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Short communication

Isolation and identification of plant growth-promoting bacteria from rhizomes of *Arachnitis uniflora*, a fully mycoheterotrophic plant in southern Chile

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ABSTRACT

Fully mycoheterotrophic plants have attracted attention due to their ability to obtain carbon and mineral nutrients directly from fungal hyphae. However, research on bacteria associated with full mycoheterotrophs is limited. This study identifies rhizosferic and endophytic bacteria associated with *A. uniflora* rhizomes and analyzes their ability to produce microbial metabolites of various functions. Eight bacterial OTUs were revealed, and their potential roles in plant-growth promotion and antimicrobial activities were demonstrated. These findings suggest that root-system associated bacteria can be considered as essential microorganisms for growth and development of fully mycoheterotrophic plants.

1. Introduction

Symbiotic associations of land plants with microorganisms may include a broad spectrum of bacteria, fungi and viruses (Ramakrishna et al., 2019; Rincón-Molina et al., 2020; Wylie et al., 2013). These microorganisms can colonize living plant cells, establishing mutualistic, commensal or parasitic interactions (Glazebrook and Roby, 2018; Paszkowski, 2006). Such associations can be essential to plants, playing a key role in supporting several vital processes such as nutrient mineralization, improving stress tolerance, plant-growth promotion and protection against pathogens (Thirkell et al., 2016).

The most widespread symbiotic microbiota associated with land plants are arbuscular mycorrhizal fungi (AMF) as well as bacteria (Santoyo et al., 2016; Smith and Read, 2010; Bonfante and Desirò, 2017). The free living rhizospheric bacteria with beneficial effects on plant growth are usually referred to as plant growth promoting bacteria (PGPB) (Glick, 2012; Beneduzi et al., 2012). Mycorrhizal fungi and mycorhizosphere-associated bacteria live under the influence of the metabolic activity of the host plants via the colonization of their roots and establishing a symbiotic relationship where both partners may derive benefits (Saia et al., 2015). It is expected that such benefits are greater in non-photosynthetic plants by the dependence on such microorganisms to complete critical life stages such as seed germination and plantlet growth and development (Herrera et al., 2019a, 2019b).

The most studied plant-growth promotion mechanisms are related to phosphorous solubilization, phytohormone production, nitrogen fixation and the supply of essential nutrients to the host plants (Rashid et al., 2012). Studies about growth promotion of fully mycoheterotrophic plants have examined mostly the beneficial effect of mycorrhizal fungi, whereas research in bacteria associated with achlorophyllous plants is limited (Kinoshita et al., 2016; Selosse et al., 2017).

Arachnitis uniflora Phil. (Corsiaceae), commonly known as a spider flower, is a fully mycoheterotrophic plant growing in native forest understorey in South America. Most of the vegetative period *A. uniflora* spends in form of rhizomes colonizing upper layer of the soil (about 10 cm). After a short flowering period, a single capsule containing thousands of dust like seeds is formed (Ibish et al., 1996). Research regarding root microbiological interactions of *A. uniflora* has focused on characterizing AMF associated with the plant, whereas studies focusing on associations with PGPB have been neglected (Domínguez et al., 2006; Domínguez et al., 2009; Renny et al., 2017).

The aim of the present study was to isolate and molecularly identify rhizospheric and endophytic bacteria associated with the rhizomes of *A. uniflora* growing in native ecosystems of southern Chile. Furthermore, various metabolites produced by the isolated bacteria that can contribute to the growth and development of the plants were detected. On the base of our knowledge, this is the first study of a fully mycoheterotrophic plant and the closely associated bacteria.

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2. Materials and methods

Rhizosphere and rhizomes of *A. uniflora* were collected near Tolhuaca National Park, Curacautín, Region of La Araucania, southern Chile (38°17′42.2"S 71°46′14.9"W; October 2018). Five collecting sites were established and four rhizomes with adhesive soil were taken from each site. Isolation of rhizospheric and rhizome-associated endobacteria was performed according to Blain et al. (2017) with some modifications. Briefly, to isolate rhizospheric microorganisms, the entire rhizomes of each collecting site together with 500 mg of adhering soil were placed in a 500 mL Erlenmeyer flask containing 300 mL of sterile phosphate-buffered saline (PBS; 1.2 g L^{-1} K_2HPO_4, 0.18 g L^{-1} KH_2PO_4, 8.5 g L^{-1} NaCl). After shaking (orbital shaker for 45 min at 180 rpm) the solution was diluted into dilutions 10^{-1} to 10^{-5} in sterile distilled water (1 mL of rhizospheric soil solution diluted in 9 mL of sterile distilled water). Then, 500 µL of the dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated in triplicate onto Petri dishes containing 30 mL of modified Luria Bertani Agar (LBA) plus 100 mg L^{-1} cycloheximide. Plates were incubated at 25 ± 1 °C until bacterial colonies were visible. Then they were subcultured to obtain pure cultures. All rhizospheric bacteria were stored at 4 °C for further analysis.

In the case of endophytic bacteria, a similar protocol was followed with addition of rhizome disinfection as the first step. The entire rhizomes were immerged into a solution of 3 mL sterile distilled water, 1 mL of sodium hypochlorite (5% chlorine) and 1 mL of 100% alcohol (for each 5 mL of working solution) for 5 min, followed by five washes in sterile deionized water. Four superficially sterile rhizomes per sampled population were suspended in 50 mL of 1/10 (m/v) sterile PBS according to Blain et al. (2017) with modifications. An aliquot of 500 µL from the last wash was added on in LBA to discount the presence of any superficial bacteria. The rhizomes suspended in PBS buffer were ground using a sterile mortar and pestle. After the mixture was serial diluted into 10^{-1} to 10^{-5} in sterile distilled water. Then, 500 µL of the dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated in triplicate onto Petri dishes containing 30 mL of modified LBA. Plates were incubated at 25 ± 1 °C or until bacterial colonies were visible. All endophytic bacteria were stored at 4 °C for further analysis.

Bacterial strains with different phenotypic characters and growth rates were subjected to molecular identification. DNA was extracted from liquid cultures in Luria Bertani broth (LBB) after 3 days' culturing in 50 mL Falcon tubes in an orbital shaker (at 150 rpm and 25 ± 1 °C). An aliquot of the pure culture (1.8 mL) was centrifuged twice for 30 s at room temperature and the supernatant was discarded. DNA extraction was performed following the DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5'-GGTTACCTTGTTACGACTT-3′) were used to amplify the 16S-rRNA regions according to Miller et al. (2013). The PCR cycle consisted of an initial denaturing at 95 °C for 5 min, followed by 30 cycles of denaturing at 95 °C for 1 min each, annealing at 58 °C for 1 min, extension at 72 °C for 1 min, and final extension for 5 min at 72 °C. PCR products were checked on 2% agarose gel stained with GelRed®. Sequencing was performed by Macrogen (Seoul, South Korea). Blast searches were conducted to find the closest match, accepting the genus and species classification according to Chen et al. (2011), and the sequences were submitted to the GenBank database under accession numbers MK790632 to MK790639.

Production of siderophores by the isolated bacteria was estimated according to Milagres et al. (1999). Furthermore, phosphate solubilization and production of indole acetic acid (IAA) were estimated according to Ahemad and Khan (2010) and Khalid et al. (2004), respectively. Additionally, the production of low molecular weight organic acids was determined by RP-HPLC, as described in Herrera et al. (2018) with some modifications. Briefly, liquid bacterial cultures were performed in LBB and incubated in an orbital shaker (140 rpm and 25 ± 1 °C) for 1 day. The resulting solution was filtered (pore ø 0.45 µm), freeze-dried, re-suspended in 500 µL deionized sterile water and filtered again (pore ø 0.22 µm). Calibration curves were prepared using the organic acids kit (47264, Supelco, Bellefonte, PA). Chromatographic analysis was carried out in a HPLC (Shimadzu CTA-20 AC, Kyoto, Japan) equipped with a UV–visible detector. Separation of organic acids was done in a C-18 reverse phase column (MultoHigh 100 RP-18, 5 mm particle size, CS-GmbH, Langerwehe, Germany). The mobile phase was 93% (v/v) 25 mM KH_2PO_4 at pH 2.5 and 7% (v/v) methanol with a flow rate of 1 mL min^{-1} according to Cawthray (2003). Additionally, the ability of the isolated bacterial strains to restrict growth of fungal hyphae was evaluated, following the methodology proposed by Petatan-Sagahon et al. (2011), considering two potential plant pathogenic strains that we isolated from rhizomes of *A. uniflora* (*Phoma herbarum* and *Fusarium oxysporum*; Herrera et al., 2019a, 2019b).

Quantitative data was analyses by ANOVA. If the p value indicated significant differences between treatments ($p < 0.05$), post hoc pairwise comparisons were performed, using the SD of means and Tukey's multiple range test. All statistical tests were conducted using the *R* software (R Core Team 2018; https://www.R-project.org).

3. Results

In total, 331 bacterial colonies were counted in the Petri dishes and molecularly classified into eight different operative taxonomic units (OTUs). We revealed 8 different strains of rhizospheric bacteria (214 colonies) and 9 strains of rhizome-associated endobacteria (117 colonies, Table S1). The sequence analyses of the 16S rDNA gene showed that the rhizospheric bacteria were related to *Bacillus megaterium*, *Paenibacillus lautus*, *Bacillus* sp., *Microbacterium hatanonis*, *Paenibacillus tundrae*, *Chryseobacterium* sp., and one bacterium that did not match with any sequence submitted to the GenBank database (Table 1). Rhizome-associated endobacteria were related to *B. megaterium*, *P. lautus*, *Bacillus* sp. *Bacillus velenensis*, *M. hatanonis*, *Chryseobacterium* sp., and two unknown bacteria with no match in the GenBank database (Table 1). The most common bacteria isolated from the rhizosphere were *M. hatanonis* and *P. tundrae* (frequency of isolation 0.23 and 0.20, respectively); whereas in the endosphere the isolates *B. megaterium* and *P. lautus* were the most abundant (frequency of isolation 0.21 and 0.20, respectively) (Table S1, Fig. 1). The isolates *B. velenensis* and *P. tundrae* were exclusively found in the endosphere and rhizosphere of the plants, respectively.

We showed that the bacterial isolates either from rhizosphere or endosphere have a potential to promote plant growth. Phosphate solubilization and production of indole acetic acid was detected in almost all strains, with the exception of AU2, AU6 and AU9 for phosphate solubilization, and AU8 for auxin production (Table S1). The greater presence of indole acetic was detected in isolate AU1 (0.28 µg mL^{-1}), which also presented the greatest siderophore production halo. Concerning organic acid production, the bacterial isolates that showed the greatest overall exudation rate were AU9 and AU4 (Table S1). Maximum production of oxalic acid was significantly higher in AU4 (114.8 µg L^{-1}), whereas the maximum lactic acid was detected in AU10 (4006.9 µg L^{-1}). The isolate AU9 showed significantly high exudation of malic (358.7 µg L^{-1}), citric (974.5 µg L^{-1}) and succinic acid (1684.0 µg L^{-1}; Table S1).

Some bacterial strains have antagonistic effects against some potential plant pathogens isolated from the roots of *A. uniflora*. Specifically, the isolates AU1 (*B. megaterium*), AU2 (*P. lautus*), AU4 (*B. velezensis*), AU6 (*P. tundrae*), AU7 (*Chryseobacterium* sp.) and AU10 (*Chryseobacterium* sp.) were able to inhibit the growth of *F. oxysporum* and *P. herbarum.* The effectivity of this inhibition (positive in Table S1) varied between 15 and 30% of inhibition area.

Table 1
Molecular identification of bacteria isolated from rhizosphere and rhizomes of Arachnitis *uniflora*.

Isolate	Identification	Isolation source	GenBank accession	Closest GenBank relative (% identity)	Isolation source (Relative)	Reference
AU1	*Bacillus megaterium*	Rhizosphere/Endosphere	MK790632	MK618596 (100%)	Citrus endophyte	GenBank
AU2	*Paenibacillus lautus*	Rhizosphere/Endosphere	MK790633	KY352849 (100%)	*Huperzia serrata* rhizosphere	GenBank
AU3	*Bacillus* sp.	Rhizosphere/Endosphere	MK790634	MH311889 (100%)	*Theonella swinhoei*	Kuo et al. (2019)
AU4	*Bacillus velezensis*	Endosphere	MK790635	MK618602 (99%)	Citrus endophyte	Genbank
AU5	*Microbacterium hatanonis*	Rhizosphere/Endosphere	MK790636	MG458427 (100%)	Compost	GenBank
AU6	*Paenibacillus tundrae*	Rhizosphere	MK790637	KX349198 (99%)	Soil	Rickman et al. (2018)
AU7	*Chryseobacterium* sp.	Rhizosphere/Endosphere	MK790638	MG859544 (99%)	Frog	Catenazzi et al. (2018)
AU8	Unknown bacterial endophyte	Endosphere	–	–	–	–
AU9	Unknown bacterial endophyte	Rhizosphere/Endosphere	–	–	–	–
AU10	*Chryseobacterium* sp.	Rhizosphere/Endosphere	MK790639	MG859544 (99%)	Frog	Catenazzi et al. (2018)

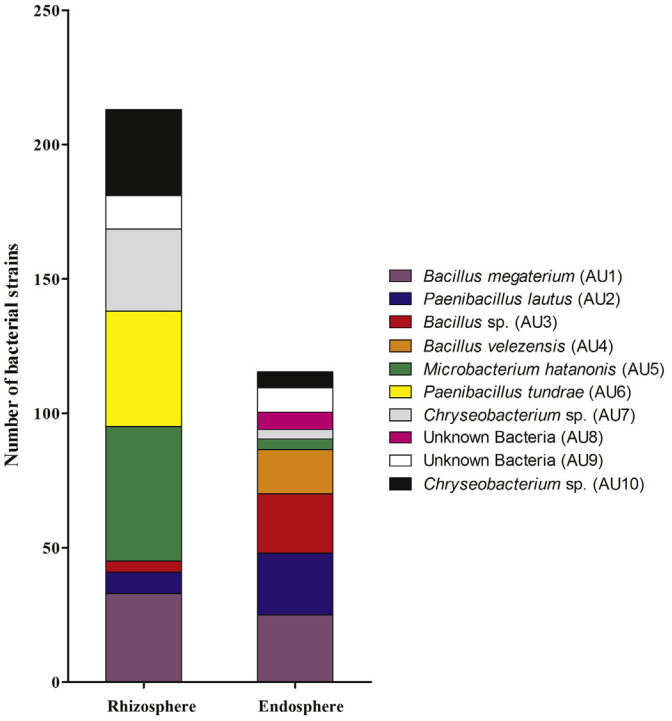

Fig. 1. Quantification and distribution of bacterial strains isolated from roots of the mycoheterotrophic plant *Arachnitis uniflora* in southern Chile.

4. Discussion

Our results revealed a broad range of bacteria occurring in rhizosphere and in endosphere of *A. uniflora* rhizomes. The obtained bacterial isolates demonstrated a significant ability to support growth of this fully mycoheterotrophic plant via various examined growth promotion traits.

Knowledge of root system-associated microbiota of mycoheterotrophic plants provides crucial understanding of their lifecycle, especially in case *A. uniflora*, which carries through the life cycle mostly in form of underground rhizomes (Dominguez and Sérsic, 2004). PGPB are essential for many different plants, providing mineral nutrients for symbionts and improving plant growth and stress tolerance (Komaresofla et al., 2019; Vurukonda et al., 2016). Plant-growth promotion traits have been reported in endophytic and rhizospheric bacteria isolated from several plants, but the studies analyzing interaction with partially or fully mycoheterotrophic plants are limited (Herrera et al., 2019a, 2019b; Santoyo et al., 2016). Our study identified diverse bacteria with potential plant-growth promotion traits, such as phosphate solubilization, IAA and siderophore production, and high exudation rates of organic acids, which agree with the studies of Pavlova et al. (2017) and Faria et al. (2013), who showed production of auxin in bacterial strains associated with *Dendrobium nobile* and *Cattleya loddigesii*, respectively. Additionally, Tsavkelova et al. (2007) showed a positive role of bacteria isolated from roots and stems of the epiphytic orchid *Dendrobium moschatum* in seed germination. Additionally, Novotná and Suárez (2018) revealed several bacterial OTUs associated with living hyphae of *Serendipita* sp., which is a potential mycorrhizal fungus of the epiphytic orchid *Stanhopea connata*. The fungal mycelium thus can provide another putative isolation source of some of the bacteria isolated in our study. In line with our results, Faria et al. (2013) did not show phosphate solubilization in any of the *Paenibacillus* spp. isolates. However, other bacterial strains such as *B. megaterium* and *B. velezensis* have a clear role in phosphate solubilization, which agrees with the study developed by Torres et al., 2019, who isolated *B. velezensis* strains from the rhizosphere of *Juncus effuses*. Several symbiotic relationships between plants and microorganisms are based on the reciprocity of benefits for both partners, but these processes are certainly different in mycoheterotrophic plants, because the lack of photosynthesis makes mycoheterotrophic plants dependent on fungal-origin carbon (Field et al., 2015; Smith and Read, 2010). Hence, the symbiotic benefits for bacteria in the symbiosis established with mycoheterotrophic plants are limited. It is likely that the safety of living inside roots in exchange for bacteria-induced benefits for the associated plant is key to allowing bacteria in the vital tissues of mycoheterotrophic species, as is reported in plantlet of the orchid *Dendrobium catenatum* (Wang et al., 2016).

Our study showed that *B. megaterium* was able to produce metabolites with a potential role in plant-growth promotion, which is consistent with the report of Silambarasan et al., 2019, who showed that *B. megaterium* strains isolated from the rhizosphere of *Lactuca sativa* and *Beta vulgaris* plants contribute to plant-growth promotion and stress tolerance in *Vigna radiata* inoculated plants. The ability of these bacteria to promote plant growth through phosphate solubilization, organic acid exudation and siderophore production have been reported in several locations and plants (Nehra and Choudhary, 2015; Santoyo et al., 2016), as well as their ability to avoid the growth of pathogenic fungi (Bolívar-Anillo et al., 2019). Similarly, Petatan-Sagahon et al. (2011) showed that rhizospheric bacteria isolated from corn crops have

the ability to restrict the growth of some pathogenic strains that affect plants. Likewise, some bacterial strains of *Paenibacillus* spp. can reduce the deleterious effect of the parasitic nematode *Meloidogyne incognita* and the pathogenic fungus *F. oxysporum* in ginseng roots (Sang et al., 2018), as well as some *Bacillus* spp. strains that inhibit fungal phytopathogens (Cawoy et al., 2015), which coincides with our result of the restriction of fungal growth inhibition obtained in the analyses.

The inhibition of fungal growth by some of the isolated bacteria was detected in our study and may play an important role in regulating fungal growth inside roots. Considering that fully mycoheterotrophic plants need to be colonized by different fungi to complement their nutritional demands, the association of the roots of these plants with microorganisms able to restrict fungal growth must be considered a key strategy developed by the plants to avoid the negative effect of fungi on plant metabolism, as is the case with *Arachnitis uniflora* (Herrera et al., 2019a, 2019b). The potential of endophytic or rhizospheric bacterial strains to control the growth of pathogenic microorganisms have been reported in several locations and plants (Berg et al., 2005; Eljounaidi et al., 2016). This is the case of *Chryseobacterium* sp. isolated from pepper soils and their effect on the control of the Phytophthora blight of pepper caused by *Phytophthora capsici* (Kim et al., 2012; Sang et al., 2018), as well as *Chryseobacterium nankingense*, which effectively controls *Ralstonia solanacearum,* a bacterial strain that causes disease in tomato plants (Huang et al., 2017). Similarly, *Chryseobacterium* sp. strains have been reported as antifungal bacteria able to inhibit the growth of pathogenic fungi affecting the skin of frogs and boreal toads (Muletz-Wolz et al., 2017; Park et al., 2014).

Usually, the emphasis of research into mycoheterotrophic plants is the fungi, despite bacteria having been reported as among the most important microorganisms for plants contributing to nutrient solubilization, stress tolerance, defense against phytopathogens and disease, among others (Eljounaidi et al., 2016; Ryan et al., 2008). The habitat of *A. uniflora* is characterized by big trees and a lot of decaying leaves in which saprophytic fungi usually grow in the organic residues. Here, *A. uniflora* associates preferentially with arbuscular mycorrhizal fungi (Domínguez et al., 2009), but it is expected that free-living fungi belonging to different genera can also play a role in the germination of the dust-like seeds (Herrera et al., 2019a, 2019b). These fungi need to be restricted if negative effects on the developed embryo or young plantlets need to be avoided. At this developmental stage, the association with antifungal and plant growth-promoting bacteria, like some of the isolates in our study, can be essential and their role in seed germination and plantlet-growth promotion certainly need to be tested. The microbiological association of *A. uniflora* plants is necessary to complement their nutritional demands, especially in the time when the plant lives as underground organs, a characteristic of this plant which only emerges from the soil for short periods (usually two or three weeks) to produce seeds. Certainly, the plant-growth promotion ability of the isolated bacterial strains in the first life stages of this plant should be tested, but to date there has been a paucity of data on the critical life cycle steps of the plant (seed germination and plantlet growth), which makes it difficult to study the effect of the isolated bacterial strains in *Arachnitis uniflora* using in-vitro or in-vivo assays.

5. Conclusion

In this study we successfully isolated and identified various bacterial OTUs associated with rhizomes of the fully mycoheterotrophic plant *A. uniflora* from southern Chile. Moreover, several plant growth promoting traits were examined and demonstrated. Additionally, some of our isolates showed inhibitory effect on growth of plant pathogenic fungi. However, further studies are needed to understand the specific role of root-associated bacteria in the physiology of fully mycoheterotrophic plants.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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