum-parsimony tree-making algorithms. Only bootstrap values above 60% are shown. The respective scale bar represents the number of substitutions per nucleotide position. Evolutionary distances were calculated with the Kimura-2-parameter model (Kimura, 1980) for the neighbor joining and maximum parsimony algorithm and the General Time Reversible model (Lanave et al., 1984) for the maximum likelihood algorithm.

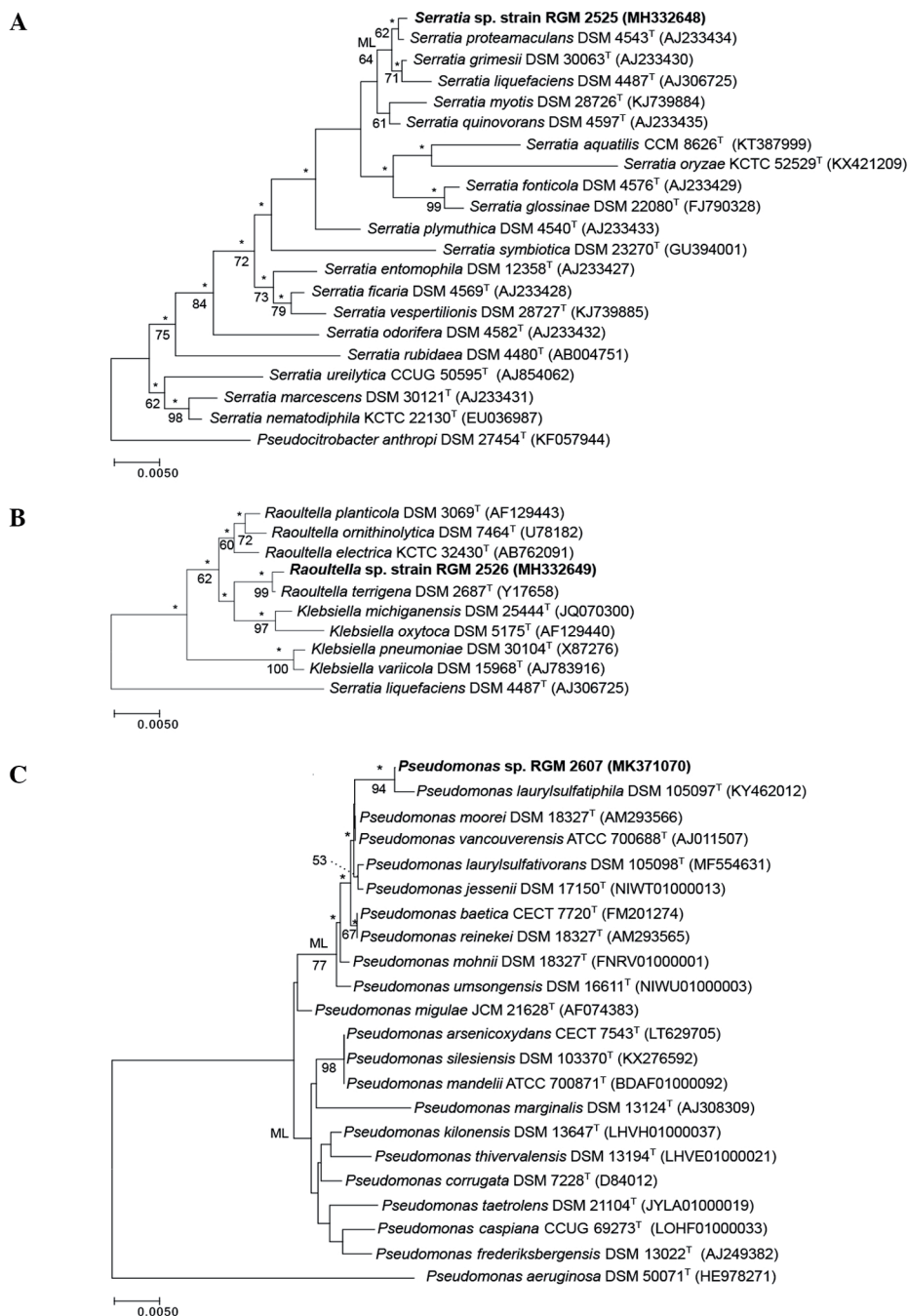
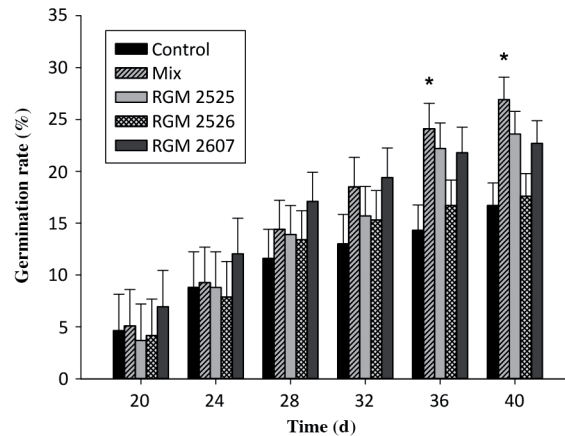


Figure 4. Evaluation of the use of isolates *Serratia* sp. RGM 2525, *Raoultella* sp. RGM 2526, and *Pseudomonas* sp. RGM 2607 and the mixture of these isolates (Mix) on the germination rate of seeds of *Solanum fernandezianum* over time.



Asterisks indicate treatments significantly higher than control ($p < 0.05$).

In terms of the taxonomical affiliation of the strains, the isolate *Serratia* sp. RGM 2525 forms a consensus clade with members of the *S. liquefaciens* complex, species that are frequently associated to plants (Grimont et al., 1981; Petersen and Tisa, 2013). The isolate *Raoultella* sp. RGM 2526 was grouped with the type-strain *R. terrigena* DSM 2687^T, a strain also isolated from rhizosphere soil. The strain *Pseudomonas* sp. RGM 2607 was similar to the type-species *S. laurysulfatiphila* DSM 105097^T, for which there is little information regarding growth promotion traits since it has been recently described as a novel species (Furmanczyk et al., 2018).

The use of *Serratia* sp. RGM 2525 and *Pseudomonas* sp. RGM 2607 as individual bioinoculants improved the seed germination rate of *S. fernandezianum*, although a better result ($p < 0.05$) was achieved with the bacterial mix, obtaining a 26.9% germination rate, which is 10.2% higher than the control without bacteria. On the other hand, the strain *Raoultella* sp. RGM 2526 displayed no effect on the germination rate when inoculated alone, being similar to that obtained with the control treatment; there was potentially a synergistic effect when the three isolates were inoculated together, suggesting there could exist others bacterial metabolites that may be influencing the germination process (Figure 4). Other reports have shown that a mixture of microbial strains used as bioinoculants has no effect on plant-growth promotion as some strains can affect or inhibit the growth of other strains in the formulation, thus it is important to check the compatibility of the strains before using them as bioinoculants (Kang et al., 2014).

Microbial inoculants have become of interest for scientists as a solution to agronomic and ecological problems (Mahanty et al., 2017). The results showed in this work indicated that the treatment of *S. fernandezianum* seeds with IAA producing PGPR increased the germination rate. Several reports have concluded that auxin production by bacterial strains associated with the rhizosphere plays an essential role in regulating different functions in plants, such as germination, growth, and development of the plant (Miransari and Smith, 2014). For instance, bacteria species of *Serratia* spp. have been reported with capacities to IAA synthesis and beneficial properties in plant growth promotion. *Serratia proteamaculans* isolated from vegetal tissues of poplar, exerted beneficial plant-growth promotion properties on poplar plants, giving rise to well-developed roots and shoot compared to the non-inoculated plants (Taghavi et al., 2009). The nearest species to the strain *Raoultella* sp. RGM 2526, *R. terrigena*, displayed increased shoot and root DM of wheat and barley plants when using the microorganism with B under cold stress and ambient conditions (Turan et al., 2013). *Pseudomonas* species have been regarded to harbor genes related to plant-growth promotion traits, for which there are several examples of their use as bioinoculants, such as *Phaseolus vulgaris* L. (Yadegari, 2014), *Triticum aestivum* L. (Shaharoon et al., 2008), and *Solanum lycopersicum* L. (Adesemoye et al., 2008). Plant-growth promotion has been reported for members of the genera *Serratia* spp. and *Raoultella* spp. in *Pueraria thunbergiana* Benth. (Selvakumar et al., 2008), and *Achyranthes aspera* L. (Devi et al., 2016), as a result of phosphate solubilization, production of phytohormones and siderophores. Additionally, improvement of seed germination parameters by PGPR has been reported for endangered plants (Galdiano Júnior et al., 2011; Gumiere et al., 2014; Liu et al., 2017).

Strategies that involve the use of PGPR could be used as complementary tools in conservation programs of endangered native plants from Robinson Crusoe Island, considering the current critical state of some plant species. Future work is needed to verify whether these strains could also promote the seed germination rate of other endangered plants of Robinson Crusoe Island.

CONCLUSIONS

We conclude that rhizosphere soils of plants of Robinson Crusoe Island remain an untapped local source of plant growth-promoting rhizobacteria (PGPR) microorganisms with the capacity of indoleacetic acid synthesis, which improve the germination rate of the endangered plant *Solanum fernandezianum*. The bacteria characterized in this work could be used as a tool for *in situ* conservation and recovery of endangered plant species of Robinson Crusoe Island.

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