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ORIGINAL ARTICLE

Mycorrhizal fungi isolated from Chilean orchids as biocontrollers of the pathogen *Rhizoctonia solani*

Hongos micorrícicos aislados de orquídeas chilenas como biocontroladores del patógeno *Rhizoctonia solani*

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ABSTRACT

Phytopatogenic fungi cause severe economic losses worldwide. *Rhizoctonia solani* Kühn is a pathogenic fungi affecting several crops, controlled mainly by agrochemicals. Biological control has arisen as another option for managing this pathogen. In this study, we evaluated the biocontroller potential of five orchid mycorrhizal fungi (OMF) isolated from terrestrial Chilean orchids on *R. solani*. We compared the biocontroller effect of these OMFs with that of *Trichoderma harzianum* Rifai in an *in vitro* dual culture experiment. We found that *R. solani* can be controlled *in vitro* by OMF isolated from native orchids. The OMF isolated from *Chloraea virescens* and *C. lamellata* showed the best biocontroller results, which were similar, or even higher, than with *T. harzianum*. Thus, OMFs could become a relevant alternative for the integral control of *R. solani*, contributing to the reduction in the use of agrochemicals in crops.

Keywords: biological control, *Ceratobasidium*, dual culture, orchid fungi, pathogenic *Rhizoctonia*.

RESUMEN

Los hongos fitopatógenos causan severas pérdidas económicas a escala global. *Rhizoctonia solani* Kühn es un hongo fitopatógeno que afecta varios cultivos, controlado principalmente por agroquímicos. El control biológico ha aparecido como otra opción para el manejo de este patógeno. En este estudio evaluamos el potencial biocontrolador de cinco hongos micorrícicos orquidioides (OMF), aislados de orquídeas chilenas terrestres, sobre *R. solani*. Comparamos el efecto biocontrolador de estos OMF con el de *Trichoderma harzianum* Rifai en un experimento *in vitro* de cultivo dual. Encontramos que *R. solani* puede ser controlado *in vitro* por OMF aislados de orquídeas nativas. Los OMF aislados de *Chloraea virescens* y *C. lamellata* mostraron los mejores resultados de biocontrol, los que fueron similares, o incluso superior, al de *T. harzianum*. De esta manera, los OMF podrían convertirse en una alternativa relevante para el control integral de *R. solani*, contribuyendo a la reducción del uso de agroquímicos en cultivos.

Palabras clave: *Ceratobasidium*, control biológico, cultivo dual, hongos orquidioides, *Rhizoctonia* patógena.

INTRODUCTION

Soil phytopatogenic fungi cause severe economic losses worldwide (Savary *et al.* 2012, Syed Ab Rahman *et al.* 2018). They negatively impact agriculture and forestry by reducing plant yield and survival (Agrios 2005, Oerke 2006). These fungi can attack crops and nurseries producing rot, damping-off, and blight, among other diseases (Lamichhane *et al.* 2017, Landis 1989). *Rhizoctonia solani* Kühn is a cosmopolitan phytopathogenic fungus that affects several economically important crops (Ma *et al.* 2013, Otero *et al.* 2013, Sneh *et al.* 1991, Wigg & Goldman 2020). Its control has been largely based in the use of agrochemicals, with the consequent detrimental effect on human health and the environment (Gunnell *et al.* 2007, Mir-Tutusaus *et al.* 2014, Park *et al.* 2020). In Chile, *R. solani* is usually controlled with methyl bromide (SAG 2020), also known as bromomethane, a very dangerous agrochemical that is hazardous for humans (i.e. Barry *et al.* 2012, Park *et al.* 2020) and damages the ozone layer (Ristaino & Thomas 1997). Moreover, it is included in the Montreal Protocol and Clean Air Act and its use is restricted in developed countries (EPA 2019, WHO 1995).

Biological control of pathogenic fungi has been proposed as an effective control method with low impact on human health and the environment (Berg 2009, Romanazzi *et al.* 2016). Biocontrol of *R. solani*, in particular, has been proven as an effective and sustainable method (Barnett *et al.* 2017, Brewer & Larkin 2005, Sahu *et al.* 2020). For example, *Trichoderma harzianum* Rifai, a commonly used biocontroller fungi, can be used effectively against *R. solani* (Benítez *et al.* 2004, Sood *et al.* 2020, Strashnow *et al.* 1985). Additionally, isolates of non-pathogenic *Rhizoctonia* strains with binucleate hyphae can also have a high biocontroller effect on *R. solani* (Burns & Benson 2000, Mosquera-Espinosa *et al.* 2013, Otero *et al.* 2013, Sneh *et al.* 2004). Some *Rhizoctonia* strains are naturally found in symbiosis with orchids (Herrera *et al.* 2017, 2019, Otero & Bayman 2009, Pereira *et al.* 2014, 2018). The symbiosis is essential for seed germination (Otero & Bayman 2009). Additionally, this mutualistic association can also occur in adult plants, where pelotons (clusters of hyphae) can be found inside parenchyma cells of the roots (Pereira *et al.* 2014, 2018). Species belonging to the form-genus *Rhizoctonia* D.C. are a group of filamentous fungi with a non-spored imperfect state, known as the *Rhizoctonia* anamorph (González *et al.* 2006). These fungi that associate typically with orchids are called orchid mycorrhizal fungi or OMF. In Chile, studies on OMF are still limited to a few orchid species and sites (Atala *et al.* 2015, Durán *et al.* 2007, Herrera *et al.* 2019, Pereira *et al.* 2014, Steinfort *et al.* 2010) and their potential as biocontroller of pathogenic fungi is unknown.

We hypothesize that OMF isolated from native Chilean orchids can be used as biocontrollers of *R. solani*. To test this, we evaluated the *in vitro* potential of OMF naturally associated to the roots of the Chilean orchids *Chloraea lamellata* Lindl., *C. virescens* (Willd.) Lindl., *Codonorchis lessonii* (Brongn.) Lindl. and *Gavilea longibracteata* (Lindl.) Sparre ex Navas as biocontrollers of the pathogenic fungi *Rhizoctonia solani*. We compared their putative biocontroller effect with that of *T. harzianum*, a known biocontroller of *R. solani*.

MATERIAL AND METHODS

Isolation of studied fungal strains

With the aim of isolating possible biocontroller OMFs, roots of adult plants of the terrestrial orchid species *Chloraea lamellata*, *C. virescens*, *Codonorchis lessonii* and *Gavilea longibracteata* were collected from the field in different sites in Southern Chile. During spring (Sep-Oct) 2014, we sampled three plants per site and two roots per plant. Plant were collected from the following sites: 1) Road to Antuco Volcano, Bío-Bío Region; *C. lamellata* (Cl) (37° 25' 44" S - 72° 51' 27" O), *C. lessonii* (Cle1) (37° 21' 31" S - 71° 51' 33" W), and *G. longibracteata* (Gl) (37° 21' 31" S - 71° 51' 33" O), 2) Pucón airdrome, Araucanía Region; *C. virescens* (Cv) (39° 17' 40" S - 71° 54' 25" O), and 3) Villarrica National Park, Araucanía Region: *C. lessonii* (Cle2) (37° 23' 20'' S - 71° 24' 22'' W). In site 1 and 2, no license is required for collecting biological samples according to Chilean law. In site 3 we had verbal authorization by CONAF staff. Root sampling does not kill the plants if done carefully, according to our previous experience.

Root segments washed with distilled water were cleared of rhizodermis and then transversal sections of approximately 0.4-0.5 mm were cut and sterilized by immersion in serial ethanol baths (Otero & Bayman 2009). Samples with presences of pelotons in their root cortical cells (Fig. 1a) were sown in the laminar flux chamber into 4 cm Petri dishes with potato dextrose agar (PDA) medium, previously autoclaved at 121 °C and 1 atm for 20 min. The medium pH was set to 5.8. Growing hyphae from the root segments were isolated and grown in three separate Petri dishes with PDA medium (triplicated) and incubated for 10 days at 24 °C in the dark. Growing fungi were observed with a light microscope (Olympus CX30, Tokio, Japan) and digital images were taken with an attached digital camera (Moticam 2000, Motic Inc., Hong-Kong, China). We observed hyphae type, branching, hyphae constrictions, and presence of monilioid cells for preliminary identification (see Fig. 1b). All isolated OMF showed *Rhizoctonia*-like traits. The OMFs

isolated from *C. virescens* and *C. lessonii* were also identified using standard molecular techniques based on amplification of the internal transcriber spacer by using the ITS1 and ITS4 primers (Bidartondo *et al.* 2004, Gardes & Bruns 1993, Taylor & McCormik 2008, White *et al.* 1990). The sequence were submitted to the GeneBank database (Cv: accession number MN199626, Cle1: KT003600, Cle2: KT003605), and were identified by BLAST analysis as *Ceratobasidium* sp. with 100% similitude (Table I).

Isolation of *R. solani* (Rs) was done *in vitro* in the laboratory directly from an infected potato (*Solanum tuberosum*) (Table I). In the laminar flux chamber, we extracted small fragments of the epidermis of a potato with evidence of sclerotia of *R. solani*. These fragments were superficially sterilized with ethanol previous to the extraction. Fragments were put in 4 cm Petri dishes with agar-water (AW) medium and were incubated at 24 °C for 10 days Pure colonies were then transferred to PDA in three separate plates (see Fig. 1c). Additionally, we obtained a previously isolated strain of the known biocontroller *T. harzianum* (Th) from the Laboratorio de Biorremediación, Facultad de Ciencias Agropecuarias y Forestales, Universidad de la Frontera, Temuco, Chile. This fungi was previously identified using the ITS region of the 18S rRNA gene (GenBank accession number HG940501), showing 100% similarity with a soil *T. harzianum* strain (Table I).

Thus, we used 7 fungal strains in our study (Table I), including *R. solani*, *T. harzianum*, and 5 OMFs isolated from terrestrial Chilean orchids.

Radial growth and dual culture experiment

The antagonistic capacity of the isolated OMF and *T. harzianum* on the pathogenic *R. solani* was tested using the dual culture technique in 9 cm Petri dishes containing PDA medium. We took 5 mm disc of agar from plates containing actively growing mycelia from the fungal species to be tested. These disks were put in the surface of the 9 cm Petri dishes in the outer edges, 1 cm away from the border. At each end of the plate we put one antagonist; on one end, a disk with *R. solani* and, in the other end, a disk with the OMF strain to be tested or with *T. harzianum* following a previously reported protocol (Guédez *et al.* 2012, Howell 2003). Additionally, as a control to evaluate radial growth when alone, we had Petri dishes with disks of agar with mycelium of each tested fungi (*R. solani*, all OMFs and *T. harzianum*) separately (only one fungus per plate). We had 4 separate plates per treatment. Plates were incubated at 24 °C ± 1 °C in the dark for 3 days. We measured radial growth (mm) every 36 h with a ruler. We then evaluated the percentage of inhibition of radial growth (PIRG; Ezziyyani *et al.* 2004, Guédez *et al.* 2012) as follow:

$$PIRG\ (\%) = \left[\frac{R1 - R2}{R2}\right] \times 100$$

Where R1 is the radius of the colony of the pathogen growing alone, and R2 is the radius of the pathogen in the dual culture, growing with the antagonist.

The overall differences in radial growth and PIRG of the different fungal strains were analyzed using a Kruskal-Wallis test. Differences between each strain was analyzed *a posteriori* with the Tukey test. All statistical analyses were conducted using JMP 7 software (SAS Institute Inc., NY, USA).

Table I. Fungal strains used in this study./ Cepas de hongos usadas en este estudio.

Source	Identity	Abbreviation in this study	Genebank accession n°	Reference
Roots of *Chloraea viresces*	*Ceratobasidium* sp.	Cv	MN199626	Pereira *et al.* 2020[(1)]
Roots of *Chloraea lamellata*	Rhizoctonia-type	Cl	-	-
Roots of *Gavilea longibracteata*	Rhizoctonia-type	Gl	-	-
Roots of *Codonorchis lessonii* (site 1)	*Ceratobasidium* sp.	Cle1	KT003600	Pereira *et al.* 2018[(2)]
Roots of *Codonorchis lessonii* (site 2)	*Ceratobasidium* sp.	Cle2	KT003605	Pereira *et al.* 2018[(2)]
Sclerotia from infected potato	*Rhizoctonia solani*	Rs	-	-
Previously isolated laboratory sample	*Trichoderma harzianum*	Th	HG940501	-

[(1)]Pereira G, Herrera H, Arriagada C, Cid H, García JL, Atala C. 2020. Controlled mycorrhization of the endemic Chilean orchid Chloraea gavilu (Orchidaceae). Plant Biosystems doi: 10.1080/11263504.2020.1801875

[(2)]Pereira, G., Suz, L. M., Albornóz, V., Romero, C., García, L., Leiva, V., & Atala, C. (2018). Mycorrhizal fungi associated with *Codonorchis lessonii* (Brongn.) Lindl., a terrestrial orchid from Chile. *Gayana Botánica*, 75(1), 447–458.

RESULTS

The isolated colonies of OMF showed characteristics typical of the form-genus *Rhizoctonia*. All isolated fungi had right-angle branching, hyphae constriction in the septum, and monilioid cells (Fig. 1b).

Radial growth differed between studied fungi (Kruskall-Wallis test, $p < 0.05$). When grown alone, the radial growth of the OMF isolated from *C. virescens* (Cv) and *T. harzianum* (Th) was higher compared to the other OMF (Fig. 2a; Tukey test, $p < 0.05$), but similar to *R. solani* (Fig. 2a; Tukey test, $p > 0.05$). This OMF was identified as *Ceratobasidium* sp. (Table I), and showed 100% similarity to mycorrhizal fungi isolated from other terrestrial orchids (See Pereira *et al.* 2020 for further detail).

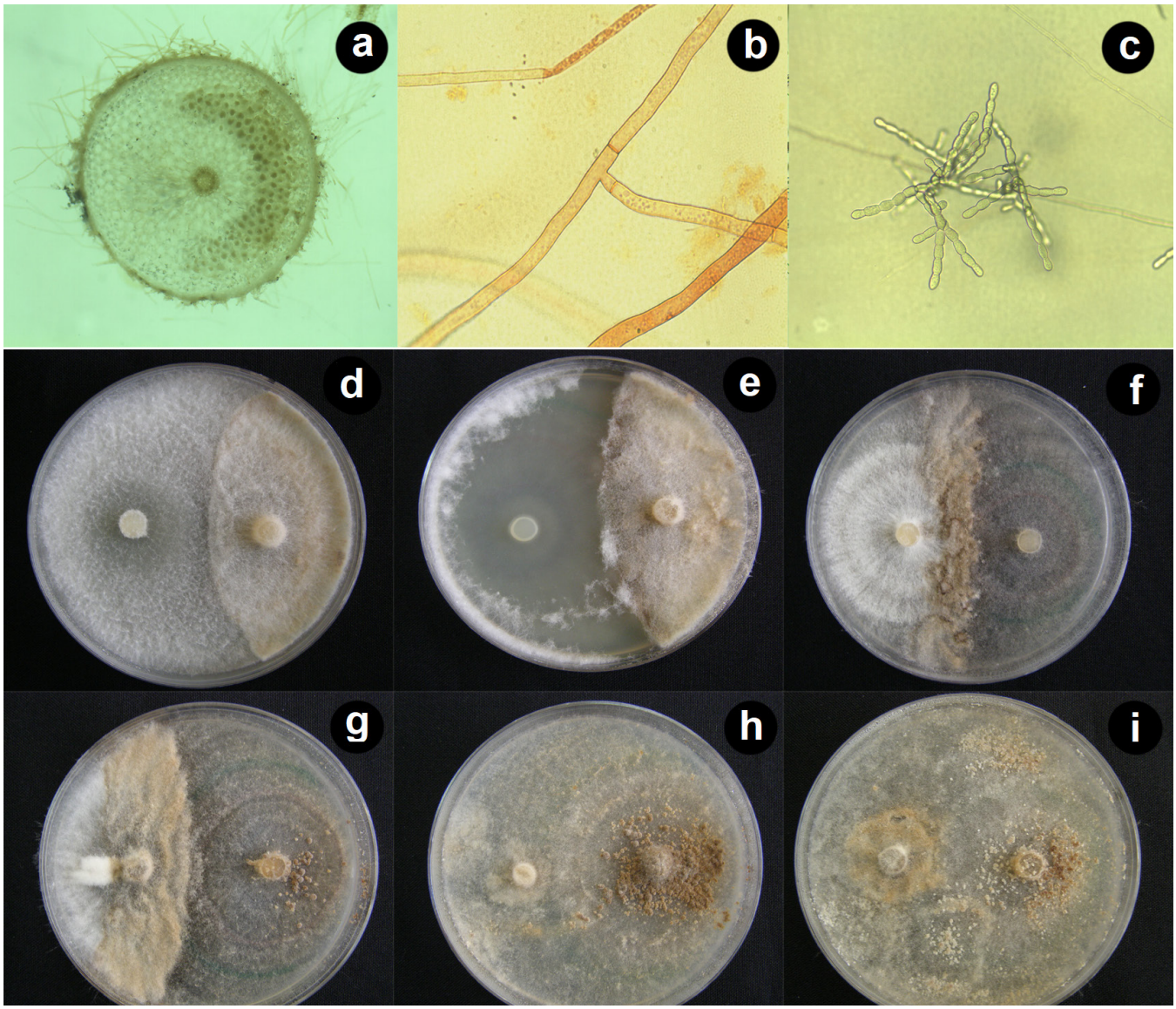

Figure 1. a: Root section with pelotons in the cortical cells. b: Hyphae with simple septum and straight-angle branching in isolated OMF (100×). c: *Rhizoctonia solani* hyphae and moniloid cells (40×). d: Dual culture (DC) of OMF isolated from *Chloraea virescens* and *R. solani*. e: DC of *Trichoderma harzianum* and *R. solani*. f: DC of OMF isolated from *C. lamellata* and *R. solani*. g: DC of OMF isolated from *Codonorchis lessonii* 1 and *R. solani*. h: DC of OMF isolated from *C. lessonii* 2 and *R. solani*. i: DC of OMF isolated from *Gavilea longibracteata* and *R. solani*. / a: Secciones de raíces con pelotones en las células corticales. b: Hifas con septo simple y ramificación en ángulo recto en el OMF (hongo micorrícico orquidioide) aislado (100×). c: Hifas de *Rhizoctonia solani* y células monilioides (40×). d: Cultivo dual (DC) de los OMF aislados de *Chloraea virescens* y *R. solani*. f: DC de OMF aislados de *C. lamellata* y *R. solani*. g: DC de OMF aislados de *Codonorchis lessonii* 1 y *R. solani*. h: DC de OMF aislados de *C. lessonii* 2 y *R. solani*. i: DC de OMF aislados de *Gavilea longibracteata* y *R. solani*.

In the dual cultures, the colonies of Cv and Th made contact with the colonies of *R. solani* after 3 days (Fig. 1d, 1e). The colonies of all other OMF (see Table I) made contact with *R. solani* one day after that (Fig. 1f-1i). PIRG varied significantly between fungal strains (Kruskall-Wallis test, $p < 0.05$). Cv showed a higher PIRG of *R. solani* compared to Th, or the other tested OMF (Fig. 2b; Tukey test, $p < 0.05$). This strain reached almost a 50% of inhibition of the pathogen (Fig. 2b). The second-best antagonistic fungi in inhibiting *R. solani* was Th with a PIRG of 30.5% (Fig. 2b), although it did not significantly differ from some of the tested OMF (Fig. 2b; Tukey test, $p > 0.05$). The lowest PIRG was evidenced for the OMF isolated from *C. lessonii* 2 (Cle2) and *G. longibracteata* (Gl). These treatments resulted in a significantly lower PIRG compared with the other tested fungi (Tukey test, $p < 0.05$). Furthermore, in the dual cultures, plates with these species were completely overgrown by the pathogen in subsequent days (Fig. 1h, 1i).

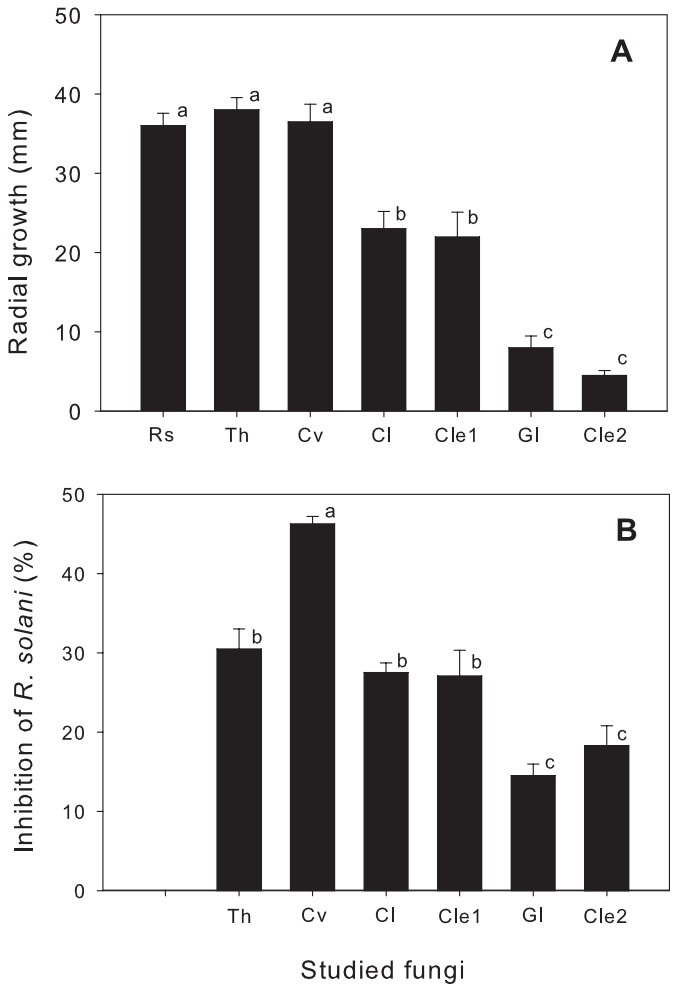

Figure 2. a: Radial growth (mm/d) of the different fungal strains used in this study in PDA medium. Different letters indicate statistically significant differences (Tukey test, $p < 0.05$). b: Percentage of inhibition of radial growth (PIRG) of *Rhizoctonia solani* growing with different antagonistic fungi in dual culture. Different letters indicate statistically significant differences between columns (Tukey test, $p < 0.05$). / a: Crecimiento radial (mm/d) de las diferentes cepas fúngicas usadas en este estudio en medio PDA. Letras diferentes indican diferencias estadísticas significativas (Tukey test, $p < 0.05$). b: Porcentaje de inhibición del crecimiento radial (PIRG) de *Rhizoctonia solani* creciendo con diferentes hongos antagonistas en cultivo dual. Letras diferentes indican diferencias estadísticas significativas entre columnas (Tukey test, $p < 0.05$).

DISCUSSION

We found that OMF growing associated with the native Chilean orchids *C. virescens* and *C. lamellata* show potential as biocontrollers of *R. solani in vitro*. The obtained inhibition (PIRG) of *R. solani* with those OMF surpassed or equaled that of *T. harzianum*. Previous studies have shown that binucleate *Rhizoctonia* OMF have been successful in controlling phytopathogenic fungi like *R. solani*, *Fusarium* sp., *Phytophthora* sp., and *Pythum* sp., among others (Cardoso & Echandi 1987, Carvalho *et al.* 2015, González *et al.* 2006, Jiang *et al.* 2016, Mosquera-Espinosa *et al.* 2013). The mechanism for their biocontrolling effect is still unknown, but could be related to an induced systemic resistance, production of antibiotics, mycoparasitism, or competition for resources as seen in other biocontrolling fungi (see Chaibub *et al.* 2016, Sood *et al.* 2020, Verma *et al.* 2007, Villajuan-Abgona *et al.* 1996). Other non-pathogenic *Rhizoctonia*-like strains have been found to extensively colonize plant tissues and increase the production of phenolic compounds, pectic substances, phytoalexins and several proteins that inhibit the growth of phytopathogenic fungal strains (Jiang *et al.* 2016). Thus, the strains tested in our study could have a similar induced resistance mechanism for their biocontrol effect. Studies in tropical South America show that orchid fungi can be relevant biocontrolling agents of valuable crop fungal diseases, contributing to the reduction in the use of agrochemicals in the currently affected tropic ecosystem (Mosquera-Espinosa *et al.* 2013, Otero *et al.* 2013). As orchids can form mycorrhizae with potential phytopathogenic and saprophytic fungal strains, they must possess strong molecular defense mechanisms against fungal attacks, which joined to the potential of endophytic microorganisms to reduce fungal growth, may serve as key mechanisms in the development of orchid mycorrhizae (Jiang *et al.* 2019, Valadares *et al.* 2014). Such restriction potential has been detected even in bacteria associated with native

mycoheterotrophic species, including orchids (Herrera *et al.* 2020a, 2020b), suggesting that microorganisms associated to mycoheterotrophic species can be a key source of biocontrolling agents of natural soil-borne phytopathogens such as *R. solani*.

If the OMF isolated from native Chilean orchids are to be considered as biocontrolling agents of *R. solani ex vitro*, it needs to be tested if they show any pathogenic response in plant hosts different from orchids. Isolates of binucleate *Rhizoctonia* that are usually root endophytes are able to establish mutualistic or pathogenic relationships with their hosts depending on their host's susceptibility (Sen *et al.* 1999). The health status of the host plant and environmental conditions when evaluating their potential pathogenic effect should be taken into account (Bayman & Otero 2006). For example, despite the fact that OMF inoculation resulted in pathogenicity symptoms in rice plants, these were less severe compared to the produced by *R. solani* (Mosquera-Espinosa *et al.* 2013, Otero *et al.* 2013).

In the current context of global demands for the reduction of pesticides and agrochemicals (i.e. United States Environmental Protection Agency 2017), the identification of new biocontrol agents is essential for a sustainable agricultural practice (Berg 2009, Es-Soufi *et al.* 2020). Native biodiversity can be a constant source of these new bioresources (i.e. Yadav *et al.* 2017), hence the relevance of their conservation and study. Knowing the diversity and biological characteristics of OMF isolated from native Chilean orchids could be essential for the advancement of new biocontrolling fungi for agriculture and forestry, two relevant economic sectors in Chile (ODEPA 2020, Chilean government), with the associated advantages to traditional methods like the added value of organic food production and complying with the increasing environmental demands of international markets like Europe.

The applied use of microorganisms for the biological control of plant pathogens has caught up recent attention due to the increasing incidence of pathogens resistant to several pesticides and the restrictions for their use in many countries. The use of biocontrollers has several merits over agrochemicals, including being more specific, cheaper, more environmentally friendly. Its value also resides in been an effective plague control with a mid- to long-term action, and having a low environmental risk compatible with a sustainable production, which likely will become increasingly important in the near future of agriculture and forestry.

In conclusion, orchid fungi isolated from terrestrial Chilean orchids can effectively reduce the growth of *R. solani* in dual culture, showing high potential as biocontrollers of this pathogenic fungi. Some OMF strains performed even better than *T. harzianum*, a known and common biocontroller, in the dual culture experiment. Thus, these orchid fungi show a high biotechnological potential as biocontrollers of *R. solani*. However, further field studies are required to confirm their applicability.

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