UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Programa de Doctorado en Ciencias en Recursos Naturales

ADAPTATION AND TOLERANCE MECHANISMS DEVELOPED BY MYCORRHIZAL ORCHIDS IN A METAL(LOID) CONTAMINATED SOIL

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DOCTORAL DEGREE IN NATURAL RESOURCES SCIENCES

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TEMUCO-CHILE

2017

ADAPTATION AND TOLERANCE MECHANISMS DEVELOPED BY MYCORRHIZAL ORCHIDS IN A METAL(LOID) CONTAMINATED SOIL

Esta tesis fue realizada bajo la supervisión del Dr. Cesar Arriagada Escamilla, perteneciente a la Facultad de Ciencias Agropecuarias y Forestales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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ACKNOWLEDGMENTS

.My thesis project was accomplished with the aid of many people to whom I am very grateful. Specially, I would like to thank my advisor **Dr. César Arriagada Escamilla** for his guidance and for the fruitful discussions we had regarding the improvement of the results of my Doctoral Thesis, and for some valuable life tips. I would also like to thank **Dr. Rafael Valadares** for his dedicated and skilled help both in the theory and in the laboratory. His support has been much appreciated, especially when I was in Sao Paulo, the place in which I felt impressed by and fascinated with orchid mycorrhizas. Thanks to **Yoav Bashan** for his kind help with manuscript preparation and submissions. Finally, I would like to thank the **Doctoral Program in Natural Resources Sciences** for their support to develop and fulfil my Doctoral Thesis.

Thanks to **Dr. Fernando Borie**, **Dr. Pablo Cornejo** and **Dr. Claudia Castillo**, my thesis evaluation committee for their comments and support for improving this Doctoral Thesis. And also thanks to **Dr. Domingo Contreras** for his invaluable help during plant sampling. I am also grateful to have been part of the "**Laboratorio de Bioremediación**", which gave me the opportunity to participate and develop my undergraduate and Doctoral Thesis. I would especially like to thank **Dr. Leonardo Almonacid** and **Dr. Alejandra Fuentes**, **Claudio Lagos**, **Javiera Soto** and **Javier Ortiz**; our meetings were always very rewarding both in a professional and in a social sense. Furthermore, I would like to thank the **Vale Research Institute for Sustainable Development** (Belem, Brazil) for the technical support of during my doctoral internship.

Finally, I am very grateful to friends and family for their support, patience and help when needed. To **Lujan**, **Patricia**, **Katherine**, **Mathias, Cristobal**, **Luz, Lucas** and **Martina**… your love and patience have been priceless… than you!!!!

Funding was provided by the **FONDECYT project n°1130662** and the **CONICYT fellowship n°21130491** to H. Herrera.

THESIS OUTLINE

Orchidaceae is one of the most numerous families among flowering plants, accounting roughly by 35,000 species distributed in all terrestrial ecosystems with the exception of extremely hot/cold environments such as deserts, Arctic and Antarctic poles, and the highest mountain ranges on Earth. Orchids are associated with free living fungus of the polyphyletic *Rhizoctonia*-like fungi complex, which is an artificial fungal grouping of diverse unrelated species that share anatomical characteristics during their anamorphic stages. These mycorrhizal fungi include genera of diverse nature (e.g. saprophytic, pathogenic, and ectomycorrhiza) that do not develop their normal infectious processes in symbiotic orchids. Chilean Orchidaceae includes at least 70 species, many of which are endangered, because human activities and climate change are altering their distribution range. Orchid life cycle starts when the seeds are dispersed into the environment; then, a compatible mycorrhizal fungus must be associated with orchid seeds, providing organic nutrition to the embryo for advancing to further developmental stages. Briefly, the fungus-orchid seed interaction is extremely specific and mandatory for orchids; under natural conditions without appropriate fungal infection during seed germination, there is no orchid establishment.

Studies in orchid mycorrhizas are limited, and they are mainly focused on the isolatation and identification of mycorrhizal fungi, with many unexplored issues (molecular and ecological) that will improve our knowledge in this especial mycorrhizal symbiosis. Nowadays, there is no knowledge about the symbiotic mechanisms developed by orchids, which allow plants to colonize diverse adverse environments. Understanding mycorrhizal compatibility of endemic orchids performance under the restrictive conditions of metal(loid) polluted soils will improve our knowledge about the

mechanisms developed by terrestrial orchids to fit into diverse hostile environments, such as rock surfaces, terrestrial oil spills and wildfire affected soils.

In **Chapter I,** general introduction, hypotheses, general, and specific objectives are presented. The general objective of this Doctoral Thesis was **to study and characterize some adaptation mechanisms developed by mycorrhizal orchids in order to achieve plant establishment in a metal(loid)s contaminated soil**.

Chapter II corresponds to a review entitled "**Plant host or fungal host? Mycoheterotrophy in orchid mycorrhizal symbioses**". In this manuscript, a background of studies regarding orchid mycorrhizas dynamics was presented, summarizing the research that has been done on orchid mycorrhizal symbioses related to nutrient transfer, gene expression, proteomics, and metabolic fluxes between orchids and mycorrhizal fungi, highlighting the ecological role of underground mycorrhizal networks established at different trophic stages.

Chapter III corresponds to the manuscript entitled "**Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in South Central Chile**". In this chapter, an analysis of root fungal endophytes was carried out in order to identify the most common mycorrhizal fungi associated with some endemic orchids, in specific Chilean ecosystems of south-central Chile. Mycorrhizal fungi from *Bipinnula fimbriata*, *Chloraea chrysantha*, *Chloraea gavilu* (Constitución), *Chloraea bletioides*, *Chloraea crispa*, *Chloraea longipetala*, *C. gavilu* (San Clemente), and *Chloraea grandiflora* were isolated, cultured, and identified. Furthermore, their ability to induce seed germination was assessed in symbiotic germination trials. Main results showed Tulasnellales as the main mycorrhizal fungi associated with the sampled orchids. These mycorrhizal fungi have the non-specific

ability to promote seed development of different *Chloraea* spp. Data obtained from these experiments are invaluable for the symbiotic conservation of endemic orchid flora.

Chapter IV corresponds to the manuscript entitled "**Evidence of the orchid-mycorrhizal fungi (*Bipinnula fimbriata-Tulasnella calospora*) symbiosis adaptation in a metal(loid) polluted soil, determined by proteomic analysis and organic acid exudation**". In this chapter, proteomics and root exudation were used to unravel physiological changes in the wild orchid *Bipinnula fimbriata* established in a soil contaminated with metal(loid)s, and ascertain whether the mycorrhizal fungi *T. calospora* affects root metabolic responses. Higher phosphorus and heavy metal accumulations were detected inside mycorrhizal roots growing under stress. In mycorrhizal and non-mycorrhizal roots, a differential protein accumulation was detected, which points out different metabolism strategies on colonized roots. The *B. fimbriata* proteome shows that under metal(loid) stress, roots tend to facilitate mycorrhizal colonization in order to alleviate metal(loid)s negative effects in plants. Substantial part of these analyses was performed in the Vale Technological Research Institute for Sustainable Development (Belem, PA, Brazil).

Chapter V corresponds to the manuscript entitled "**Dynamics of mycorrhizal associations of endemic orchids under soil contaminated with metal(loid)s".** This chapter summarizes all experiments that were carried out, to better understand the mycorrhizal interaction dynamics for the endemic orchid *B. fimbriata*, adapted to a metal(loid)s contaminated soil, exploring molecular and mycorrhizal processes in naturally established plantlets, specifically, to test the symbiotic interaction under *in-vitro* conditions. First, an *in-vitro* assay to test the potential of mycorrhizal fungi to promote seed germination was developed, isolating mycorrhizal fungi from plantlets

developed in metal(loid) contaminated soil, and assessing their ability to improve growth and differentiation of orchid protocorms in a contaminated media. Second, *B. fimbriata* root proteomic regulations were evaluated in one of their natural distribution environments, in order to depict changes in mycorrhizal and non-mycorrhizal single plant roots. Finally, a set of degenerate primers was designed in order to study antagonistic and mutualistic markers in orchid roots developed in metal(loid) contaminated soil. Main results showed that the symbiotic interaction between *T. calospora* and *B. fimbriata* seeds, under metal(loid)s stress, improve protocorm growth and embryo differentiation. Furthermore, we showed that the restrictive conditions of Puchuncaví soil stimulate mycorrhizal fungi colonization, forming condensed infected areas, with high rates of degraded pelotons, which are a potential source of mycorrhizal fungi-derived nutrients for the orchid, as well as a physical place to store metal(loid)s. It was also demonstrated that orchids have different metabolism for mycorrhizal and non-mycorrhizal roots; specifically, mycorrhizal roots act as a source of mycorrhizal fungi-derived nutrients, provided that there are some transitory control mechanisms to avoid fungal spread to vital plant tissues. Gene expression, evaluated by RT-PCR, suggested that some of the mutualistic markers were visibly over-expressed in mycorrhizal roots similarly to other mutualistic interactions. Amplicon intensity stimulation of pathogenic markers may derive from the enhancement of mycorrhizal fungi colonization, to avoid the infection of vital plant tissues. All these analyzed issues allow understanding unexplored aspects of orchid mycorrhizal interactions, at local and global level. It is crucial to mention that all mycorrhizal fungi were stored and saved as a measure to reintroduce orchid seeds and fungi to their natural environments, which is an especial strategy to avoid extinction of some endangered endemic orchids, such as *B. gabriel* and endemic orchids from La Araucania Region (**Figure 1**). All these subjects are currently

under preparation and they will be submitted to different scientific journals, specified in **Chapter V**.

Finally, **Chapter VI** corresponds to general discussion, conclusions, and future directions that must be taken in orchid mycorrhizal researches.

In summary, it was concluded that endemic orchids preferably associated with mycorrhizal fungi from *Tulasnella* genus. This association is broadly distributed between the Andean regions and is also present in degraded ecosystems such as the case of the Ventanas industrial complex, Puchuncaví valley, Region de Valparaiso. At this contaminated ecosystem, orchid mycorrhizal fungi play a crucial role to promote plant establishment. Specifically, *in-vitro* media protocorm growth was achieved even under metal(loid) stress. Adverse soil conditions of the Puchuncaví Valley contaminated soil affect mycorrhizal performance in symbiotic established plantlets, changing the dynamic of mycorrhization processes, as well as root metabolic responses (proteomics and root exudates).

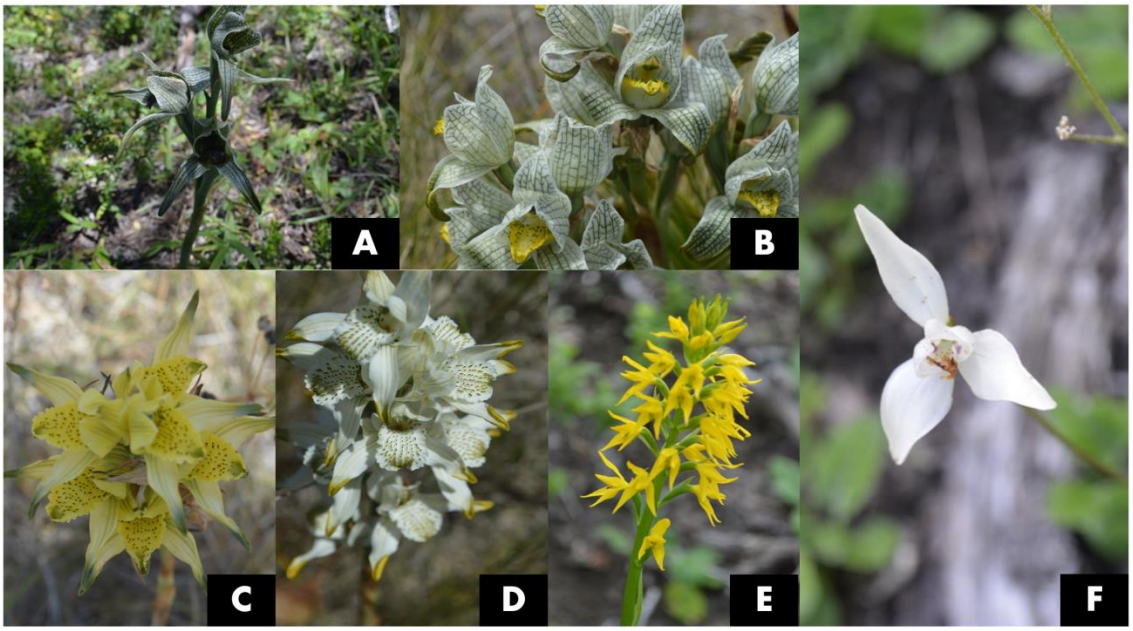

Figure 1. Endemic orchids from Región de La Araucanía colonizing a segment of Cordillera Las Raíces in Southern Chile. (**A**) *Chloraea grandiflora*, (**B**) *Chloraea maguellanica*, (**C**) (**D**) *Chloraea* sp. (hybrids), (**E**) *Gavilea lutea*, and (**F**) *Codonorchis lessonii*.

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CHAPTER I

General introduction, hypothesis and objectives

1.1 GENERAL INTRODUCTION

Orchid mycorrhizas (OM) are particular relationships that orchids establish specifically with different fungal clades (Otero et al., 2011). The term *Mycorrhizae* involves a mutualistic relationship in which both symbionts obtain benefits from each other, based in a complementary molecular mechanism (Perotto et al., 2014). These mechanisms have been widely studied in arbuscular mycorrhiza symbioses (AM), although little is known about OM (Oldroyd et al., 2009; Valadares, 2014). In mycorrhizal associations, different plant families establish a symbiosis with several fungal groups, with variable degrees of specificity (Bending et al., 2006). In mutualistic symbioses (plant-fungi), a soil fungus contributes with mineral nutrition to a plant and the plant contributes with photosynthetically fixed carbon back to the fungus through root system (Smith & Read, 2010). The dynamic of the mycorrhizal symbiosis is different in OM, in which orchid seeds must be infected by an appropriate free living fungus to germinate their tiny seeds (Leake, 1994; Tsai et al., 2008). At seedling stage, orchids are parasites on the fungi, exploiting carbon and other nutrient resources directly from the fungal hyphae (Merckx et al., 2009; Motomura et al., 2010). These parasitic interactions are broken-up when the first green leaf emerges and the orchid turns partially autotrophic (Barrett et al., 2010). Nowadays, it is unclear whether orchids establish a mutualism (Cameron et al., 2008; Cameron et al., 2006) or a mycophagous interaction in which fungi are "parasitized by the orchid" (Cameron et al., 2009; Rasmussen & Rasmussen, 2009). Mycoheterotrophy is essential for orchids and the fact they have developed it is certainly an ecological advantage that helps improving their nutritional uptake (Leake, 1994; Yam & Arditti, 2009).

OMs are symbiotic interactions found only in Orchidaceae family, showing particular ecological characteristics, because orchid seeds must be infected by an

appropriate fungus to germinate their small seeds. Orchids obtain nutrients from the fungus, but there is little evidence about the reverse process (Cameron et al., 2006; Rasmussen, 1995; Rasmussen & Rasmussen, 2009). Studies in OM symbioses are mainly focused in isolating and characterizing root fungal endophytes, and exploring the ability that these fungi have to germinate orchid seeds (Tešitelová et al., 2012; Valadares et al., 2011), which is known as potential and ecological specificity, respectively (Steinfort et al., 2010). Certainly, identifying mycorrhizal fungi is a topic of great importance for designing strategies to reintroduce endangered species in their natural environments (Brundrett, 2002; Cozzolino et al., 2006). Currently, there is still lack of knowledge about issues such as the mechanisms that allow selection and attraction of the mycorrhizal fungi and how orchids turn symbiosis into a unilateral relationship, at least during the seed germination stage, known as protocorm (Valadares et al., 2011), when mycoheterotrophic processes are entirely active.

Orchid mycorrhizal fungi are usually Basidiomycetes (Dearnaley, 2007) and belong to the genera *Rhizoctonia*-like fungi (Otero et al., 2007), which is an artificial grouping of unrelated fungi based on anamorphic life stage (*Ceratobasidium*, *Sebacina* and *Tulasnella*) (Otero et al., 2002). Many of these fungi are usually saprotrophs living in soil organic matter, whereas some groups form ectomycorrhiza with trees, and others are plant parasites (Yamato et al., 2005).

With the exception of fully mycoheterotrophic orchids, the rest of orchid species develop photosynthetic tissues, after plantlet growth from protocorm; hence, orchids may transit through three main trophic stages across their life cycle: (1) a stage fully dependent on the mycorrhizal fungus (the mycoheterotrophic stage), in which some orchids remain achlorophyllous throughout their adult phase (Taylor & Bruns, 1997); (2) a transition stage in which orchids are acquiring the photosynthetic ability, turning

them partially mycoheterotrophic (Zimmer et al., 2008); and (3) the fully independent stage, when orchids obtain carbon by photosynthesis, hence, the interaction is stopped until the environmental conditions induce the symbiosis re-activation (autotrophic stage) (Rasmussen & Rasmussen, 2009).

Both, climate change and the economic ornamental potential that some orchids have are negatively affecting orchid biodiversity and conservation. Many orchid species are in danger of extinction due to growing environmental pollution and segregation of orchids only to protected areas (Schödelbauerová et al., 2009). For this reason, to known which mycorrhizal fungi associated with orchids is essential to safeguard endangered species, and thus, to design strategies to promote their reintroduction in their natural environments. Orchids may vary their mycorrhizal preferences under different environmental condition and are often linked to surrounding plant species through their root-fungal symbiont (Seaton et al., 2010; Selosse et al., 2004). Orchids are good at establishing diverse relationships with another kingdom species, so that orchids are in love "from head to toe" with symbioses (Dearnaley et al., 2012; Waterman & Bidartondo, 2008). The underground relationships are relevant for orchids, as orchids base their nutrition on these interactions to obtain nutritional benefits during specific life stages (Kuga et al., 2014).

Chilean Orchidaceae include 70 species, according to Novoa et al. (2015). Recently Pereira et al. (2014) reported that the Chilean Orchidaceae family is represented by 7 genera and 63 species, 27 of which are endemic to the country. To date, all Chilean orchid species are classified as photosynthetic and nothing is known about their nutritional modes, an issue that has been addressed at global level (Bougoure et al., 2014). However, their mycorrhizal fungi are almost entirely unexplored (Herrera et al., 2017; Pereira et al., 2015; Pereira et al., 2014; Steinfort et

al., 2010). Results of the experiments carried out during this Doctoral Thesis, have contributed to improve the knowledge about orchid mycorrhizal association, and there are published in Herrera et al., (2017). Despite Chilean orchid habitats are mainly protected areas, there are some orchids that colonize hostile substrates such as sand, rocks, and understory forest of introduced exotic species. It is expected that some of these tolerant orchids activate mechanisms to obtain nutritional benefits of their fungal partners. Research related to mechanisms that OM develop to tolerate detrimental environmental conditions are scarce (Shefferson et al., 2008).

It is known that all orchids base their life on the initial fungal infection of orchid seeds to develop the mycoheterotrophic organ known as protocorm (Beyrle et al., 1995; Yam & Arditti, 2009). Vestiges of this interaction can be found in orchid radical tissues, in specific structures called pelotons. These structures are the physical place in which orchids restrict the growth of the fungal mycelium, mainly by the production of plant-derived antifungal compounds (Shimura et al., 2007). Besides, pelotons are the main place of nutrient interchange that is possible by the production of hydrolytic enzymes (Bougoure et al., 2014; Kuga et al., 2014). Peloton formation does not mean dynamic mycorrhizal associations because many of them are inactive most of the time (Kristiansen et al., 2004).

Considering the particular characteristic of OM interactions, there are some issues that have not been explored yet, which will provide applicable and valuable ecological information about OM symbioses, such as the particular symbiotic interaction established between *B. fimbriata* and *T. calospora* under the metal(loid) contaminated ecosystem, which was analysed in depth in this Doctoral Thesis.

1.2 HYPOTHESES

As orchids depend on the mycorrhizal fungi for their mineral nutrition to germinate their seeds, all processes related to orchid resistance are developed at this orchid life stage. This juvenile characteristic could be retained in some orchids and may be determinant to colonize degraded or nutrient-impoverished environments. Considering that some orchids have the potential to grow in degraded areas, the following hypotheses have been performed.

The working hypotheses supporting this research are:

- The presence of metal(loid)s will promote metabolic changes in orchids in order to improve root adaptations of the orchid *Bipinnula fimbriata* growing in contaminated soils.
- The germination of orchid seeds in symbiosis with compatible mycorrhizal fungi, under metal(loid)s stress, will enhance the morphological protocorm adaptation to contaminated media.

1.3 GENERAL OBJECTIVE

To study and characterize some adaptive mechanisms developed by mycorrhizal orchids in order to achieve plant establishment in a metal(loid)s contaminated soil.

1.4 SPECIFIC OBJECTIVES

- To know compatibility and specificity between fungi and some endemic orchids.
- To study root exudate patterns of orchid plantlets established in a metal(loid) contaminated soil.
- To assess the expression of some reported mutualistic and antagonistic genes in orchid roots growing in a metal(loid)s contaminated soil.
- To evaluate the effect of mycorrhizal fungi on the establishment of orchid protocorms in a metal(loid)s contaminated media.

CHAPTER II

Plant host or fungal host? Mycoheterotrophy in orchid mycorrhizal symbioses

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Review to be submitted to Planta

Plant host or fungal host? Mycoheterotrophy in orchid mycorrhizal symbioses

2.1 ABSTRACT

Researches related to orchid mycorrhizas have been focused on characterizing root fungal endophytes and phylogenetic relationships established between symbionts. However, there are few studies that analyse nutrient fluxes between orchids and their fungal partners, as well as to identify key molecules for recognition and selection of appropriate mycorrhizal fungi. Despite the Orchidaceae is one of the highest family of flowering plants, little is known about the nutrient dynamics in orchid mycorrhiza. However, the use of OMICs approaches has provided insights about the molecular mechanism involved in the maintenance of the orchid-fungus relationships. In this context, fluxomic approaches have emerged as a new and interesting choice to analyse nutrient movement between symbionts, providing contradictory data about the nutritional dependence of certain orchids with different nutritional modes. Hence, the main discussion if orchid mycorrhiza is mutualistic or if it is a unilateral relationship in favours of the plant (mycoheterotrophy) depend on the way in which nutrients are supplied to the orchid. The aims of this review are to summarize the research that have been done on OM symbioses with emphasis in metabolic fluxes between orchid and fungi and to analyse the ecological role of underground mycorrhizal networks established at different trophic stages.

Keywords: Mycorrhiza, *Rhizoctonia*-like fungi, Orchidaceae, OMICs.

2.2 AN OVERVIEW OF THE ORCHID MYCORRHIZAL PROCESS

Plants with mycorrhizal associations predominate in most natural ecosystems, where they are usually in a mutualistic relationship. The term of *Mycorrhizae* encompass a broad spectrum of interactions, in which plants present endophytes growing inside plant tissues without causing any symptoms of disease (Kasiamdari et al., 2002). The different kinds of mycorrhizas are associated with fungi belonging to aseptate endophytes of the phylum Glomeromycota and septate endophytes of the Ascomycetes and Basidiomycetes. Glomeromycota are present in arbuscular mycorrhizas, whose are presents in the majority of phyla Bryophyta, Pteridophyta, Gymnospermae and in Angiospermae (Smith & Read, 2010). The septate fungi of the other kinds of mycorrhiza (ecto-, ectendo-, ericoid, arbutoid, monotropoid and orchid mycorrhizas) include members of all orders of Basidiomycetes and many Ascomycetes associated with the corresponding plant species (Smith & Read, 2010). In a typical mutualistic mycorrhizal association a soil fungus contributes with mineral nutrition to a plant, and the plant contributes with photosynthetically fixed carbon back to the fungus through root system; however, these processes are different in orchid mycorrhizas (OM) symbioses, in which orchid seeds must be infected by an appropriate free living fungus to germinate their tiny and embryo lacking seeds (Leake et al., 2004; Tsai et al., 2008). In early developmental stages, all orchids are parasites on the fungi, exploiting carbon and other nutritional resources directly from the colonizing hyphae (Valadares et al., 2014; Valadares, 2014). These parasitic interactions are broken-up when the first green leaf emerges and the orchid turns partially autotrophic, controlling directly the interaction processes (Barrett et al., 2010). Nowadays, it is unclear if orchids establish a mutualism (Cameron et al., 2008; Cameron et al., 2006) or if there is a mycophagous interaction in which fungi are “parasitized by the orchid” (Rasmussen & Rasmussen,

2009). The aims of this review are to summarize the researches that have been done on OM symbioses related to nutrient transfer, gene expression, proteomics and metabolic fluxes between orchid and fungi and to analyse the ecological role of underground mycorrhizal networks established at different trophic stages.

Mycorrhizae are trophic symbiotic associations that the majority of vascular plant species establish with defined taxa of fungi. According to Bending et al. (2006) encompasses a number of morphofunctional types, involving different plant families and corresponding fungal groups with variable specificity degrees. The OMs are a group with some peculiarities: it is a form of interaction found only in Orchidaceae family; it is unique because of its morphology; orchids must be colonized by an appropriate fungus to germinate their small seeds; and there is evidence that seeds obtain nutrients from the fungus, but there is little evidence about the reverse process (Cameron et al., 2006; Rasmussen, 1995; Rasmussen & Rasmussen, 2009; Selosse & Roy, 2009). More than sixty years of researches in OM symbioses have improved our knowledge in topics related to the isolation and characterization of root fungal endophytes associated with orchids and the ability that these fungi have to germinate orchid seeds (Tešitelová et al., 2012; Valadares et al., 2011), known as potential and ecological specificity, respectively (Steinfort et al., 2010). Certainly, to identify mycorrhizal fungi is a topic of great importance for designing strategies to reintroduce endangered species or plants that have problems to germinate their seeds under natural conditions (Brundrett, 2002; Cozzolino et al., 2006). Currently, still lack of clarity about issues such as the mechanisms that allow selection and attraction of the mycorrhizal fungi and how orchids turn the symbiosis into unilateral relationship, at least during the seed germination stage to protocorm (Valadares et al., 2011).

Orchids belong to the Orchidaceae family, which is one of the largest in the world with about 700 genera and more than 25,000 species distributed in different growth habitats (Atwood, 1986; Dressler, 2005; Hopper & Brown, 2007). Members of this family can be found growing in three main substrates: (1) soil dwelling, when light is sufficiently available and soil conditions are optimal for plant establishment (terrestrial); (2) on other plants, coating tree branches and stems (epiphytic); and (3) colonizing rock surfaces (lithophytic), where orchids are the main elements of succession between pristine and intervened ecosystems, in most cases (Kottke et al., 2010; Valadares et al., 2011). The ability of orchids to establish a relationship with a broad spectrum of fungi is necessary for completing their life cycle (Rasmussen & Rasmussen, 2009; Smith & Read, 2010), allowing growth during their early development stages and reproduction, since orchid seeds are minuscule; hence, producing a large number of seeds in each floral capsule (Athipunyakom et al., 2004). Orchid seeds lack endosperm, needing a particular nutrition way to sustain growth during the first developmental stages. This nutritional mode is known as mycoheterotrophy (Merckx et al., 2009). In this process seed germination and further growth to protocorm requires the infection of an appropriate mycorrhizal fungus to obtain carbohydrates and other nutrients, until its first green leaf emerges (Leake, 1994; Smith, 1966; Yam & Arditti, 2009). This juvenile characteristic (mycoheterotrophy) can be retained, or can be stopped until environmental conditions stimulate the participation of the fungal associate (Gebauer & Meyer, 2003; Shefferson et al., 2008). Mycorrhizal fungi remain present in pelotons of adult plant roots, probably providing carbohydrates and other simple compounds (Smith & Read, 2010). Fungi that form OM are usually Basidiomycetes (Dearnaley, 2007; Rasmussen, 2002) and belong to the *Rhizoctonia*-like complex (Bougoure et al., 2005; Otero et al., 2002; Waterman & Bidartondo, 2008), which is an artificial grouping of

unrelated fungi based on anamorphic life stage (*Ceratobasidium*, *Thanatephorus*, *Sebacina* and *Tulasnella*) (Otero et al., 2002; Smith & Read, 2010). Many of these fungi are usually saprotrophs living in organic matter of soils; whereas some groups form ectomycorrhiza (ECM) with trees and others are plant parasites (Bougoure et al., 2009; Yamato et al., 2005).

Most orchid species eventually develop photosynthetic tissues, hence across its life cycle, orchids may transit through three main trophic stages: (1) a stage fully dependent on the mycorrhizal fungus (the mycoheterotrophic stage), in which some orchids remain achlorophyllous throughout their adult phase and continue exploiting their mycorrhizal fungi without a return of organic carbon or other metabolic compounds derived from plant metabolism (Taylor & Bruns, 1997); (2) a transition stage, when orchids acquire the photosynthetic ability, obtaining carbon by both fungal and autotrophic way (the mixotrophic stage), turning them partially mycoheterotrophic (Zimmer et al., 2008); and (3) the fully independent stage, when orchids obtain carbon by photosynthesis and the symbiosis is stopped until the environmental conditions induce the symbiosis re-activation (autotrophic stage) (Cameron et al., 2006; Rasmussen & Rasmussen, 2009). In nature, there are two kinds of orchids: the photosynthetic ones (green orchids), which depend on the mycorrhizal fungi only to germinate their seeds and to supplement carbon that they themselves fix through photosynthesis (Merckx et al., 2009); and the non-photosynthetic ones (fully mycoheterotrophic), which depend on the mycorrhizal fungi even during the adult stage, due to the absence of photosynthetic tissues, retaining this juvenile characteristic throughout their entire life cycle. Thus, orchids remain obligate parasites on fungi (Barrett et al., 2010).

Morphologically, *Mycorrhizae* form typical structures between their symbionts, often related with interchange and store of nutritional compounds. In this context, it is

well known that AM form arbuscules and vesicles as interfaces of interchange and store nutrients, respectively. In the same way, ECM form a sheath with appropriates trees and shrubs (Smith & Read, 2010). Correspondingly, all orchids have pelotons in their radical tissues as vestige of the relationship established in the seedling stage (Valadares et al., 2011). In root tissues of adult orchids, the peloton presence, both intact and degraded, is characteristic of OM (**figure 2.2**) (Pecoraro et al., 2012) and both can transfer nutrient to their symbionts (Kuga et al., 2014). Hence, pelotons are considered as mycoheterotrophic organs with a key role in orchid nutrition (Bougoure et al., 2014). They are developed during the protocorm (mycoheterotrophic organ) stage and correspond to hyphal coils in which mycorrhizal fungi are restricted and stored for enzymatic degradation, mainly to obtain carbon and other mineral nutrient (Smith & Read, 2010). In adult orchids, the intracellular growth of fungal hyphae is mainly promoted by depletion of flavonoids (Chomicki et al., 2014) and the fungal hyphae also can spread to the growth substrate throughout radical hairs (Sathiyadash et al., 2012). Despite the importance of pelotons in the orchid nutrition, little is known about the physiology controlling the activation of the mycoheterotrophic processes.

2.3 BROAD SPECTRUM OF FUNGI ASSOCIATED WITH ORCHIDS

Several fungi influence directly the orchid metabolism, participating in processes such as seed germination (physiological bases entirely unknown), peloton derived nutrient exchange (required for seed development) and likely a wide range of functions that have not been detected yet, such as protection against pathogens or probably the photosynthetic flow between orchids and other higher plants. The versatility of root fungal endophytes is very high; we can find the typically OM symbionts (*Rhizoctonia*-like fungi) (Otero et al., 2004; Otero et al., 2007; Otero et al., 2002), and a broad spectrum of fungi that do not have a specific role on orchids metabolism attributed so

far (see **Table 2.1**). To identify the most common orchid root fungal endophytes, fungal strains have to be isolated and described morphologically (Valadares et al., 2011). In this context, the most effective technique to isolate orchid symbionts is the peloton isolation method (Rasmussen & Whigham, 2002; Shefferson et al., 2005; Warcup & Talbot, 1967). Fungi directly isolated from pelotons are potential mycorrhizas because the fungi grew from the cortical cells, containing living pelotons and the morphological and molecular characteristics match with the reported OM characteristics (Nontachaiyapoom et al., 2010). This technique is useful to identify culturable endophytes, but there are still some fungi recalcitrants to in-vitro culture, and identification using morphological characteristics turns difficult (Xing et al., 2013). However, with the use of molecular techniques the spectrum of fungi associated with orchid has grown-up, allowing a better understanding of orchid root fungal endophytes.

In green orchids, the most common mycorrhizal symbionts are the *Rhizoctonia*-like fungi. The most common techniques used to study these interactions are the microscopic ones, which have been widely used to identify the morphological characteristics of *Rhizoctonia*-like fungi isolated from pelotons (uninucleate, binucleate or multinucleated hyphal cells, septum with 90º branching, no spore formation). The advent of fluorescent staining has allowed visualizing fungal structures such as nucleus, presence of septum, or monilioid cell formation in optical microscopy (Schelkle et al., 1996; Valadares et al., 2011).

Different orchid species can be associated with a large variety of fungi (Rasmussen, 1995; Shefferson et al., 2008; Xing et al., 2013). The specificity degree of orchids is variable and is influenced by environmental conditions and the surrounding associated plant species (Waterman & Bidartondo, 2008). The same orchid specie might vary its mycorrhizal preferences according to different geographic zones and even

individual orchids has more than one mycorrhizal fungus inside their roots (Jacquemyn et al., 2010; McCormick et al., 2004; Shefferson et al., 2008). Probably orchids can host a large number of fungi and this ability could be a fitness to survive together with other plants, even in degraded environments (Jurkiewicz et al., 2001). If we analyse the compatibility webs formed by the orchid hyphosphere, we can show that orchids can act as opportunist hosting more than one fungus to obtain the metabolic requirements throughout its life cycle (**Figure 2.1**). Orchids can form mycorrhizas with pathogens, with dark septate endophytes, fungi that can acts as ECM with trees, etc. (see **Table 2.1**). These diverse interactions are directly controlled by the orchid metabolism and suggest that different species are very versatile to accept endophytes in their tissues in order to have more than one way to obtain nutrients and exploit this characteristic if environmental conditions are adverse. Probably the non-peloton forming fungi are more difficult to identify because they do not form typical peloton structures and may be often considered as contamination in the classical isolation techniques (peloton isolation method).

ECM forming fungi are the most common endophytes in mycoheterotrophic orchids (MHO) and certainly play an important role in the soil environment. These fungi are well known by its role for improving water and mineral contents to the associated plant, in return the fungal partner receives about 10 to 20% of plant assimilates as retribution (Wiemken & Boller, 2002). The colonization by ECM has been usually reported (Barrett et al., 2010; Cowden & Shefferson, 2013; Girlanda et al., 2011; Taylor & Bruns, 1997; Xing et al., 2013), in special in MHO roots, indicating that autotrophy deficiency is compensated by the colonization with other fungal clades to complement their nutritional requirements. These fungi can form a ‘wood-wide web”, connecting individual plants through the fungal mycelium (Simard et al., 1997).

Furthermore, the saprotrophs that are usually living in rhizospheric soils, with a strong metabolism and playing a crucial role for decomposing lignocellulosic residues and some recalcitrant xenobiotic compounds (Khadrani et al., 1999), can also be found colonizing orchid roots (Fan et al., 1996; Guo et al., 1997; Martos et al., 2009; Pecoraro et al., 2012; Selosse et al., 2010). In these cases, they may be contaminants or facultative biotrophic encounters, forming mycorrhizal structures on very small root portions or colonizing tissues as endophytes (Selosse et al., 2010). Similarly, plant fungal endophytes are usually found colonizing orchid tissues (Martos et al., 2009; Ogura-Tsujita & Yukawa, 2008; Roy et al., 2009a) and are often beneficial for the host. They can live asymptomatically within plant tissues and are certainly inherited components of the host plant, thus, they increase not only phenotypic but also genotypic diversity of the host (Faeth & Saari, 2012). Different kinds of fungi usually found colonizing orchid roots are dark septate endophytes (DSE). The role of DSE in plants has not been elucidated. According to Jumpponen, (2001) effects of DSE may vary from negative to neutral and to positive, analysing the host responses to growth or nutrient concentration, and must be considered as mycorrhizas by its ranging from parasitism to mutualism (Cázares et al., 2005; Fernando & Currah, 1996; Jumpponen et al., 1998; Jumpponen & Trappe, 1998). Sometimes these dark septate endophytes can act as pathogens, but orchids have improved the ability to accept plant pathogens in their tissues, controlling the pathogenic property of certain fungal strains (Cowden & Shefferson, 2013; Kennedy et al., 2011; Steinfort et al., 2010.

The specificity of orchids to select their mycorrhizal fungi is variable, some orchids show a high specificity, associating only with certain fungal taxa, and others can modify their mycorrhizal preferences (Dearnaley, 2007; Waterman & Bidartondo, 2008). This processes are more evident in mycoheterotrophy, in which orchid depends

entirely on colonized fungi for carbon throughout their lives (Leake, 1994). Most MHO associate with ectomycorrhiza-forming fungi (Smith & Read, 2010); therefore, the orchid, their ectomycorrhizal fungi and the ectomycorrhiza-forming trees are associated with one another in such tripartite (or multipartite if we consider saprotrophs or endophytes) relationships (Taylor & Bruns, 1997), likely moving nutrients or metabolic compounds, derived from tree metabolism (photosynthesized carbon) to the orchid, through the fungal mycelia of the orchid hyphosphere (defined as to the analogous region surrounding individual fungal hyphae from fungi associated with orchid roots) (Bidartondo et al., 2004; Julou et al., 2005; Veresoglou et al., 2012). The orchid hyphosphere acts exploiting different nutrient sources, transporting photosynthates and water toward orchids, but the feasibility of these processes need to be better addressed.

2.3.1 The role of mycorrhizal fungi in conservation of endangered orchids

Many orchid species are in danger of extinction due to growing environmental pollution, human induced habitat loss and segregation of orchids only to protected areas (Schödelbauerová et al., 2009). Furthermore, some orchids lack of protection due to the increase of commercial demand (Dutra et al., 2009). The mycorrhizal fungus certainly plays a predominant role in orchid preservation, by the dependence of orchids to germinate their seeds. The specificity of OM associations is relevant to orchid physiology restoration and conservation (Dearnaley, 2007). In nature, soil and its fungal diversity are essential for orchid development and colonization of diverse environments. Therefore, the consideration of saving endangered orchids needs to isolate, identify and store appropriates mycorrhizal fungi to germinate their seeds (Jacquemyn et al., 2014). According to Jacquemyn et al. (2010) a high specificity is unlikely in most ecological interactions, because orchid species with the ability to associate with a wider range of fungi have a broader range of ecological options to survive and colonize diverse

habitats. However, when high specificity involves generalist fungi, that associate with most other orchid species and that are widely distributed, high specificity does not necessarily have to be associated with limited distribution (Shefferson et al., 2005). The ability of orchids to accept a soil fungus broadly distributed could be a strategy to improve the fitness to a new habitat or to adverse environmental conditions. On the other hand, the "rare" orchids, with limited distributions, may have associated fungi that are not frequent in soils, acting as constraint to orchid distribution. Orchid species could be either generalist or specialist in the selection for mycorrhizal fungi, although the association with a broadly distributed fungus may allow a wider distribution range than orchid species that associate with only a few or rare fungal species (Otero et al., 2007). A full understanding of the associated mycorrhizal fungi is necessary to protect orchid populations or recovery actions of each declined or endangered species, because these fungi could be used for symbiotic orchid propagation (Brundrett, 2002; Brundrett, 2007; Jacquemyn et al., 2005; Kull & Hutchings, 2006).

2.3.2 Potential of orchids to grow under adverse environmental conditions

Ecological advantages for orchid of having a broad spectrum of fungal symbionts, is that basing their nutritional demands on their fungal partners, orchid may be set in diverse degraded environments, in which soil natural processes are completely altered. Orchids are well known not only for their fascinating beauty, but also for their specialization to particular habitats (Shefferson et al., 2008). They have relatively limited distribution range and the diverse orchids habitats are often colonized by a few species. The particular characteristics of OM, indicate that the fungi give orchids new ways to obtain nutrients, at different life stages and even under stress conditions (Bidartondo & Read, 2008; McCormick et al., 2006). Researches on the ability of orchids to alleviate contaminated soils have not been performed yet, but a few works

have been done: (1) to characterize the subset structure of mycorrhizal fungi growing in mine tailing sites to explore the ecology of OM fungi in the context of primary succession and conservation (Shefferson et al., 2008); (2) to find if the presence of fungal mycelium affects heavy metal distribution within orchid roots (Jurkiewicz et al., 2001); (3) to study the colonization processes of orchids in lignite mining areas (Esfeld et al., 2008); and (4) to elucidate how orchids respond to environmental stresses (Shefferson et al., 2005). In habitats where nutrients are limited, the main constraint to plant development are phosphorous and nitrogen deficiency, and the selection of several mycorrhizal partners might orchids improve their nutritional uptake (Bidartondo et al., 2004; Gebauer & Meyer, 2003). Besides, it is expected that a bigger number of mycorrhizal associates allows orchid species to cope better with the associated stress condition in degraded or impoverished environments (McCormick et al., 2006).

Some orchids have the ability to grow in nutrient-impoverished soils and this suggests a strong metabolism that allow orchid survive and colonize habitats without or with few plant species. As adaptive strategy, orchids have developed a strong metabolism to face and act as primary succession elements, especially the lithophytic ones, which are able to grow in rocks where water and nutrient conditions are deficient, the UV radiation is extreme and rhizospheric microbiological communities are reduced (Herrera et al., 2017; Kottke et al., 2010). The same strategies developed by orchids to survive under these adverse conditions are probably the same to act as primary elements to colonize these habitats and it could be useful to explore strategies of acclimation and colonization of degraded soils.

Considering that orchids are plants that have a huge number of fungi inside their root tissues and that there are reports of orchids growing in degraded and/or contaminated soils, their associated microorganisms could facilitate both peloton

derived nutrient movement, and pollutant uptake by the plant, through absorption and relocation into other plant tissues (Jurkiewicz et al., 2001), which might be stored or removed recovering soil health of the degraded soils.

2.4 METABOLIC AND MOLECULAR ANALYSES ARE ESSENTIAL TO UNRAVEL THE TROPHIC NATURE OF ORCHID MYCORRHIZAS

The OMICS related technologies are considered as powerful techniques to describe the complexity of plants and soil microorganisms under symbiosis. In a perfect mutualistic relationship, both partners obtain benefits from each other, based on a molecular mechanism that allows improve the growth or nutritional content of the symbionts. Such mechanisms have been widely studied in arbuscular mycorrhizal (AM) symbioses (Capoen et al., 2010; Hause & Fester, 2005; Küster et al., 2004; Massoumou et al., 2007; Oldroyd et al., 2009; Reinhardt, 2007) and there are few reports about the cellular and molecular bases of plant–fungus interaction in OM (Perotto et al., 2014; Valadares, 2014; Zhao et al., 2014). Analysing the works that have been done on OM, there are issues that need to be assessed to have a better understanding of the benefits of each partner, especially for mycorrhizal fungi, to clarify whether orchids are symbionts or parasites of the fungi. Thus, the so called OMICS technologies can be useful tools to assess the molecular mechanisms that are influencing the symbionts. These OMICS approaches can be applied at several scales that are relevant for studying OM symbioses. If we adopted the new technologies such as the called high-throughput technologies to analyse the transcriptome, proteome or metabolome of mycorrhizal plant, we would unravel key mechanisms involved in OM symbioses.

2.4.1 Genomics applied to ecological studies in orchid mycorrhiza

The most used molecular tools, in OM studies, are the genomics ones, specially the related to fungal partner identification, because a finer distinction between strains is possible and induction of a teleomorphic stage is not necessary (Nontachaiyapoom et al., 2010). The OM symbionts are difficult to characterize using only morphological characteristics and the amplification of certain genes is necessary to know the identity of fungal partners (Shefferson et al., 2005; Taylor & Bruns, 1997). It has been reported that a minor fraction of the total microbial taxa are suitable to culture in an appropriate medium and it can be identified using culture independent methods (Pham & Kim, 2012). In the study of OM, DNA based analyses through PCR amplification of ribosomal internal transcribed spacer (ITS) has been widely used to characterize the mycorrhizal fungi inhabiting orchid roots (Chen et al., 2012; Selosse et al., 2010; White et al., 1990). To date it is still unclear how specificity varies in different orchid species, some authors have suggested a narrow specificity (Irwin et al., 2007; McCormick et al., 2006) and others mentioned a variable range of fungi (Waterman & Bidartondo, 2008). Similarly, orchids can be analysed using some specific primers to establish phylogenetic relationship among orchids inhabiting a given habitat (da Silva, 2013). To establish evolutionary studies the PCR amplification of nuclear rDNA must be performed, specifically, sequences from plasmid trnL-F or matK region (Chemisquy & Morrone, 2012; Kocyan et al., 2008) and sequences from mitochondria (nad1b-c) (Freudenstein & Chase, 2001; Freudenstein et al., 2000) or nucleus (18S rDNA) (Cameron & Chase, 2000) have been applied. Collecting both molecular data from fungi and from orchids, the compatibility webs between orchids and the different fungi inhabiting their roots could be design (Barrett et al., 2010; Bonnardeaux et al., 2007; Jacquemyn et al., 2010). In terms of microbial ecology, the application of methods to have a general overview of

the microbiological communities (DGGE or TGGE) (Joynt et al., 2006; Kubartová et al., 2009) could be a useful approach to assess the composition of microorganisms associated with orchids, and how the orchid root exudates influences the microbiological communities. More specific methods are phylogeny based in pyrosequencing and related technologies (Jones et al., 2009). These approaches facilitate microbiological studies, although they are limited to characterize individual microbes. More efficient approaches to identify fungal endophytes are the sequence analysis of clone libraries (from PCR amplified genes or metagenomic DNA libraries) (Dunbar et al., 2002; Timke et al., 2005) or pyrosequencing (Roesch et al., 2007), that is able to generate a huge number of sequences without a cloning step. Jacquemyn et al. (2010) through application of novel DNA array technology identified OM associations of different temperate photosynthetic orchid species, which were composed of multiple- rather than single-species associations and those associations exhibited a significantly nested pattern; Lievens et al. (2010) (with DNA array based analysis) recommended the fungal primer set ITS1-OF and ITS4-OF (Taylor & McCormick, 2008) to accurately identify OM symbionts (*Basidiomycetes* and *Tulasnella* species) (Nontachaiyapoom et al., 2010).

2.4.2 Expressed genes and their relevance to elucidate physiological changes in orchids

To identify which genes are expressed in OM symbioses and their relevance to symbiosis, the analysis of whole mRNA sequences (transcriptome) coupled to sequencing technologies has been applied (Perotto et al., 2014; Zhao et al., 2014). This technology is useful to have a better understanding about the molecular mechanisms that are involved in this particular symbiosis. The first report using transcriptomic approaches was focused on gene expression of *Cypripedium parviflorum* roots

(inoculated with an OM fungus) by Watkinson and Welbaum (2002) through differential display technique. The selected genes trehalose-6-phosphate synthase phosphatase (Tps) and nucleotide binding protein (NuBP) showed a differential expression. These genes can act as regulators of the sugar transport in symbiotic roots or accommodating the fungus in mycorrhizal tissues, respectively. More recently, Zhao et al. (2014) developed the first work focused on identifying genes expressed in symbiotically germinated seeds of *Dendrobium officinale* through suppression subtractive hybridization. Some of the up-regulated genes included defence and stress response, metabolism, transcriptional regulation, transport and signal transduction pathways, providing a set of candidate genes to be used in further experiments related to molecular events that control OM symbioses. Besides, the analysis of the transcriptome of photosynthetic orchid protocorms has recently been done by Perotto et al. (2014). They analysed the gene expression of *Serapias vomeracea* protocorms symbiotically germinated, through 454 pyrosequencing of cDNA libraries (expressed genes) and qPCR to quantify either, mutualistic or antagonistic related genes. The main results of this study were that none of the genes related to pathogenesis or stress were up-regulated and some nodulin genes (NOD), selected as mutualistic marker, were up regulated, hence, suggesting a high level of compatibility with minimal activation of defence related genes (**Figure 2.2**). Summarizing the works reported here, we can conclude that the low development of defensive strategies against the fungus colonizing orchid roots are key mechanisms to maintain this symbiotic interaction. Probably, the up-regulated genes of stress and pathogen defence are results to compare the expression with non-germinated seeds, when obviously the fungal symbiont cause activation of minimal defence or stress responses, and the corresponding activation of metabolism mainly due to the gradual transition of mycoheterotrophy to autotrophy.

2.4.3 Biomolecules synthetized at orchid-fungus interface

Proteomic approaches are powerful tools that have been applied to characterize the catabolic processes influenced by the establishment of a plant-fungi symbiosis (Dumas-Gaudot et al., 2004; Louarn et al., 2013). However, analysing the works that have been done previous to the OMICs development, related to proteins involved in OM physiology, they have been mainly focused on the analyses of the antioxidant enzyme contents (oxidase, ascorbic acid oxidase, peroxidase, and catalase) (Blakeman et al., 1976), cytochemical location of phenol oxidases (Andersen & Rasmussen, 1996); activity of phenylalanine ammonia lyase (PAL) (Beyrle et al., 1995) or to characterize some mannose binding proteins (MBP) with antifungal properties (Sattayasai et al., 2009; Wang et al., 2001). Despite the relevance of the identification of single protein, a global overview of the protein content of orchids under symbiosis is necessary for having have insights about the principal biomolecules implied in OM symbioses. A few remarkable works have addressed the previous statement; thus, to discuss the latest proteomic advances that have been done in OM issues we need to refer to Valadares et al. (2014). Considering that orchids are obligate colonized by *Rhizoctonia*-like fungi to germinate their seeds, this study applied two dimensional electrophoresis (2D-LC-MS/MS) coupled to isobaric tagging for relative and absolute quantification (iTRAQ), to identify which proteins are differentially expressed in orchid protocorms at different stages of development (mycoheterotrophic and autotrophic). The main results of this experiment were that transition from mycoheterotrophy to autotrophy enhances the activation of proteins involved in metabolic pathways related to carbon metabolism, modulation of reactive oxygen species homeostasis, defence-related responses, and phytoalexins and carotenoid biosynthesis (**Figure 2.2**); this is mainly explained by the processes that orchids activate to acquire the carbon-autonomy. These novel results

should be applied to track some key biomolecules throughout seed development through design of monoclonal antibodies against the selected biomolecules (Paduano et al., 2011).

2.4.4 Metabolomics; key to study nutrient interchange between orchids and fungi

Transcriptomics and proteomics approaches do not provide a comprehensive view of the molecular processes that OM develop. Thus, the metabolic profiling and metabolic response of orchids under symbiosis might be a useful tool to determine whether final cellular processes products influence both partners. Despite the term *Mycorrhizae* is commonly accepted as a mutualistic association, the studies of nutrient transfer between orchids and fungi have revealed insights of both a unilateral interaction in which the nutritional benefits are only for the orchid (Dearnaley et al., 2012; Rasmussen & Rasmussen, 2009; Rasmussen & Rasmussen, 2007; Taylor et al., 2002); and a mutualism, where both partners get benefits mainly due to the return of photosynthetic compounds to the degraded fungal hyphae (Cameron et al., 2008; Cameron et al., 2006), generating a discussion of the real trophic nature of this interaction. Thus, metabolomics related technologies are promising tools to answer whether the orchid-fungus symbiosis varies the nutritional fluxes or if these processes are mainly regulated by the orchids. According to Bougoure et al. (2014) the main ways for nutrient movement between OM symbionts are: biotrophic transfer of nutrients across the active interface formed between plant and fungus (uni or bi-directional); necrotrophic transfer of nutrients after fungal pelotons lysis (fungus to plant); or mixotrophic, that is a combination of both processes. Metabolomics has been widely used to study principal alteration related to chemical compound contents in *Mycorrhizae* (Schliemann et al., 2008; Schweiger et al., 2014). Such studies can be used to study OM interactions and establish a chemical fingerprint differentially found metabolites and

also characterizing their role in the maintenance of the relationship established between orchids and fungi. Metabolites with a fundamental role in AM are isoflavonoids, hydroxycinnamoyl amides, apocarotenoids, fatty acids (Schliemann et al., 2008) and, as in OM (Watkinson & Welbaum, 2002), metabolites from fungal origin related to carbohydrate and lipid metabolism, that allows accumulate trehalose in roots has been reported (Douds et al., 2000). The above mentioned metabolites may also have a crucial role in OM and needs to be addressed to have a complete understanding about the metabolic fluxes in OM interactions.

The metabolome of orchids and their mycorrhizal fungi are largely unknown. Specifically, the analysis of chemical composition and complexity of an orchid root exudate has not been performed yet. It is unclear how carbon passes from these fungi to orchids; they could be parasites, saprotrophs or mycorrhizal associates of other plants. According their trophic nature (mixotrophic or mycoheterotrophic), the use of labelled isotopes has been applied to identify the metabolic fluxes between some orchids and their appropriate mycorrhizal fungi, making fluxomic a promising tool to unravel the trophic nature of the interaction. Previously works indicated that orchids degrade fungal hyphae apparently without (or little) benefits for the fungi (Alexander & Hadley, 1985; Hadley & Purves, 1974); and recently, evidence from a mutualism has been published (Cameron et al., 2008; Cameron et al., 2006; Martos et al., 2009). In MHO, carbon nutrition is entirely provided by the mycorrhizal fungi and in some mixotrophic orchids both photoassimilated and fungal derived carbon is presented (Waterman & Bidartondo, 2008). Orchids gain carbon and probably other nutrients through enzymatic peloton degradation, although Kuga et al. (2014) revealed that the two kinds of peloton (intact and degraded) can transfer carbon and nitrogen to their fungi associates (Bougoure et al., 2014; Kuga et al., 2014). Recently, it has been reported that the fungal control is

related to the flavonoid content in cortex, in structures named “passage cells”. These zones (mainly epiphytic orchids) are depleted in flavonoids and restricted to the distal radical tissues, an strategy to maximize carbon gain from photosynthesis, while enabling further trophic exchanges from mycorrhizal associations (Chomicki et al., 2014).

Few remarkable works have been carried out to identify metabolites with a key role in OM processes. Specifically, an antifungal compound with a critical role in the control of pathogenic fungi at cellular level it is has been identified (Shimura et al., 2007). In addition, the characterization of some phytoalexins, that are secondary metabolites usually secreted by plant against pathogen attack, has been published, specifically, the presence of orcinol (orc) (Beyrle et al., 1995), lusiathrin (lus) and chrysin (chr) (Fisch et al., 1973; Shimura et al., 2007) that are able to inhibit growth of the fungal mycelium has been studied in OM symbioses. Besides, the characterization of some phenolic compounds like stilbenoids (Stb), that are usually synthetized in plant fungal interactions, can act as effective regulator on OM (Münzenberger et al., 1990). Furthermore, the pectin (pec) location as modulator of interaction at peloton level has been reported (Paduano et al., 2011). The authors analysed the pectin localization between two kinds of mycorrhizal fungi, reporting that the cellular responses against two different mycorrhizal fungi are different, showing that pectin is exclusively found in the interface formed around genus *Ceratobasidium* and not in *Russula* symbiont.

Fluxomic, defined as the whole set of reaction in a cell, comprising conversion and transport rates of metabolites (Klein & Heinzle, 2012), is a powerful tool to unravel crucial pathways that control and lead the transition from mycoheterotrophy to autotrophy. The application of stable isotopes containing isotopic labelled substrates is currently the best way to study fluxomic in a cellular complex. To determine the

dependence of the fungal derived carbon stable isotope, natural abundance analysis has been applied and allows distinguishing C, P and N fluxes (^{13}C, ^{33}P and ^{15}N) between MHOs and their mycorrhizal fungi (Liebel & Gebauer, 2011; Liebel et al., 2010). The basis of the methods is related to analysis of the isotope pattern of fungal associates and compares it with the orchid and the neighbouring autotrophic plants, which are often slightly different (Liebel et al., 2010). Thus, different orchid species that depending the carbon or nitrogen supply on the fungi, are enriched in the same way as their fungal associate (Trudell et al., 2003). The natural abundance of stable isotopes has been used to characterize the nutrient gains in mixotrophic (Bidartondo et al., 2004; Gebauer & Meyer, 2003; Girlanda et al., 2011; Roy et al., 2009a; Zimmer et al., 2008) and mycoheterotrophic orchids (Bougoure et al., 2014; Gebauer & Meyer, 2003; Liebel et al., 2010; Ogura-Tsujita et al., 2012). McKendrick et al. (2000), reported ectomycorrhizal fungal contribution of carbon to the orchid *Corallorhiza trifida* through the common hyphae that form endomycorrhiza in orchids and ECM in *Pinus silvestris*, and little carbon derived from photosynthetic activity has been reported (Cameron et al., 2009). In addition, Liebel and Gebauer (2011) reported the nitrogen and carbon gain in the fully MHO *Epipogium aphyllum*. The ability of MHO to associate with ectomycorrhizal forming fungi is often reported (Roy et al., 2009a; Selosse et al., 2004; Taylor & Bruns, 1997) and pathogens or saprotrophs for mixotrophic orchids, although there are some orchids that change their mycorrhizal preferences (Ogura-Tsujita & Yukawa, 2008). This view of changing mycorrhizal pattern is supported by Bidartondo et al. (2004). They reported that some photosynthetic orchids inhabiting a forest understory are able to form ECM, indicating distinctive pathways for nitrogen and carbon acquisition through the stable-isotope fingerprint, which was similar to the signatures found in non-photosynthetic orchids. Bougoure et al. (2009) reported that the

orchid *Rhizanthella gardneri* shares ECM with *Maleluca* species, and acquires nutrients through hyphae connections from a typical ECM shrub and directly from the soil via the same fungus, establishing a particular tripartite relationship. Sommer et al. (2012) developed an experiment to assess the nutritional mode of 27 orchid species by C and N isotope abundance analysis to confirm the fully autotrophy through $^{13}CO_2$ pulse labelling. They reported that the partial mycoheterotrophy processes are less common in Australian orchids than in other countries (Abadie et al., 2006; Girlanda et al., 2006), attributing these findings to the selection of saprotrophic fungi rather than to ECM as symbionts. Gebauer and Meyer (2003) revealed the natural enrichment of stable isotopes of different orchid species providing insights of carbon or nitrogen gain, particularly in the mycoheterotrophic ones. Besides, Girlanda et al. (2011) analysed the nutrient acquisition of four Mediterranean photosynthetic orchid species. They suggested that the mycorrhizal specificity is influenced by the symbiosis requirements, especially the nutritional dependence of the fungi. Liebel et al. (2010) showed that in most adult orchids, of open habitats in the Mediterranean and Macaronesia (Italy and Spain), carbon or nitrogen gains through mycoheterotrophy are weak or inexistent, indicating net plant-to-fungus carbon transfer. Cameron et al. (2006) demonstrated a bidirectional carbon transfer between a photosynthetic orchid and its fungal symbiont; and a fungus-dependent pathway for organic N acquisition by an orchid for the first time, also corroborated in Cameron et al. (2008), suggesting a dynamic transfer at an interfacial apoplast as opposed to reliance on digestion of fungal peloton. Furthermore, the acquisition of phosphorous by orchids through fungi has been reported in the terrestrial *Goodyera repens* (Cameron et al., 2007). Recently Stöckel et al. (2014) established that the partial mycoheterotrophy determined by C and N signatures is not useful for confirming partially mycoheterotrophy in orchids, which are associated with

saprotrophs, hence, the estimated number of partially mycoheterotrophic orchids may be much higher than expected.

To date, the fluxomic approaches has been used to study mixotrophic and mycoheterotrophic orchids, but the principal metabolic pathways in which the nutrient gain is utilized it still unclear. Through quantitative metabolomics using isotopic labelling compounds (flux balance analysis, which is based on the stoichiometry and to represent the connections of all the metabolites in the cellular network) (Klein & Heinzle, 2012), we can have a comprehensive analysis of the nutrient dynamics that is necessary to complement the OMICS results that have been reported hitherto (**Figure 2.2**).

2.5 CONCLUSIONS AND PERSPECTIVES

Despite the controversial data that has been published, it is unclear how orchids can explote their fungal associates to obtain nutritional benefits. It is true that the mycorrhizal fungi infect orchid seeds, and the plants have the capacity of revert the usual infection processes and form the typical structures of these mycorrhizal interactions, known as pelotons. The diversity of associated fungi is huge, which indicates more than one way to obtain nutrients from the mycorrhizal fungi. There is evidence that proves that those orchids are more similar to a mutualism than to parasitism. Thus, the production of antifungal and defence related compounds are natural processes to avoid a bigger colonization of plant vital tissues. In addition, the synthesis of metabolites related to oxidative stress control is related to metabolism activation, due to the presence of the colonizing hyphae, which is often degraded to obtain nutritional benefits. Despite the indicium of a friendly relationship, all orchids during their seedling stage establish a unilateral mycophagous interaction in which

orchid acts as parasites of the mycorrhizal fungi. The mechanisms that orchids activate and lead the change from mycoheterotrophy to autotrophy are unknown, however, is clear that the major benefits are for the orchid, which is the main "boss" in this kind of mycorrhiza, being the minuscule seeds which have the ability to control the infective processes of orchid mycorrhizas. Thus, responding the main question of this review, we can suggest that orchids are near to be parasites of the fungi, because fungi are indispensable to obtain nutrient during their seedling stage, and apparently little benefits are given to the fungal mycelium, that is often restricted to determined radical zones.

Further researches need focus on fluxomic approaches, to have a better understanding of global processes that are changing the physiology of both; fungi and orchid, and complement the genomic, transcriptomic and proteomic results. It is necessary to study the real benefits for the fungal side, in order to have more evidence from a mutualism relationship than a parasitism.

2.6 ACKNOWLEDGMENTS

The authors thank the National Commission for Scientific & Technological Research (Chile) for the research grant FONDECYT 1130662 and CONICYT scholarship Nº 21130491.

Table 2.1 Worldwide diversity of root-fungal endophytes found colonizing diverse orchid species

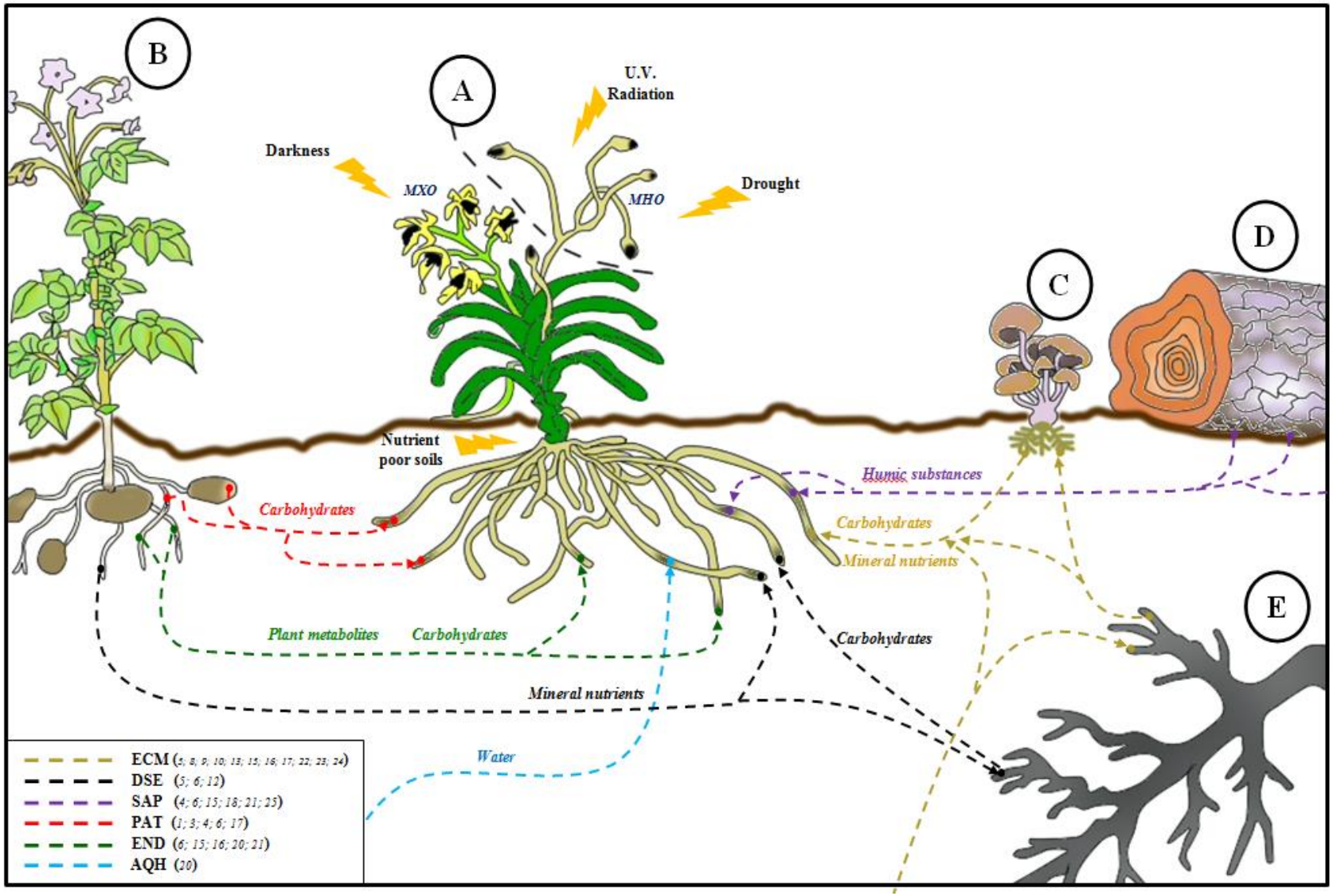
Orchid host	Associated fungi.	Fungal grouping	Nutritional mode	Location	Reference (*reference number*)
Bippinula fimbriata	*Ceratorrhiza* *Epulorrhiza* *Rhizoctonia solani*	*Rhizoctonia*-like fungi *Rhizoctonia*-like fungi Plan pathogens	Autotrophic (terrestrial)	Chile	Steinfort et al. (2010) (*1*)
Chloraea collicensis and *Chloraea gavilu*	*Tulasnella calospora* Tulasnellaceae sp.	*Rhizoctonia*-like fungi	Autotrophic (terrestrial)	Chile	Pereira et al. (2014) (*2*)
Coppensia doniana	Ceratorrhiza sp. *Uninucleate Rhizoctonia*	*Rhizoctonia*-like fungi plant pathogens	Autotrophic (Terrestrial)	Brazil	Valadares et al. (2011) (*3*)
Dendrobium sp.	principal fungal taxa*:* *Fusarium* *Acremonium* *Alternaria* *Verticillium*	 Saprotrophs Saprotrophs Plant pathogens saprophytic and parasitic	Autotrophic (epiphytic)	China	Chen et al. (2012) (*4*)
Epipactis thunbergii	*Tulasnella* sp. *Ascomycete* *Exidia* sp.	*Rhizoctonia*-like fungi Dark septate endophytes *Ectomycorrhizae*	Autotrophic (terrestrial)	Japan	Cowden and Shefferson (2013) (*5*)
Habenaria radiata	*Thanatephorus cucumeris* Sebacinales *Phialocephala sphaeroides* *Glomeromycota* *Colletotrichum* sp. *Tulasnella* sp. *Lasiosphaeria* sp.	Plant pathogens *Rhizoctonia*-like fungi. Dark septate endophytes *Endomycorrhizae* Dark septate endophytes *Rhizoctonia*-like fungi. Ligninolitic saprotrophs	Autotrophic (terrestrial)	Japan	Cowden and Shefferson (2013) (*6*)
Dendrobium officinale and *Dendrobium fimbriatum*	*Tulasnella calospora.* *Epulorhiza* sp. *Pluteus seticeps* *Ceratobasidium* sp.	*Rhizoctonia*-like fungi *Rhizoctonia*-like fungi *Ectomycorrhizae* *Rhizoctonia*-like fungi	Autotrophic (terrestrial)	China	Xing et al. (2013) (*7*)

Table 2.1 Worldwide diversity of root-fungal endophytes found colonizing diverse orchid species (continued).

Orchid host	Associated fungi	Fungal grouping	Nutritional mode	Location	Reference (*reference number*)
Dactylorhiza baltica	*Ceratobasidium albasitensis*	*Rhizoctonia*-like fungi			
Epipactis atrorubens	Unnamed Tulasnellaceae *Trichophaea woolhopeia* *Geopora cooperi* *Chalara dualis*	*Rhizoctonia*-like fungi *Ectomycorrhizae* *Ectomycorrhizae* *Ectomycorrhizae*	Autotrophic (terrestrial)	Estonia	Shefferson et al. (2008) (*8*)
Orchis militaris	Unnamed Tulasnellaceae	*Rhizoctonia*-like fungi			
Anacamptis laxiflora *Ophrys fuciflora* *Orchis purpurea* *Serapias vomeracea*	*Sebacinales*, *Tulasnella* and *Ceratobasidium*	*Rhizoctonia*-like fungi	Autotrophic (terrestrial)	Italy	Girlanda et al. (2011) (9)
	Macowanites vinaceodorus , *Terfezia* sp., *Choiromyces echinulatus*	*Ectomycorrhizae*			
Corallorhiza striata	*Tomentella* sp. *(Tormentella. fuscocinerea)*	*Ectomycorrhizae*	Mycoheterotrophic (terrestrial)	USA	Barrett et al. (2010) (*10*)
Liparis loeselii	*Epulorhiza* *Rhizoctonia*	*Rhizoctonia*-like fungi	Mycoheterotrophic (terrestrial)	Czech Republic Hungary	Illyés et al. (2005) (*11*)
Unspecified	Tulasnellales Sebacinales *Atractiellomycetes*	*Rhizoctonia*-like fungi *Rhizoctonia*-like fungi Simple-septate basidiomycetes	Autotrophic (terrestrial and epiphytic)	Ecuador	Kottke et al. (2010) (*12*)
Corallorhiza maculata and *Corallorhiza mertensiana*	Russulaceae	*Ectomycorrhizae*	Mycoheterotrophic (terrestrial)	USA	Lee Taylor and Bruns (1999) (*13*)
Stelis hallii, Stelis Superbiens, Pleurothallis lilijae and *Stalis concinna*	*Tulasnellales*	*Rhizoctonia*-like fungi	Autotrophic (epiphytic)	Ecuador	Suárez et al. (2006) (*14*)

Table 2.1 Worldwide diversity of root-fungal endophytes found colonizing diverse orchid species (continued).

Orchid host	Associated fungi	Fungal grouping	Nutritional mode	Location	Reference (*reference number*)
Aphyllorchis sp. and *Cephalanthera exigua*	*Thanatephorus* sp. Russalaceae Clavulinaceae *Resinicium* sp.*; Malassezia* sp.	*Rhizoctonia*-like fungi *Ectomycorrhizae* Endophytes Saprotrophs	Mycoheterotrophic (terrestrial)	Thailand	Roy et al. (2009a) (*15*)
Epipactis helleborine	Pezizales *Wilcoxina* sp. *Tuber* sp. *Exophiala*	*Ectomycorrhizae* Endophytes	partial Mycoheterotrophic	Japan	Ogura-Tsujita and Yukawa (2008) (*16*)
Hexalectris sp.	*Sebacinales*, Ceratobasidiaceae Russalaceae *Ascomicetes*	*Rhizoctonia*-like fungi *Ectomycorrhizae* Plant pathogens	Mycoheterotrophic (terrestrial)	USA	Kennedy et al. (2011) (*17*)
Apostacia sp.	*Botryobasidium* *Ceratobasidium* and *Tulasnella*	Saprotrophs *Rhizoctonia*-like fungi	Autotrophic (terrestrial)	Japan	Yukawa et al. (2009) (*18*)
Wullschlaegelia aphylla	Unspecified	Saprotrophs *Rhizoctonia*-like fungi	Mycoheterotrophic (epiphytic)	Italy	Selosse et al. (2010) (*19*)
Orchis militaris	*Tetracladium* sp.	Asexual aquatic hyphomycetes (potential plant endophytes)	Autotrophic (terrestrial)	Italy	Vendramin et al. (2010) (*20*)
Wullschlaegelia aphylla	*Mycenoid* sp.*;* Stereaceae sp. *Gymnopoid* sp.	Saprotrophs Endophytes	Mycoheterotrophic (Terrestrial)	France	Martos et al. (2009) (*21*)
Epipogium aphyllum	*Epipogium* sp.	*Ectomycorrhizae*	Mycoheterotrophic (Terrestrial)	France, Russia and Japan	Roy et al. (2009b) (*22*)
Gymnadenia conopsea	*Peziza Terfezia, Morchella, Geopyxis* and *Wilcoxina*	*Ectomycorrhizae*	Autotrophic (Terrestrial)	Germany	Stark et al. (2009) (*23*)
Lecanorchis sp.	*Lactarius*, *Russula* and Atheliaceae	*Ectomycorrhizae*	Mycoheterotrophic (Terrestrial)	Japan	Okayama et al. (2012) (*24*)
Orchis tridentate	*Fusarium* sp. *Candida* sp.	Saprotrophs	Autotrophic (Terrestrial)	Italy	Pecoraro et al. (2012) (*25*)


B
A
U.V.
Radiation
Darkness
MXO
MHO
Drought
C
D
Nutrient
poor soils
Humic substances
Carbohydrates
Carbohydrates
Mineral nutrients
E
Plant metabolites
Carbohydrates
Carbohydrates
Mineral nutrients
Water
ECM (5; 8; 9; 10; 13; 15; 16; 17; 22; 23; 24)
DSE (5; 6; 12)
SAP (4; 6; 15; 16; 21; 25)
PAT (1; 3; 4; 6; 17)
END (6; 15; 16; 20; 21)
AQH (20)

◀ **Figure 2.1** Schematic representations of some underground orchid-fungi connections and their possible fluxes under different environmental conditions. (**A**) Orchid. It represents all orchids reviewed, independent from its trophic strategy; MXO (mixotrophic); or MHO (partially mycoheterotrophic). (**B**) Plant. It represents a plant affected by pathogenic fungi. (**C**) Mushrooms from an ectomycorrhizal fungus. (**D**) Lignocellulosic residue. (**E**) Ectomycorrhizal forming tree root. ECM (ectomycorrhizal fungi); DSE (dark septate endophytes); SAP (saprobes); PAT (plant pathogens); END (plant endophytes); AQH (asexual aquatic hyphomycetes). Numbers in parenthesis indicate the reference number from **Table 2.1**.

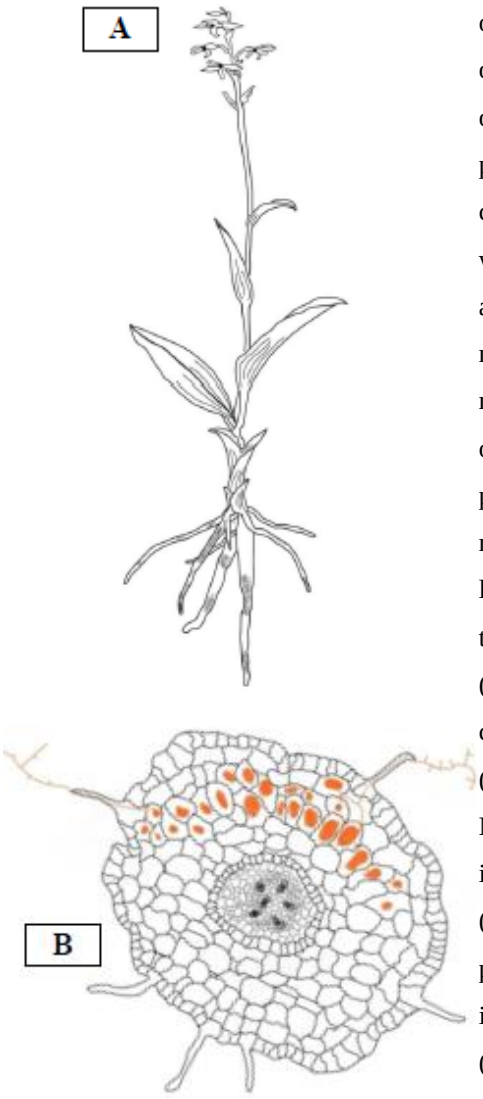
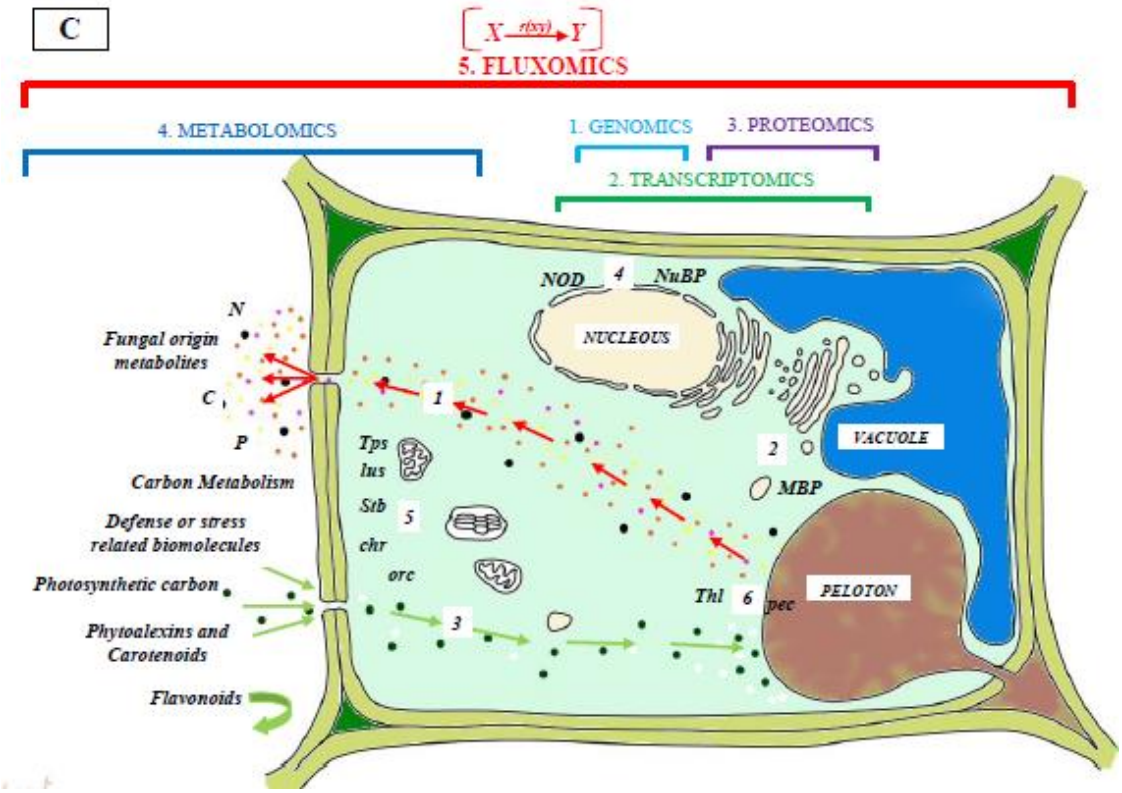


Figure 2.2 Schematic representations of metabolic interactions in orchid mycorrhiza interactions. (**a**) Partially mycoheterotrophic orchid root showing infected zones. (**b**) Representation of an orchid root showing infection through radical cortex and their pelotons as typical orchid mycorrhizal structures. (**c**) Main orchid compounds synthetized at orchid-fungus interface. Number in white box indicates the physiological process that has been appropriately cited in chapter 3 (OMICS in the study of orchid mycorrhizas. (**1**) Plasmodesmal nutrient (C, N, and P) and metabolite fluxes (related to trehalose accumulation) from fungal origin to orchid. (**2**) Production of hydrolytical enzymes and other proteins related to peloton degradation and hyphae control (i.e. mannose binding proteins, phenol oxidases; phosphate ammonia lyase, antifungal proteins). (**3**) Principal biomolecules identified in the colonized cell, related to pathogen control and stress (phytoalexins, carotenoids, oxidative burn control). The colonized cells are characterized by the depletion of flavonoid compounds. (4) Gene expression of mutualism related genes. NOD (nodulin); NuBP (nucleotide binding proteins). (**5**) Secondary metabolites involved in orchid mycorrhizal interactions: Lus (lusiathrin), Chr (chrysin), Orc (orchinol), Stb (stilbenoids), Tps (trehalose-6-phosphate synthase phosphatase). (**6**) Principal carbohydrates involved in orchid mycorrhizal interactions Thl (trehalose), Pec (pectin). OMICS showed in color letters and their hierarchies in the networks (numbers) were adapted from Klein & Heinzle, (2012).



CHAPTER III

Mycorrhiza 2017

doi:10.1007/s00572-016-0733-0

Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in South Central Chile

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Associated to specific objective 1.

Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in South Central Chile

3.1 ABSTRACT

Little is known about Orchidaceae plants in Chile and their mycorrhizal associations, a key issue for designing protective actions for endangered species. We investigated root fungi from seven terrestrial orchid species to identify potential mycorrhizal fungi. The main characteristics of *Rhizoctonia*-like fungi were observed under light microscopy and isolates were identified through PCR-ITS sequencing. Molecular identification of fungal sequences showed a high diversity of fungi colonizing roots. Fungal ability to germinate seeds of different orchids was determined in symbiotic germination tests; 24 fungal groups were isolated, belonging to the genera *Tulasnella*, *Ceratobasidium* and *Thanatephorus*, but also dark septate and other endophytic fungi were identified. The high number of *Rhizoctonia*-like fungi obtained from adult orchids from the Coastal mountain range suggests that, after germination, these orchids may complement their nutritional demands through mycoheterotrophy but beneficial associations with other endophytic fungi could also co-exist. In this study, isolated mycorrhizal fungi had the ability to induce seed germination at different efficiencies and with low specificity. Germination rates were low, but protocorms continued to develop for 60 days. A *Tulasnella* sp., isolated from *Chloraea gavilu* was most effective to induce seed germination of different species. The dark septate endophytic (DSE) fungi did not show any effect in seed development, but their widespread occurrence in some orchids suggests a putative role in plant establishment.

Key words: Orchid mycorrhiza; *Rhizoctonia*-like fungi; Root fungal endophytes; Symbiotic germination

3.2 INTRODUCTION

Orchid mycorrhizas are specific orchid-fungi interactions where orchids establish symbiosis with different fungal clades to sustain seed germination and establishment of the orchid (Gebauer & Meyer, 2003; Selosse et al., 2004). Waterman and Bidartondo (2008) listed some remarkable facts about orchid biology: (1) the Orchidaceae family is one of the largest plant families, with over 700 genera and an estimated 30,000–35,000 species in different habitats, about one-tenth of flowering plants (Dressler, 1993; Schultes, 1990); (2) orchids depend on mycorrhizal fungi for organic nutrients to germinate their seeds (Smith & Read, 2010); and (3) orchids obtain nutrients from the fungus, although little is known about the benefits to the fungus from the orchid (Cameron et al., 2007; Rasmussen, 1995).

The ability of orchids to establish a relationship with an appropriate mycorrhizal fungus is necessary to complete their life cycle and enable growth during the early stages of their reproduction and development (Rasmussen, 1995; Smith & Read, 2010). Several fungi participate and contribute to diverse vital processes in orchids: (1) the *Rhizoctonia*-like fungi (RLF) complex, which are the mycorrhizal fungi commonly associated with orchids; (2) ectomycorrhizas, which have been associated with green-leaved orchids (putatively autotrophic species) and also with fully mycoheterotrophic forest orchids; (3) Wood- or litter-decomposing fungi have been identified as mycorrhizal fungi in a range of fully mycoheterotrophic orchids; and (4) a broad spectrum of other different endophytic fungi without a specific role (Bayman & Otero, 2006; Roy et al., 2009b; Selosse et al., 2004). Orchid seeds are minuscule, produced in large numbers, each with minimal nutritional reserves (Rasmussen & Rasmussen, 2009; Smith & Read, 2010). This renders orchids dependent on mycorrhizal fungi for organic nutrient acquisition during the mycoheterotrophic stage, known as protocorm

(Sathiyadash et al., 2012). Under natural conditions, the orchid life cycle starts when dust-size seeds are dispersed by several mechanisms. During germination, a compatible mycorrhizal fungus provides carbohydrates to promote seed development (Bonnardeaux et al., 2007; Brundrett, 2002). All orchids have pelotons in mycorrhizal roots, which contains hyphae of mycorrhizal fungi that are separated from the root cell cytoplasm by the plasma membrane (Peterson et al., 1996). Pelotons can exchange nutrients between symbionts and are often digested and absorbed by the plant cell, providing beside carbon, mineral nutrients and water (Valadares 2014). The presence of intact and degraded pelotons are typical in mycorrhizal roots and both are a source of nutrients to the associated orchid; therefore, pelotons are mycoheterotrophic organs with a key role in orchid nutrition (Kuga et al., 2014; Smith & Read, 2010). Also, mycorrhizal colonization in later developmental stages of the plant may also include fungal species outside the RLF complex (Bayman & Otero, 2006; Hou & Guo, 2009).

Orchids grow in different habitats and possess different trophic strategies ranging from epiphytic to terrestrial and from autotrophic to fully mycoheterotrophic, comprising variable degrees of dependence on specific fungal and bacterial endophytes (McCormick et al., 2004; Puente & Bashan, 1994). Under natural conditions, all orchids are fully dependent on mycorrhizal fungi for germination (initial mycoheterotrophic stage), a feature that is retained for life in fully mycoheterotrophic orchids (Leake, 1994). In partially mycoheterotrophic orchids, the mycobiont supplies essential minerals and organic nutrients until its first green leaf emerges, when the plant can acquire carbon from two sources i.e. photosynthesis and fungal-derived carbon (Gebauer & Meyer, 2003; Merckx et al., 2009). Autotrophic orchids may become less dependent on mycorrhizal associations after the rapid development of the autotrophic apparatus in

leaves and photosynthetic roots of the seedlings (McCormick et al., 2004; Shefferson et al., 2008).

In Chile, Orchidaceae includes at least 63 species within seven genera (Pereira et al., 2014). All of these orchids are terrestrial with a high density of root tissue. We expected that partial mycoheterotrophism would be a key factor to compensate any deficit on energetic metabolism when orchids defoliate. Recently, some fungal strains associated with the orchids *Bipinnula fimbriata*, *Chloraea gavilu*, and *C. collicensis* in Chile, have been described (Pereira et al., 2014; Steinfort et al., 2010). However, given the large number of Chilean orchids and the lack of knowledge of their mycorrhizal associations under different environmental conditions, comprehensive studies regarding mycorrhizal status are important for safeguarding endangered species in symbiotic propagation strategies, since propagation of symbiotic plantlets is essential for orchids with narrow mycorrhizal associations (Bidartondo & Read, 2008; Swarts et al., 2010; Vendramin et al., 2010). Knowledge of diverse mycorrhizal fungi in Chile will be helpful for orchid propagation and understanding symbiotic mechanisms underlying colonization of different ecological niches in Coastal mountains and Andean habitats.

The purpose of this study was to isolate and identify mycorrhizal fungi associated with seven Chilean orchids and to test the ability of the isolated fungi to induce seed germination by examining roots of orchids growing in the Maule Region of central-southern Chile, six from the Andes and two from the Coastal Range.

3.3 MATERIALS AND METHODS

3.3.1 Study sites and sampling

Samples were collected during the flowering stage (November 2014) in the Maule Region of Chile. Eight locations with high orchid occurrence were selected for this

study (**Figure 3.1**). Different populations colonize different areas in the Andes and Coastal Range. At each site, the soil samples of rhizosphere were collected (0–20 cm deep) and brought to the laboratory where they were sieved at 2 mm and kept at 4ºC until further analysis. Their main characteristics are shown in **Table 3.1**. Orchid roots occupied the top 10 cm of soil or grew in organic matter accumulated above rocks. To avoid unnecessary damage to orchids, only a 5 cm segment of the active root system of each plant was excised and placed in paper bags.

3.3.2 Biochemical characterization of soil rhizosphere

In order to evaluate biochemical microsite differences between sampling locations, enzymatic assays were conducted. Acid phosphatase activity was measured using p-nitrophenyl phosphate (PNPP) as a substrate. β-glucosidase activity was determined by detection of p-nitrophenol (PNP) released from p-nitrophenyl-β-D-glucopyranoside (PNG). In both assays, the p-nitrophenol formed was determined in a spectrophotometer at 398 nm (Tabatabai & Bremner, 1969). Fluorescein diacetate (FDA) hydrolysis was assessed and expressed as μg fluorescein released per g of dry soil (Adam & Duncan, 2001). The final concentration of FDA was measured as absorbance at 490 nm.

3.3.3 Fungal isolation and characterization

Isolation of fungi was performed according to Valadares et al. (2011), with minor modifications. One day after sampling, root samples were washed in distilled water and cut into 5 cm segments. The surfaces of the fragments were disinfected by washing in 70% ethanol for 1 min and 4 min in 20% sodium hypochlorite solution (0.5% active chlorine), followed by five washings with sterile water. Fragments were transversely sliced and observed for presence of pelotons (hyphal coils) under a stereoscopic

microscope (40×). Root slices containing pelotons had the velamen (outer cell layer of the root cortex of orchids) removed; the remaining cortical fragments were washed in sterile water. Eight to ten fragments were placed on Petri dishes and incubated at 25 ± 1 °C in potato dextrose agar (PDA) supplemented with streptomycin (100 mg L^{-1}) to isolate orchid mycorrhizal fungi. Roots not containing pelotons were also incubated in PDA to isolate other endophytes unrelated to pelotons.

Critical steps in this isolation technique are the detection of features of orchid mycorrhiza inside living roots by observing pelotons inside brownish zones of roots followed by the observation of characteristics of RLF on Petri plates. Fungi with 90° hyphal branching, constrictions close to the branching point, and no spore formation (on PDA media) were classified as RLF (Otero et al., 2011; Valadares et al., 2011). Colonies were observed daily and radial growth was measured, and expressed in mm day^{-1}. Each selected fungus was transferred to Petri dishes containing fresh PDA plates and modified oatmeal agar (OMA medium) (4 g oats L^{-1}, 10 g agar, pH 5.6, supplemented with streptomycin 100 mg L^{-1}) for confirmation of RLF (Warcup, 1981). Other fungi that did not follow these criteria were classified as endophytes.

Plugs of each fungal colony were placed in the centre of Petri dishes containing OMA and PDA and incubated at 25 ± 1 °C. Diameter of the growing colony was measured daily. Fungal characteristics were observed at 10 and 20 days incubation and colour and growth rate were recorded. The growth rate was calculated as the mean difference between measurements of daily diameters, expressed in mm day^{-1}. Richness of the fungal species was estimated by the Margalef index, $Ri = (S - 1) / \ln N$, where S is the number of species in the assemblage, N is the number of isolates, and ln is the natural logarithm (Suryanarayanan & Kumaresan, 2000; Yuan et al., 2009). To compare endophyte assemblages inside orchid roots, the Jaccard similarity coefficient was

calculated: Sc = C (A + B – C)–1, where A and B are the total number of fungal species isolated from two orchid species and C is the number of fungal species common to both orchids; results were expressed as a percentage (Yuan et al., 2009).

Morphological features of isolated fungi were used to describe and classify the fungal diversity and also to select fungal groups to obtain DNA sequences. Microscope slides were placed on a paper towel inside Petri dishes and autoclaved. The paper towel was then moistened with 1 mL of sterilized deionized water and 300 µL of filtered and sterile (0.45 µm) liquid OMA medium spread across the slide. Mycelia plugs of each isolate were incubated on the slides at 28 ± 1 °C for 7 d. Samples were viewed under a fluorescence microscope. Each fungus was treated with 0.01% lactophenol cotton blue stain (LPCB) (61335, Sigma-Aldrich, St. Louis, MO) as the staining agent and Hoechst dye 1% solution (33342, Sigma-Aldrich) for 10 min to observe hyphal features. Mycorrhizal infections were estimated (Schatz et al., 2010), using the LPCB staining agent.

3.3.4 Molecular identification of fungal isolates

DNA extraction was performed from liquid cultures of the different isolates, using the DNeasy Plant Mini Kit (69104, Qiagen, Hilden, Germany). Oligonucleotide primers were used to amplify the internal transcribed spacers of the ITS1 region (5′ - TCCGTAGGTGAACCTGCGG) and ITS4 region (5 ′ - TCCTCCGCTTATTGATATGC) of genomic rDNA (White et al., 1990). The PCR cycle was initial denaturing at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 1 min each, annealing at 55.5 °C for 1 min, extension at 72 °C for 1 min, and final extension for 5 min at 72 °C. PCR products were purified using the QIAquick PCR

purification kit (28104, Qiagen) prior to sequencing. Sequencing was performed by Macrogen (Seoul, South Korea).

3.3.5 Sequence analysis

A BLAST search was conducted to find the closest known sequences with the ITS region of nuclear rDNA. All sequences were deposited in the GenBank (accession numbers KP278146 to KP278169). Multiple sequence alignment was carried out and visually checked with the following modifications: fungal sequences were aligned, using ClustalX with default conditions for gap opening and gap extension penalty (Larkin et al., 2007). All positions containing gaps and deletion data were eliminated from the dataset, using BioEdit software (Hall, 1999). Phylogenetic trees were constructed with the neighbour-joining method, using the MEGA 6 software (Chen et al., 2011; Tamura et al., 2013).

3.3.6 Symbiotic germination tests

In order to know the effect of different isolated mycorrhizal fungi on seed development, a multiple germination test was performed. Seeds were sowed in OMA and potential specificity was tested for different fungal strains. Prior to the symbiotic seed germination tests, a tetrazolium test was conducted to assess seed viability (Lakon, 1949). The surface of 200 µg of seeds from one mature fruit capsule were disinfected according to Dutra et al. (2009), with minor modifications. Seeds were immersed in an 8:1:1 solution of 8 mL of sterile deionized water, 1 mL sodium hypochlorite (5% chlorine), 1 mL 100% alcohol for 3 min, followed by five washes in sterile deionized water. Seeds were placed in a suspension of 50 mL deionized water. Then, 500 µL of this suspension was placed in Petri dishes containing 20 mL modified OMA medium (4 g oats L^{-1} and 10 g agar at pH 5.6, supplemented with 100 mg L^{-1} streptomycin) and a

plug of mycelia for fungal inoculation. A randomized design was used, with each fungal isolate as a treatment, with ten replicates per treatment, and a control of ten un-inoculated plates. The percentage of germination per plate was measured (Steinfort et al., 2010). Plates were incubated in the dark at 25 ± 1 °C for 8 weeks. Each Petri dish was then analysed under a microscope (20–40×) and germination stages of 100 seeds per plate were recorded (Stewart & Kane, 2006). The growth index was calculated (Valadares et al., 2011): $GI = (N_1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4) / (N_0 + N_1 + N_2 + N_3 + N_4)$, where N_0 is the number of seeds in stage 0, N_1 is the number of seeds in stage 1, and so on.

3.3.7 Statistical analyses

All data were analysed using one-way ANOVA to compare each treatment according to measurements of enzyme activity. Prior to statistical analysis, data were tested for normality and homogeneous variances. For the symbiotic germination test, ANOVA was performed between germination stages, where stages 2 and 3 were likely to produce protocorms, with the potential to become adult plants, as defined by Mitchell (1989). All statistical tests were conducted using R software (Ihaka & Gentleman, 1996). The results were compared using the SD of means and Tukey's multiple range tests. Statistical significance was set at $p < 0.05$.

3.4 RESULTS

3.4.1 Sampling

Seven orchid species were found colonizing different soil types (**Table 3.1**). Two species came from the Andes and six from the Coastal Range. The specimens were found growing at the ground surface, with their roots penetrating the first few centimetres of soil, sand, or penetrating organic matter derived from herbaceous plants

on rocks. The chemical and general enzymatic characterizations of the soils are summarized in **Table 3.4**. In general, soil substrates were very similar, with the exception of phosphorus content, with the highest content in 'Constitución' and 'San Clemente' soils (~4-fold compared to the lowest content). Soil enzyme activity was statistically different between soils. San Clemente soils had the highest enzyme activity and organic matter content. The soil biochemical properties are statistically different between soils sampled. The San Clemente soils had the highest microbial activity, as reflected in higher rates of FDA hydrolysis, acid phosphatase, and β-glucosidase activity ($p < 0.05$).

3.4.2 Fungal isolation and morphological characterization

All orchid roots evaluated in this work were colonized by mycorrhizal fungi. Estimates of mycorrhizal colonization were dependent on orchid habitat, being *Chloraea bletioides* and *Bipinnula fimbriata* which were found growing on rocky substrates showing the highest mycorrhizal infection (50 ± 8%), with higher frequencies of degraded pelotons throughout the cortical region. There were fewer pelotons and few brownish root zones (a sign of mycorrhization) in fragments from orchids growing in loose soil. In both cases, colonized root fragments were easily distinguished from non-colonized fragments (**Figure 3.2**). We made a preliminary classification of isolated fungi as RLF (mycorrhizal fungi) and endophytes, mainly based on morphological features, such as colony and hyphal characteristics and absence of spore formation, as seen in **Figure 3.2** (Valadares et al., 2011). At least one RLF was isolated from each orchid and a variable number of endophytes were found (**Table 3.2**). In total, 224 mycobionts were isolated, of which 96 isolates correspond to the RLF complex (isolates ORK1 to ORK13), whereas 128 were classified as endophytes and other unidentified fungi (isolates ORK14 to ORK24). Margalef's diversity index of endophytic fungi in

the eight orchids were significantly greater in *C. longipetala* (1.55) and *C. gavilu* (1.41) from San Clemente soil and was lowest in *C. chrysantha* (0.69) and *C. gavilu* (0.62) from Constitución soil (**Table 3.2**). Based on morphological characteristics, orchid source and growth rate on PDA and OMA media, 24 groups of fungal isolates were formed (see **Table 3.5**). After 10 and 20 days of incubation at 25 °C on PDA and OMA media, fungal colonies had either black, white, cream, or brown colours. The technique we used to isolate RLF was very successful. Without prior identification of colonized root segments and pelotons, a relatively large proportion of endophytes may be isolated from the orchid roots. The composition of the endophytic fungal community varied according to the orchid species, with different similarity coefficients even at the same sampling location (see **Table 3.6**).

3.4.3 Molecular identification and phylogenetic analyses

BLAST searches are summarized in **Table 3.3.** According to phylogenetic analyses fungi colonizing particular orchids are not clustered (**Figure 3.3**). However, among orchid mycorrhizal fungi, two clades were observed (orders *Tulasnellales* and *Ceratobasidiales*). Isolate ORK6 showed high homology with a Tulasnellaceae isolated from *Gavilea australis*, a genus with 17 species among Chilean orchids (Novoa et al., 2015). Sequence identities ranged from 95% to 99%; the species were accepted when identity between query and match was >99%, and the genus was accepted when >95% (Chen et al., 2011; Sánchez et al., 2008). According to morphological and molecular data, 13 different fungal sequences, corresponding to the RLF complex and 11 different endophyte sequences were identified, with three matching with dark septate endophytes (DSE) (*Leptotidium orchidicola*, *Cadophora* sp., and *Phialocephala fortinii*).

For *Rhizoctonia*-like fungi, ITS sequences of isolates ORK1 and ORK2 (from *C. crispa* and *C. longipetala*, respectively) were classified as *Ceratobasidium* spp., based on their closest match in the GenBank database. Similarly, the sequence of isolate ORK3 (from *C. longipetala*) was classified as *Thanatephorus* sp. Sequences from isolates ORK4 to ORK13 (from *C. crispa*, *B. fimbriata*, C. *gavilu, C. chrysantha*, *C. grandiflora*, *C. bletioides*, and *C. longipetala*) were of variable homology with different fungi in the order *Tulasnellales* (**Table 3.3**, references therein). We also found other fungal endophytes in cortical tissues, especially from the orchids of the Andes (52 isolates). ITS sequences of isolates ORK 14 and ORK 16 (from *C. longipetala* and *C. crispa*, respectively) were classified as *Phomopsis columnaris*. Sequences ORK17 and ORK18 (from *C. gavilu* and *C. grandiflora*) were classified as *Leptodontidium* spp. Sequence ORK19 (from *C. grandiflora*) was classified as *Cadophora* sp. Sequences ORK20 and ORK 21 (from *C. chrysantha* and *C. bletioides*, respectively) were classified as *Chaetomium* sp. and *C. globosum*, respectively. Sequence ORK24 was classified as *Phialocephala fortinii*. Based on their low homology, sequences ORK15, ORK22, and ORK23 were designated as unidentified fungi (Chen et al., 2011). All information regarding molecular identification of fungal isolates is shown in **Table 3.3**.

3.4.4 Potential of mycorrhizal fungi to induce seed germination

To test the specificity of the mycorrhizal interactions, a multiple germination test was used over 8 weeks. The tetrazolium test revealed variable viability of seeds, with *B. fimbriata* having the highest viability (65%) and *C. longipetala* having the lowest (45%). The results of the germination test of the seven fungal isolates are shown in **Figure 3.4**. All isolated RLF fungi have potential to promote seed germination to varying degrees, even when the fungus does not come from the same orchid species. The overall germination index was greatest for ORK13 strain (see **Table 3.7**). This

fungus can promote sprouting to further stages of development with very low specificity to orchid species. In the germination test, there were no significant differences among fungal isolates in the first stages of germination ($p < 0.05$). However, when later stages were analysed, significant differences were found (**Figure 3.4**). The potential specificity (potential of mycorrhizal fungi to induce germination of other orchid species) was highly variable. Seeds of *B. fimbriata* did not exhibit any specificity for mycorrhizal fungi, reflected in its high germination index (see **Table 3.7**). Germination of *C. gavilu* and *C. grandiflora* was more stimulated by specific mycorrhizal fungi (**Figure 3.4**). In control treatments, we observed that some embryos may break the seed coat similar to inoculated treatments; nevertheless, no further development occurred. Additionally, the effect of the different endophytic strains (including dark septate endophytes) was assessed but no evidence of statistically significant differences were observed, resulting in minimal development and with many decayed embryos.

3.5 DISCUSSION

Growth of orchids in an ecosystem depends on environmental factors, including temperature, soil type, competition, pollination, and the presence of mycorrhizal fungi (Gregg & Kéry, 2006; Huber et al., 2005; Swarts et al., 2010). Mycorrhizal fungi are essential for successful symbiotic development of orchids under natural conditions, to reintroduce endangered species to their natural environment, or cultivate species of ornamental and medical interest (Chen et al., 2011; Nontachaiyapoom et al., 2010; Pereira et al., 2003). Roots of most green orchids host a variety of fungi, mainly including species of *Tulasnella*, *Ceratobasidium*, *Sebacina*, and *Thanatephorus* (Smith & Read, 2010; Sneh, 1996). In our study, the most common mycobiont belonged to *Tulasnella* spp. which have been reported as commonly associated to terrestrial orchids (Ogura-Tsujita et al., 2012). We also identified strains of *Ceratobasidium* and

Thanatephorus, described as typical mycorrhizal fungi in terrestrial orchids (Steinfort et al., 2010; Valadares et al., 2011).

The degree of specificity between orchids and their mycorrhizal fungi is highly variable and is influenced by environmental conditions and surrounding plant species (Otero et al., 2011; Selosse & Roy, 2009; Waterman & Bidartondo, 2008). Fungi associated with *C. gavilu* were different in the two sampling areas. This was determined in other studies that show mycorrhizal preferences according to different habitats and even individual orchids, which can have more than one mycorrhizal fungus inside its roots (Jacquemyn et al., 2014; Jacquemyn et al., 2010; McCormick et al., 2004; Shefferson et al., 2008). The form of fungal associations vary considerably, from broad, with orchids that are compatible with several fungal groups (*C. longipetala*), to narrow, with orchids that are compatible with a few groups of fungi (*C. gavilu* from the Coastal range of Chile). Similar results are reported by Steinfort et al. (2010) who identified at least five different mycorrhizal fungi colonizing roots in *B. fimbriata* and by Pereira et al. (2014) who identified two fungi colonizing roots of *C. collicensis* and *C. gavilu*. It is crucial in the classification of mycorrhizal fungi to perform germination tests, using isolated fungi that promote germination under in vitro conditions (Shimura & Koda, 2005; Tešitelová et al., 2012; Valadares et al., 2011).

Symbiotic and asymbiotic seed propagation have been tested in the Chilean orchid *B. fimbriata* to find effective laboratory strategies to preserve this endemic orchid. These reports do not include orchids from the *Chloraea* genus (Pereira et al., 2015; Pereira et al., 2014; Steinfort et al., 2010). Our study showed that all isolated mycorrhizal fungi (RLF) have the ability to induce seed germination and orchid development to different degrees with the presence of protomeristem and rhizoids, especially species of *Tulasnella* and *Ceratobasidium*. Terrestrial orchids associated with

fungi from the polyphyletic *Rhizoctonia* group are effective in promoting seed development (Batty et al., 2006; Valadares et al., 2011). In this case, the strain that is closely related to *Thanatephorus* was not able to induce rhizoid formation, but the presence of a protomeristem is an indication of seed development. Although most mycorrhizal fungi were able to induce seed germination up to the third phase of development, the strain *Tulasnella* ORK13 was the only one able to induce seed development and promote growth of protocorms to promote later developmental stages; thus, it is a candidate for conservation and reintroduction programs.

On the other hand, our results have shown that some seeds continue to develop in the control media (OMA without mycorrhizal fungus) a process previously described by Fracchia et al. (2014). These authors suggest that some embryos have the ability to develop without specific nutrients; however, those seed are not able to continue protocorm development. Our experiments showed that all orchids are able to form associations that are different under laboratory conditions, allowing fungi to promote seed germination in orchids that are not associated in nature with the target orchid (Esitken et al., 2005).

In our germination tests, the slow growth rate from seed to protocorm might be attributed to the physiological status of the orchids used as source of mycorrhizal fungi since we used colonized roots of adult plants rather than orchids at young seedling stages. Despite isolating fungi from adult orchids is considered to be a successful strategy to study mycorrhizal diversity in terrestrial orchids (Jiang et al., 2015; Steinfort et al., 2010; Valadares et al., 2011), using fungi from protocorms (young seedling stages) is often more suitable to study seed germination (Rasmussen, 1995). In our experiments seeds start to break the testa after 30 days in darkness, which disagrees with shorter times reported for some related orchids (Fracchia et al., 2014). This

observation suggests a possible fungal switch under natural conditions, with mycorrhizal fungal lineages being different within plant development stages (Masuhara & Katsuya, 1994; McCormick et al., 2006). However, orchid seed development using fungi isolated from adult roots slowly continues. The mycobionts are candidates to be considered in recovery programs by their ability to germinate orchid seeds without fungal switch after protocorm stage, especially the isolate *Tulasnella* ORK13, which was identified as most effective mycorrhizal fungus by its ability to promote seed germination non-specifically until later developmental stages.

Endophytic growth of non-mycorrhizal fungi has often been reported in some orchids, but little attention has been given to the beneficial or detrimental effect on orchid metabolism (Bayman & Otero, 2006; Chen et al., 2011; Yuan et al., 2009). We observed that at least five strains of fungi belonged to genera of non-orchid mycorrhizal fungi, especially fungi isolated from *C. grandiflora* and *C. gavilu* from the Andes. Among terrestrial orchids, different DSE have been reported to promote seed germination or establish pelotons after inoculation of asymbiotic germinated plantlets (Fracchia et al., 2014; Hou & Guo, 2009). Abundance of several plant species in the same habitat and also the climatic conditions characteristic of the Andean region, promote the establishment of plant linkages through fungal mycelium, as shown in mycoheterotrophic orchids (Bidartondo et al., 2004; Dearnaley, 2007; McKendrick et al., 2000; Simard & Durall, 2004), but the role of non-mycorrhizal fungi in orchid development has not been explored.

This is the first study of the Pacific side of the Andes to identify fungi belonging to the polyphyletic *Rhizoctonia* group and root fungal endophytes. We showed that seed germination is promoted by mycorrhizal fungi associated with terrestrial orchids in a section of two mountain ranges in South Central Chile.

3.6 CONCLUSIONS

The terrestrial orchids studied here did not have a preference for a specific mycorrhizal fungus, showing associations ranging from typical orchid mycorrhizas to endophytes, with the fungal order *Tulasnellales* as the most common mycorrhizal symbionts and *Leptodontidium* sp. as the most common fungal endophyte. It was possible to promote development of orchid seeds with different mycorrhizal fungi which are well known to contribute to plant fitness. Many of the isolated dark septate fungal clades have been reported, but their widespread occurrence in orchid radical tissues from the Andes deserves more attention.

3.7 ACKNOWLEDGEMENTS

Funding was provided by FONDECYT project 1130662 and CONICYT fellowship 21130491 to H. Herrera. The authors thank Karina Godoy for her invaluable technical assistance in image acquisition and Ira Fogel at CIBNOR, Mexico for English and editorial services.

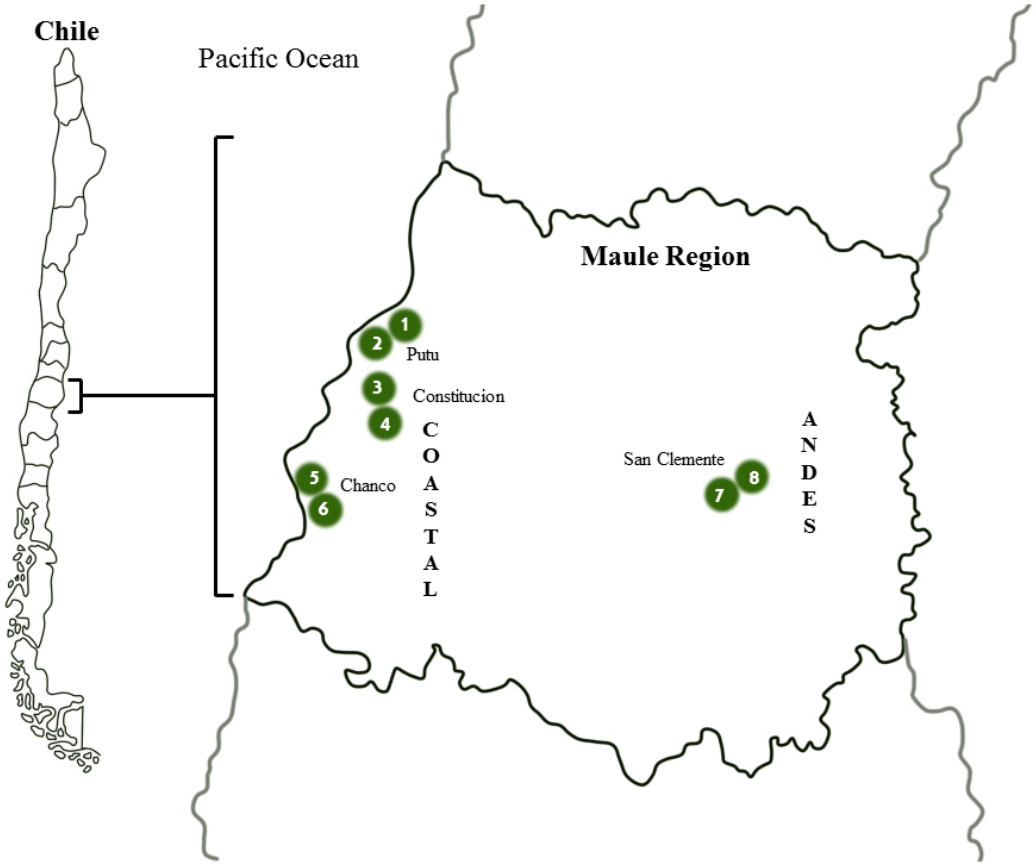

Figure 3.1 Sampling sites of the Maule region in South-Central Chile. Orchids 1 to 6 were found in the Coastal and orchids 7 and 8 were found in the Andean. *Chloraea chrysantha* = O1; *Chloraea gavilu* = O2; *Chloraea bletioides* = O3; *Bipinnula fimbriata* = O4; *Chloraea crispa* = O5; *Chloraea longipetala* = O6; *Chloraea grandiflora* = O7; and *Chloraea gavilu* = O8.

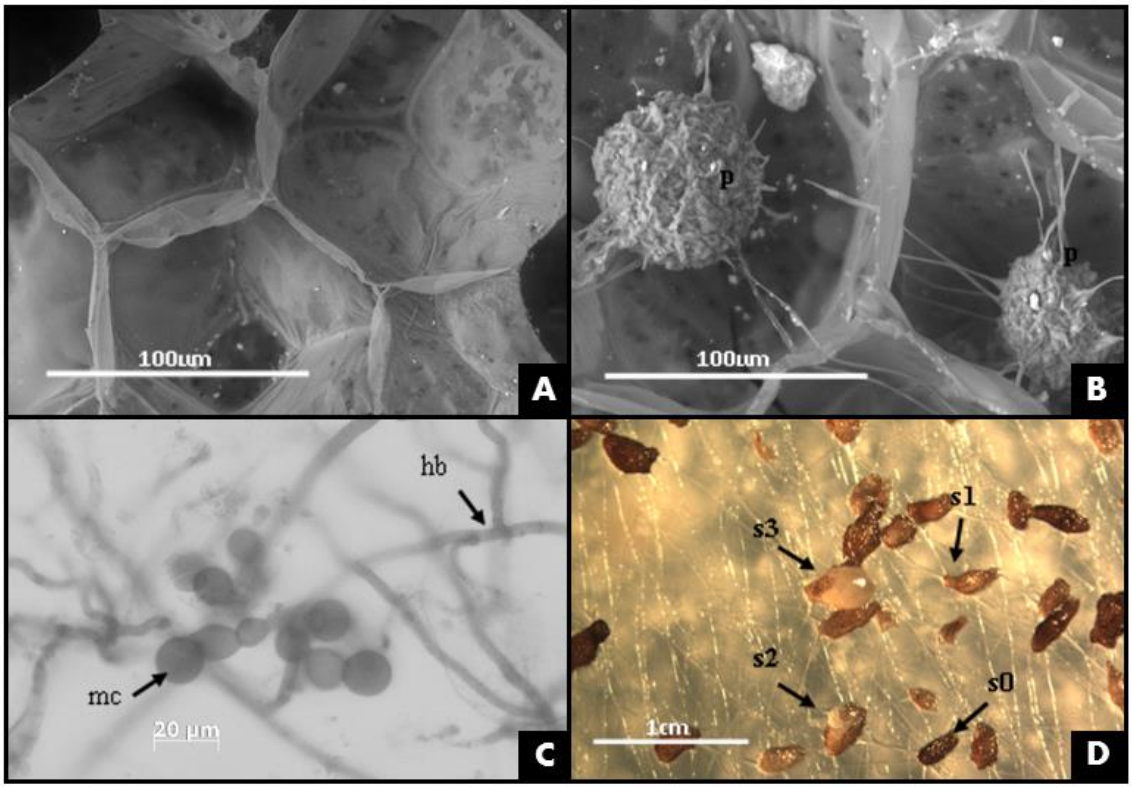

Figure 3.2 *Chloraea grandiflora* roots not colonized (**A**); and colonized, showing pelotons (p) (**B**) by mycorrhizal fungi; (**C**) Monilioid cells of *Tulasnella* sp. growing in PDA medium (hb = 90° hyphal branching; mc = monilioid cells); and (**D**) Symbiotic seed germination of *Chloraea chrysantha* seeds showing different development stages, after 6 weeks in OMA medium (s0 = stage 0, s1 = stage 1, s2 = stage 2, s3 = stage 3).

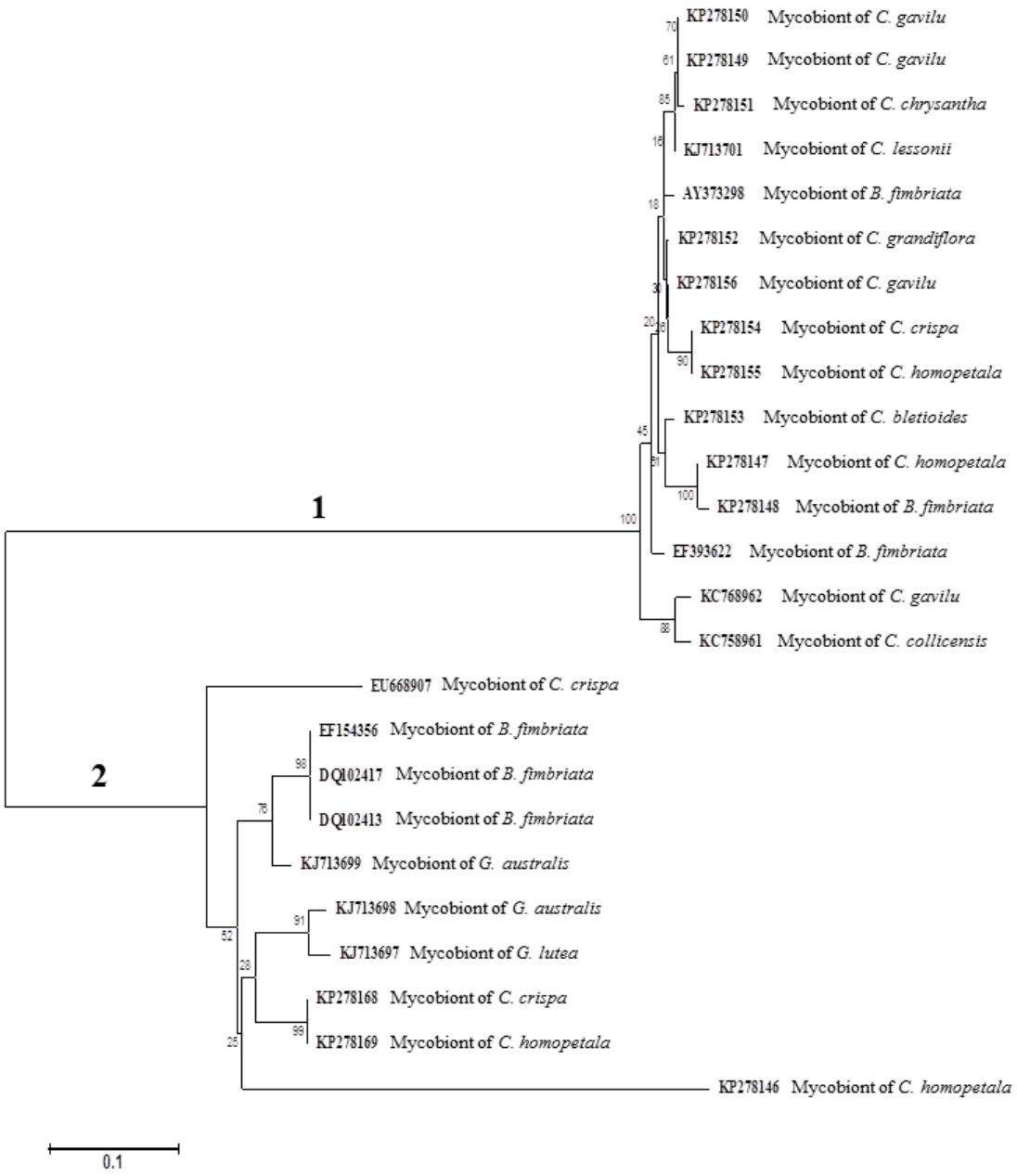

Figure 3.3 Maximum like hood tree of its sequences of *Rhizoctonia*-like isolates showing relationships in terms of distance. Numbers near branches refer to bootstrap support values. Numbers in boxes indicate principal clades (**1** = *Tulasnellales*; **2** = *Ceratobasidiales*). The tree also includes fungal sequences isolated from related orchids.

Figure 3.4 Germination assay showing effects of the isolated fungi in protocorm development of orchid seeds in OMA media. Same letters in each subfigure are not significantly different, according to Tukey's multiple range tests ($p < 0.05$). Shaded bars show fungi isolated from the same orchid. ORK1 and ORK2 *Ceratobasidium* spp., ORK3 *Thanetophorus* sp. and ORK4 to ORK13 *Tulasnella* spp. ▶

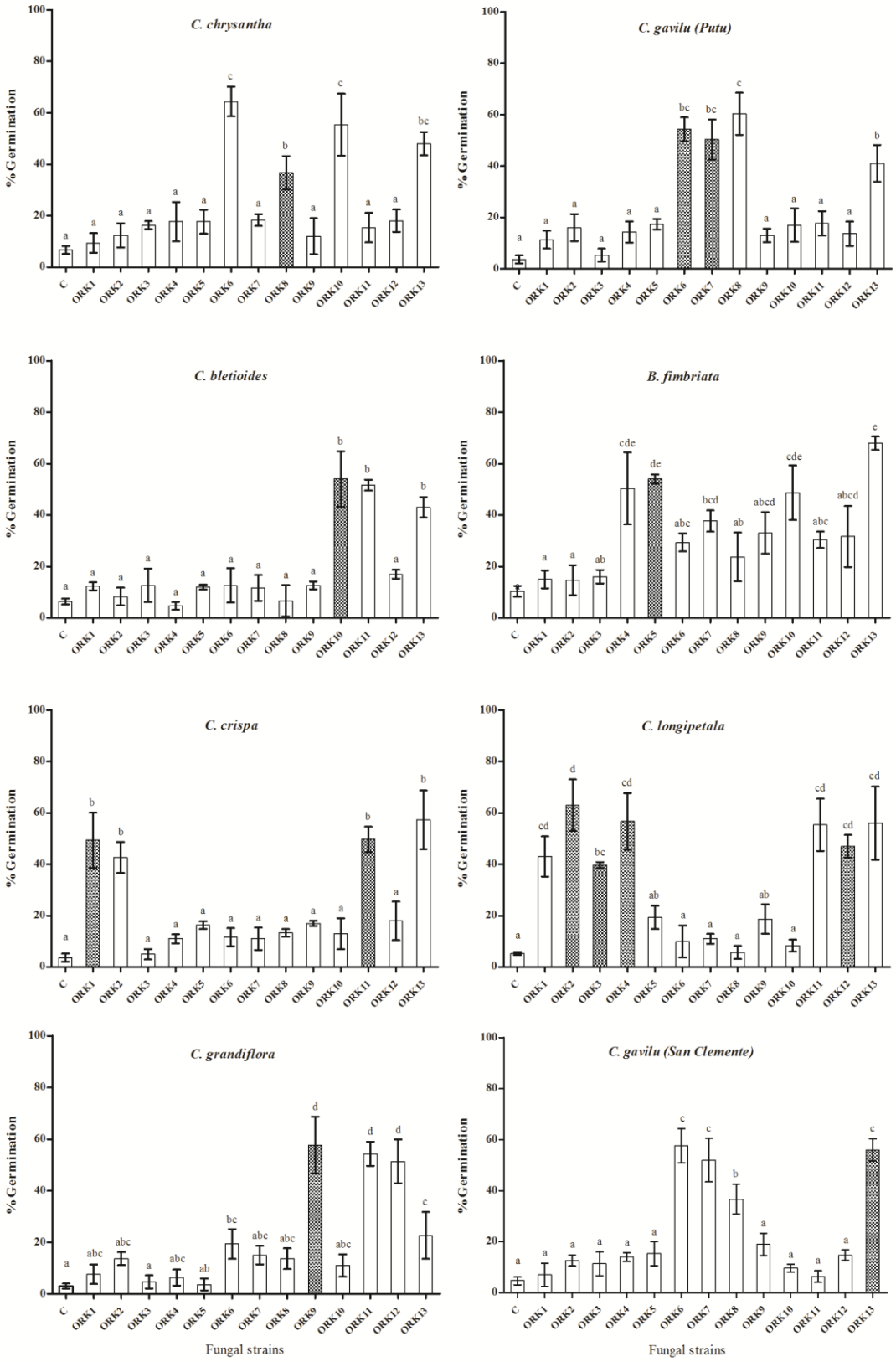
C. chrysantha
C. gavilu (Putu)
C. bletioides
B. fimbriata
C. crispa
C. longipetala
C. grandiflora
C. gavilu (San Clemente)
% Germination
Fungal strains
C
ORK1
ORK2
ORK3
ORK4
ORK5
ORK6
ORK7
ORK8
ORK9
ORK10
ORK11
ORK12
ORK13

Table 3.1 List of species and locations of orchids sampled in the experiments.

Plant species /common name	Label	Location	Growth habitat	Number of sampled plants
Chloraea chrysantha Poepp.*/ Tulipán del monte (mountain tulip)	**O1**	Constitución (35°49′57″S 72°18′87″W)	Soil (Ultisol)	4
Chloraea gavilu Lindl. / Gavilu	**O2**	Constitución (35°49′57″S 72°18′87″W)	Soil (Ultisol)	4
Chloraea bletioides Lindl.* / Pico de loro (Parrot beak)	**O3**	Putu (35°24′40″S 72°36′20″W)	Organic matter over rocks	4
Bipinnula fimbriata Phil. I. M. Johnst.* / Flor del Bigote (Flower mustache)	**O4**	Putu (35°24′40″S 72°36′20″W)	Sand and Organic matter over rocks	4
Chloraea crispa Lindl.* / No common name	**O5**	Chanco (35°70′39″S 72°44′05″W)	Dune sand	4
Chloraea longipetala Phil.* / No common name	**O6**	Chanco (35°72′48″S 72°54′49″W)	Dune sand	4
Chloraea grandiflora Poepp. **/ Gavilu	**O7**	San Clemente (35°58′77″S 71°02′79″W)	Soil (Andisol)	4
Chloraea gavilu Lindl.** / Gavilu	**O8**	San Clemente (35°58′77″S 71°02′79″W)	Soil (Andisol)	4

*Orchids sampled in the Coastal range; ** Orchids sampled in the Andean range

Table 3.2 Occurrence of the different fungi inside roots of orchids in The Andes and Coastal range in Región del Maule.

Source/Host	*Thanetophorus* sp.	*Ceratobasidium* sp.	*Tulasnella* sp.	*Phomopsis columnaris*	*Unidentified 1*	*Leptodontidium* sp.	*Cadophora* sp.	*Chaetomium* sp.	*Unidentified 2*	*Phialocephala fortinii*	**Margalef's richness index**
Coastal range											
Chloraea chrysantha	-	-	13	-	-	2	-	3	-	-	**0.692**
Chloraea gavilu	-	-	15	-	6	4	-	-	-	-	**0.621**
Chloraea bletioides	1	-	20	-	12	-	2	6	5	-	**1.306**
Bipinnula fimbriata	2	5	8	-	-	1	-	-	-	-	**1.082**
Chloraea crispa	-	-	3	15	-	1	-	-	-	4	**0.957**
Chloraea longipetala	1	3	6	10	-	3	-	-	-	2	**1.553**
Frequency*	0.026	0.052	0.425	0.163	0.118	0.072	0.013	0.059	0.033	0.039	
Andes											
Chloraea gavilu	-	-	11	1	2	9	10	-	-	2	**1.406**
Chloraea grandiflora	-	-	8	3	-	14	4	-	2	5	**1.395**
Frequency*	0	0	0.268	0.056	0.028	0.324	0.197	0	0.028	0.099	
Number of isolates	*4*	*8*	84	29	20	34	16	9	7	13	
									TOTAL		**224**

*Estimated frequency of isolation of each fungal species in the Coastal range and the Andean.

Table 3.3 Molecular identification of potential mycorrhizal fungi isolated from orchids in the Andes and Coastal range based on the closest match in the GenBank database.

	Fungal isolate	GenBank Accession number	Host	Close relatives (Accession number)	% identity	Source	Reference
Rhizoctonia-like fungi							
	ORK1	**KP278168**	*Chloraea crispa*	*Ceratobasidium* sp. (**HQ914092**)	99%	*Sarcochilus parviflorus*	(Gowland et al., 2013)
	ORK2	**KP278169**	*Chloraea longipetala*	*Ceratobasidium* sp. (**HQ914091**)	99%	*Sarcochilus hillii*	(Gowland et al., 2013)
	ORK3	**KP278146**	*Chloraea longipetala*	*Thanetophorus* sp. (**KJ777649**)	97%	Unspecified	Genbank
	ORK4	**KP278147**	*Chloraea longipetala*	Uncultured Tulasnellaceae (**JX545220**)	99%	*Dendrobium officinale*	(Xing et al., 2013)
	ORK5	**KP278148**	*Bipinnula fimbriata*	Uncultured Tulasnellaceae (**JX545220**)	98%	*Dendrobium officinale*	(Xing et al., 2013)
	ORK6	**KP278149**	*Chloraea gavilu*	*Tulasnella* sp. (**KJ713701**)	99%	*Gavilea australis*	(Fracchia et al., 2014)
	ORK7	**KP278150**	*Chloraea gavilu*	*Uncultured* Tulasnellaceae (**JX649080**)	99%	*Anacamptis morio*	(Bailarote et al., 2012)
	ORK8	**KP278151**	*Chloraea chrysantha*	*Tulasnella* sp. (**KJ713701**)	99%	*Gavilea australis*	(Fracchia et al., 2014)
	ORK9	**KP278152**	*Chloraea grandiflora*	Uncultured Tulasnellaceae (**JQ994398**)	99%	*Piperia yadonii*	(Pandey et al., 2013)
	ORK10	**KP278153**	*Chloraea bletioides*	Uncultured Tulasnellaceae (**JX649082**)	98%	*Anacamptis morio*	(Bailarote et al., 2012)
	ORK11	**KP278154**	*Chloraea crispa*	Uncultured Tulasnellaceae (**JQ994398**)	97%	*Piperia yadonii*	(Pandey et al., 2013)
	ORK12	**KP278155**	*Chloraea longipetala*	Uncultured Tulasnellaceae (**JQ994397**)	97%	*Piperia yadonii*	(Pandey et al., 2013)
	ORK13	**KP278156**	*Chloraea gavilu*	Tulasnellaceae sp.(**JX138565)**	96%	*Diuris magnifica*	(Sommer et al., 2012)
Endophytes							
	ORK14	**KP278157**	*Chloraea longipetala*	*Phomopsis columnaris* (**KM519653**)	99%	Olive cultivar *Cobrancosa*	Genbank
	ORK15	**KP278158**	*Chloraea bletioides*	*Catenulostroma germanicum* (**EU019253**)	95%	Stone	(Crous et al., 2007)
	ORK16	**KP278159**	*Chloraea crispa*	*Phomopsis columnaris* (**KM519653**)	99%	Olive cultivar *Cobrancosa*	Genbank
	ORK17	**KP278160**	*Chloraea gavilu*	*Leptodontidium orchidicola* (**AF486133**)	97%	*Platanthera hyperborean*	Genbank
	ORK18	**KP278161**	*Chloraea grandiflora*	*Leptodontidium orchidicola* (**GU479910**)	97%	*Trillium tschonoskii*	Genbank
	ORK19	**KP278162**	*Chloraea grandiflora*	*Cadophora* sp. (**JN859252**)	99%	*Juniperus communis*	(Knapp et al., 2012)
	ORK20	**KP278163**	*Chloraea chrysantha*	*Chaetomium globosum* (**JQ964802**)	97%	Unspecified	Genbank
	ORK21	**KP278164**	*Chloraea bletioides*	*Chaetomium globosum* (**JN209870**)	99%	Unspecified	Genbank
	ORK22	**KP278165**	*Chloraea bletioides*	*Catenulostroma germanicum* (**EU019253**)	95%	Stone	(Crous et al., 2007)
	ORK23	**KP278166**	*Chloraea gavilu*	*Penicillium chrysogenum* (**JF834167**)	95%	Sea sediments	Genbank
	ORK24	**KP278167**	*Chloraea gavilu*	*Phialocephala fortinii* (**KJ817297**)	99%	*Vaccinium vitis*	Genbank

Table 3.4 Chemical and enzymatic characterization of soil related to the sampled orchids in the Coastal range and Andes in South Central Chile.

Location:	Constitución	Putu	Chanco	San Clemente
N (mg kg^{-1})	11	11	14	18
P (mg kg^{-1})	26	7	5	25
K (mg kg^{-1})	117	160	102	211
pH (of water)	6.73	6.35	5.57	5.11
Organic matter (%)	3	4	4	20
K (cmol+ kg^{-1})	0.30	0.41	0.26	0.54
Na (cmol+ kg^{-1})	0.65	0.18	0.20	0.10
Ca (cmol+ kg^{-1})	6.40	23.4	5.04	4.73
Mg (cmol+ kg^{-1})	2.06	7.26	1.76	1.41
Al (cmol+ kg^{-1})	0.01	0.01	0.08	0.47
Al saturation (%)	0.11	0.03	1.09	6.48
CEC (cmol+ kg^{-1})	9.42	31.23	7.34	7.25
Base saturation (cmol+ kg^{-1})	9.41	31.22	7.26	6.78
Cu (mg kg^{-1})	1.49	2.61	1.63	12.69
Enzyme activities				
Acid phosphatase (µmol p-nitrophenol g^{-1} m^{-1})	63.27 ± 3.81 d*	95.01 ± 2.72 c*	158.31 ± 3.16 b*	172.45 ± 7.62 a*
β-glucosidase (µmol p-nitrophenyl g^{-1} h^{-1})	32.28 ± 2.16 c*	39.03 ± 1.59 b*	31.66 ± 1.30 c*	65.02 ± 5.25 a*
FDA-hydrolysis (µg fluorescein g^{-1})	71.05 ± 1.83 c*	52.38 ± 2.47 d*	75.82 ± 2.07 b*	121.95 ± 11.05 a*

*Results are means ± standard deviation. Different letters indicate statistical differences between soils ($p < 0.05$; n = 9).

Table 3.5 Morphological characterization of root fungal endophytes isolated from orchid roots on two growth media (Oatmeal and Potato Dextrose Agar). Results are means ± standard deviation (n = 4).

Isolate	Colour				Growth rate (mm day^{-1})		Hyphal width (µm)	Monilioid cell size	
	10 days		20 days		2 days	5days			
	OMA	PDA	OMA	PDA	PDA			width(µm)	length (µm)
Rhizoctonia-like fungi									
ORK1	White	Cream	Cream	Light brown	10.4 ± 3.4	15.5 ± 4.2	4.2 ± 1.6	8.8 ± 2.5	17.7 ± 0.6
ORK2	White	Cream	Cream	Light brown	11.7 ± 2.6	16.7 ± 2.9	5.4 ± 1.1	9.3 ± 1.8	29.4 ± 0.9
ORK3	Brown	Light brown	Brown	Brown	4.3 ± 1.2	13.2 ± 1.3	8.4 ± 0.5	12.3 ± 3.0	18.7 ± 6.5
ORK4	White	White	White	White	8.5 ± 2.4	12.3 ± 0.2	4.8 ± 0.2	6.2 ± 2.4	13.2 ± 3.5
ORK5	White	White	White	White	8.3 ± 1.6	14.4 ± 0.8	4.2 ± 0.7	6.5 ± 3.3	13.4 ± 2.2
ORK6	White	White	White	White	8.8 ± 0.2	14.3 ± 1.3	4.8 ± 0.3	8.2 ± 1.3	15.3 ± 1.3
ORK7	Colourless	White	White	White	6.9 ± 1.2	11.2 ± 0.5	3.8 ± 0.3	7.5 ± 0.8	14.3 ± 1.4
ORK8	White	White	White	White	8.2 ± 0.4	13.4 ± 0.8	4.4 ± 0.7	8.3 ± 2.4	16.5 ± 2.5
ORK9	White	White	White cream	White cream	5.2 ± 0.8	8.3 ± 0.6	4.1 ± 0.5	6.5 ± 0.4	10.3 ± 1.0
ORK10	Colourless	White	White	White	6.5 ± 2.1	11.9 ± 0.7	3.9 ± 0.8	7.3 ± 1.2	13.6 ± 0.5
ORK11	White	White	White	White cream	4.8 ± 0.4	8.8 ± 1.2	4.3 ± 1.4	7.6 ± 1.2	11.2 ± 1.4
ORK12	White	White	White	White cream	5.3 ± 0.2	7.4 ± 1.5	4.7 ± 0.5	6.8 ± 0.7	11.6 ± 0.8
ORK13	White	White	White	White	5.8 ± 0.4	7.8 ± 0.3	3.9 ± 0.2	ND**	ND**
Endophytes									
ORK14	White	White	Creamy white	Cream	8.3 ± 0.5	17.5 ± 0.3	6.5 ± 0.1	NA*	NA*
ORK15	Colourless	Colourless	Brown	Light brown	2.4 ± 0.2	4.3 ± 0.7	4.4 ± 0.2	NA*	NA*
ORK16	White	White	Creamy white	Cream	9.7 ± 0.8	15.7 ± 1.7	6.2 ± 0.5	NA*	NA*
ORK17	Grey	Grey	Black	Black	3.3 ± 1.2	6.0 ± 1.1	8.2 ± 0.9	NA*	NA*
ORK18	Grey	Grey	Black	Black	2.3 ± 0.5	5.8 ± 0.4	9.3 ± 0.2	NA*	NA*
ORK19	Grey	Black	Black	Black	1.3 ± 0.1	3.2 ± 0.6	8.7 ± 1.4	NA*	NA*
ORK20	White	White	Light brown	Light brown	6.0 ±1.3	6.7 ± 0.5	5.3 ± 1.2	NA*	NA*
ORK21	White	White	Light brown	Light brown	6.4 ± 0.3	15.3 ± 0.7	5.5 ± 0.7	NA*	NA*
ORK22	Colourless	Colourless	Light brown	Brown	2.8 ± 0.5	4.6 ± 0.5	3.8 ± 0.6	NA*	NA*
ORK23	White	White	Grey	Grey	2.3 ± 0.8	1.1 ± 0.2	5.3 ± 1.2	NA*	NA*
ORK24	Grey	Black	Black	Black	3.6 ± 1.1	8.1 ± 1.2	7.6 ± 0.4	NA*	NA*

*NA Not applicable, **ND Not detected

Table 3.6 Jaccard similarity coefficient between sampled orchids (values are expressed as %).

Orchid	*C. chrysantha*	*C. gavilu* (Constitución)	*C. bletioides*	*B. fimbriata*	*C. crispa*	*C. longipetala*	*C. gavilu* (San Clemente)	*C. grandiflora*
C. chrysantha	100.00	66.67	28.57	40.00	40.00	28.57	28.57	28.57
C. gavilu (Constitución)		100.00	28.57	40.00	40.00	28.57	50.00	28.57
C. bletioides			100.00	25.00	25.00	20.00	33.33	33.33
B. fimbriata				100.00	33.33	66.67	25.00	25.00
C. crispa					100.00	66.67	66.67	66.67
C. longipetala						100.00	50.00	50.00
C. gavilu (San Clemente)							100.00	71.43
C. grandiflora								100

Table 3.7 Germination index of each orchid species using the fungi isolated in this study.

	ORK1	**ORK2**	**ORK3**	**ORK4**	**ORK5**	**ORK6**	**ORK7**	**ORK8**	**ORK9**	**ORK10**	**ORK11**	**ORK12**	**ORK13**
Chloraea chrysantha	0.41 B* d**	0.46 C d	0.66 B cd	0.58 B cd	0.59 C cd	1.81 A a	0.51 B d	1.02 BC bc	0.60 C cd	1.44 A ab	0.64 B cd	0.80 B cd	1.57 A a
Chloraea gavilu (Constitución)	0.40 B c	0.53 C c	0.27 CD c	0.47 B c	0.51 C c	1.53 A ab	1.52 A ab	1.77 A a	0.53 BC c	0.62 B c	0.61 B c	0.57 B c	1.26 AB b
Chloraea bletioides	0.51 B bc	0.42 C bc	0.43BC bc	0.26 B b	0.43 C bc	0.51 C bc	0.53 B bc	0.36 DE bc	0.55 C bc	1.54 A a	1.46 A a	0.71 B b	1.46 A a
Bipinnula fimbriata	0.55 B f	0.58 C f	0.65 B ef	1.60 A ab	1.66 A ab	0.96 B def	1.21 A bcd	0.72 CD ef	1.10 B cde	1.46 A abc	0.82 B def	1.00 AB cdef	1.84 A a
Chloraea crispa	1.53 A a	1.54 B a	0.28 CD d	0.52 B d	1.07 B bc	0.67 BC cd	0.53 B d	0.51 DE d	0.50 C d	0.51 B d	1.41 A ab	0.65 B cd	1.49 A ab
Chloraea longipetala	1.48 A a	1.87 A a	1.41 A a	1.52 A a	0.58 C bc	0.41 C bc	0.57 B bc	0.30 E c	0.61 C bc	0.89 B b	0.32 C c	1.51 A a	1.67 A a
Chloraea gavilu (San Clemente)	0.32 B c	0.46 C c	0.38 BCD c	0.45 B c	0.55 C c	1.79 A a	1.56 A bc	1.24 B b	0.66 C c	0.59 B c	0.55 BC c	0.65 B c	1.80 A a
Chloraea grandiflora	0.45 B bc	0.47 C bc	0.09 D c	0.36 B bc	0.35 C bc	0.62 BC b	0.58 B b	0.54 DE b	1.54 A a	0.46 B bc	1.47 A a	1.51 A a	0.74 B b

Results are means of growth indexes (n = 5). Same letters are not significantly different according to Tukey's multiple range test ($p < 0.05$)

*Capital letters show post hoc analyses to determine how one species of orchid support several fungal strains ($p < 0.05$) (horizontal).

**Lower case letters show post hoc analyses to determine how one strain enhance germination of several orchids ($p < 0.05$) (vertical

CHAPTER IV

Evidence of the orchid-mycorrhizal fungi (Bipinnula fimbriata-Tulasnella calospora) symbiosis adaptation in a metal(loid) polluted soil, determined by proteomic analysis and organic acid exudation

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Manuscript submitted to Mycorrhiza

Associated to specific objectives 2 and 3

Evidence of the orchid-mycorrhizal fungi (*Bipinnula fimbriata-Tulasnella calospora*) symbiosis adaptation in a metal(loid) polluted soil, determined by proteomic analysis and organic acid exudation

4.1 SUMMARY

The adaptation and performance of orchid mycorrhizae under metal(loid)-polluted soils have scarcely been explored. In the present study, proteomic and metabolic approaches were used to unravel physiological changes in orchids established in a metal(loid)-polluted soil and to ascertain whether mycorrhizal fungi affect the metabolic responses of roots. *Bipinnula fimbriata* plantlets were established in control and metal(loid)-polluted soil. Young orchid plantlets were placed in individual pots and cultured in a greenhouse for 24 months. After the growing phase, exudation of root organic acids, phenolics, percentage of mycorrhization, mineral content and differential protein accumulation were measured. Higher exudation rates of citrate, succinate, and malate were detected in roots growing in the metal(loid)-polluted soil. Higher phosphorus accumulation and heavy metals were found inside mycorrhizal roots under stress. Under natural, non-contaminated conditions, non-mycorrhizal roots showed enhanced accumulation of proteins related to carbon metabolism and stress, whereas mycorrhizal roots stimulated protein synthesis related to pathogen control, cytoskeleton modification, and sucrose metabolism. Under metal(loid) stress, the proteome profile of non-mycorrhizal roots showed alleviation of defence mechanisms, such as flavonoid related compounds, phytoalexins, and ethylene; which, together with the stimulation of enzymes related to carotenoid biosynthesis and cell wall organization, may promote mycorrhizal fungi colonization. These results points to different metabolism strategies on mycorrhizal and non-mycorrhizal roots. The *Bipinnula fimbriata* proteome shows

that under metal(loid) stress roots tend to facilitate mycorrhizal colonization in order to alleviate negative effects of metal(loid)s in plant.

Key words: Metal(loid)s; orchid mycorrhiza; organic acid exudation; proteome; contaminated soil

4.2 INTRODUCTION

Ecosystem degradation from mining has well known detrimental effects on the environment and human health (Fang et al., 2003; Salomons, 1995). In Chile, copper extraction and processing have significant economic importance. These large scale industrial activities have led to significant changes in the integrity and diversity of nearby ecosystems (Arenson et al., 2015; Ginocchio et al., 2004; Verdejo et al., 2015). This is the case of the area surrounding the Ventanas industrial complex, in the Puchuncaví Valley in central Chile. This ecosystem is directly affected by deposition of massive gaseous and metal-rich particulate pollution from various industrial activities, but mainly from the CODELCO Ventanas copper smelter. The pollution negatively impacts local human health, vegetation, and soil characteristics (Cornejo et al., 2008; Salmani-Ghabeshi et al., 2016). Specifically, these contaminating processes have modified the range of endemic flora, including orchids.

Orchids belong to one of the largest plant families with ~25,000–30,000 species, distributed in all terrestrial ecosystems with the exception of extremely cold environments and dry deserts (Roberts & Dixon, 2008). The orchid's life cycle is associated with specific symbiotic associations with fungi of the polyphyletic *Rhizoctonia* group, supplying carbon, nutrients, and water, mainly during germination from seed to protocorm (Cameron et al., 2007; Cameron et al., 2006; Valadares et al., 2011). Production of seeds with an endosperm lacking essential nutrients for an embryo's development render orchids dependent on fungi for acquiring nutrients to sustain growth (Leake et al., 2004). Thus, the germination process requires an additional external carbon source for the embryo to germinate, which in nature is provided by compatible mycorrhizal fungi. The relationship between plant and fungus is dynamic. Some orchids are dependent on mycorrhizal fungi only for seed germination and early

seedling development, while others remain dependent throughout the entire life cycle, living as fully mycoheterotrophic orchids (Selosse & Roy, 2009). Additionally, the pathogenicity level of fungal species should be considered, as some interactions can easily and often shift from mutualism to parasitism (Bender et al., 2014; Rasmussen & Rasmussen, 2009). This requires a balance between the amount of nutrients received and the potential pathogenicity of some orchid mycorrhizal fungi (OMF) (Valadares et al., 2014). Bougoure et al. (2014) proposed three main ways for movement of nutrients between orchid mycorrhiza (OM) symbionts: (1) biotrophic transfer of nutrients across an active interface formed between plant and fungus that can be uni- or bi-directional; (2) necrotrophic transfer of nutrients from fungus to plant after lysis of fungal pelotons; or (3) partial mycoheterotrophy, which is a combination of the two.

Although molecular regulations of OM is little known, recent studies have identified genes and proteins having a role at the young developmental stages of the orchid-fungi interaction (Zhao et al., 2014). Perotto et al. (2014) demonstrated up-regulation of genes related to mutualism, which shows that the symbiotic relationship between protocorms of the orchid *Serapias vomeracea* and the fungus *Tulasnella calospora* is closer to a mutualism than to a unilateral relationship in favour of the plant. Zhao et al. (2014) reported genes expressed in symbiotically germinated seeds of the orchid *Dendrobium officinale* related to carbon metabolism, plant defence, signalling, and plant development. The proteome of the protocorms of the orchid *Oncidium sphacelatum* during transition from mycoheterotrophy to autotrophy have shown differential expression of proteins related to the stress response, energy metabolism, carotenoid and phytoalexin biosynthesis, and signalling (Valadares, 2014). López-Chávez et al. (2016) identified up-regulation of orchid proteins related to cell cycle, ribosome biogenesis, energy metabolism, and secretion; as well as up-regulation of

symbiotic proteins related to stress response, protein-protein interaction, saccharides, and protein biosynthesis. Valadares (2014) analysed the proteome and RNA profile of mycorrhizal and non-mycorrhizal of *Oeceoclades maculate* which suggested alleviation of defence response and enhanced nitrogen metabolism in mycorrhizal roots.

Under stress, several plants depend on symbiotic associations to first establish and then to improve nutrition (Belimov et al., 2015; Rajkumar et al., 2012). In this context, OM is a specific mycorrhizal association with a strong ability to support plant establishment in stressful ecological niches and to increase plant fitness (Dearnaley et al., 2012; Herrera et al., 2017). Features of OM (partially mycoheterotrophic) that allow colonization in stressful environments are: (1) changes in root morphology; (2) changes in orchid metabolism, such as enzymatic activities, exudation of low molecular weight organic acids and phenols; and (3) associations with OMF adapted to the ecological conditions where orchids become established (Dearnaley, 2007). The association of orchids with specific OMF is fundamental to find ecological niches in metal(loid)-polluted areas (Jurkiewicz et al., 2001; Shefferson et al., 2008). There are no reports of mechanisms developed by orchids to grow in metal(loid)-polluted soils. We hypothesized that the orchid *B. fimbriata* may develop specific metabolic mechanisms to achieve establishment in a metal(loid)-polluted soil and these mechanisms are different for mycorrhizal and non-mycorrhizal roots. Therefore, in this study we address how mycorrhizal colonization and exposure to metal(loid)-polluted soils affects root organic acids exudation and the proteome profile of *B. fimbriata* plantlets in order to explore molecular mechanisms that could enhance orchid resilience to stressful environments.

4.3 MATERIALS AND METHODS

4.3.1 Plant material and location of samples

Bipinnula fimbriata (Poepp) I. M. Johnst is a terrestrial orchid endemic to Chile that colonizes coastal ecosystems between Region de Coquimbo and Region del Maule (**Figure 4.1 a-b**) (Novoa et al., 2015). This orchid produces highly viable seeds and can be cultivated in symbiotic and asymbiotic media (Herrera et al., 2017; Steinfort et al., 2010). Their distribution includes the Ventanas industrial complex, with individuals growing in this polluted ecosystem, commonly associated with pseudo-metalophyte species (Ginocchio et al., 2004). Sampling was carried out during the flowering season in the Region of Valparaiso (July 2015). *B. fimbriata* populations were found colonizing different substrates (dunes, soils, and rocks) on the coast between Valparaiso (33°05′57.0″S, 71°43′35.9″W) and Zapallar (32°34′04.6″S, 71°26′40.8″W). Active young rhizomes and soil were collected near the Ventanas industrial complex and in the Concon dunes (32°56′40.6″S, 71°33′01.1″W), placed in paper bags, and transported to the greenhouse (<12 h) for individual experiments (**Figure 4.1 a-b**).

4.3.2 Soil sampling

Soil samples were collected at Concon City (32°56′35.9″S, 71°33′00.6″W), serving as controls because it promotes development of natural *B. fimbriata* plantlets and in the Puchuncaví Valley, affected by atmospheric deposition of metal(loid)-enriched particulates from the Ventanas industrial complex (32°46′08.3″S, 71°28′17.1″W) near Valparaiso (Fuentes et al., 2016).

4.3.3 Plant growth conditions

Each four-month-old *B. fimbriata* plantlet obtained by young rhizome replication of germinated seeds (Jiang et al., 2015) was transferred to plastic pots (30 cm ×15 cm; 10 per soil) with 800 g of sieved soil (2 mm) and cultivated (**Figure 4.1 d**). Microcosms were cultivated in the greenhouse for 14 months, irrigated at 60% water-holding capacity with distilled water. After extraction of root exudates, roots were examined for the presence of OMF (pelotons) (**Figure 4.1 f**, **g**) in the control (soil from Concon) and the metal(loid)-polluted soil (soil from Puchuncaví).

4.3.4 Collection and analysis of root exudates

Low molecular weight organic acids (hereafter referred to as organic acids) and phenolic compounds were extracted from individual root system under both soil conditions and quantified by RP-HPLC, as described in Meier et al. (2012) with minor modifications. The entire root system was rinsed thoroughly with tap water and then immersed in 40 mL of 0.25 mM KH_2PO_4 (pH = 5.5), under constant aeration. The solution was filtered (0.45 μm) and freeze-dried and roots were kept separately for mycorrhiza analysis. The residue was re-suspended in 500 μL deionized sterile water and filtered again (0.22 μm). Calibration curves were prepared using an organic acids kit (47264, Supelco, Bellefonte, PA) and the standard of the phenolic compound was cinnamic acid (133760, Sigma-Aldrich, St. Louis, MO). Chromatographic analysis was carried out in a HPLC (Shimadzu CTA-20AC, 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). Phenolic compounds were separated and analysed according to Meier et al. (2012).

4.3.5 Plant and soil chemical analyses

For soil analyses, total phosphorus was determined by the alkaline oxidation method (Batty et al., 2006). Total nitrogen was determined by the standard Kjeldahl method. Organic matter was determined by the method described by Walkley and Black (1934). Exchangeable cations (Ca^{2+}, Na^{+}, Mg^{2+} and K^{+}) were extracted with 1 M ammonium acetate at pH 7.0 (Hendershot & Duquette, 1986) and exchangeable Al was extracted with 1 M KCl. All were analysed by atomic absorption spectroscopy (PinAAcle 900T, Perkin Elmer, Norwalk, CT). Available K and total concentrations of heavy metals were determined, as described by Mingorance (2002). The mean values of chemical characteristics for soil samples are listed in **Table 4.1**. The mineral content in roots was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; Agilent Technologies, Santa Clara, CA) after HNO_3-$HClO_4$ digestion, as described in Arias et al. (2010) and Park et al. (2011).

4.3.6 Molecular analysis and characterization of mycorrhizal fungi

After extraction of organic acids, root tissues were analysed to identify the OMF, as described in Herrera et al. (2017). Mycorrhizal infection was estimated according to Schatz et al. (2010), using lactophenol cotton blue stain (2% dilution in water) (61335, Sigma-Aldrich). Each section of the root was classified, based on the area of infection: 0–20%, 20–40%, 40–60%, 60–80%, 80–100% infection. Fungi were cultured in solid PDA medium for 7 days at 27 °C and DNA was extracted to identify OMF and perform the phylogenetic analyses, as described by Herrera et al. (2017).

4.3.7 Protein extraction from roots

Proteins were extracted following the procedure described by Wang et al. (2006) with minor modifications. Root segments from mycorrhizal and non-mycorrhizal roots

(200 mg total) were placed in 1.5 mL tubes and filled with 0.7 mL SDS-based extraction buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol), following sonication (5 times). After that, 600 µl phenol was added,and the mixture was vortexed thoroughly for 30 s and the phenol phase was separated by centrifugation at 10,000 × g for 5 min. The upper phenol phase was transferred to fresh microtubes. Five volumes of 0.1 M ammonium acetate dissolved in cold methanol were added to the phenol phase and proteins were precipitated at –80 °C overnight. Precipitated proteins were recovered after centrifugation at 10,000 × g for 5 min, and washed with cold 80% acetone twice and then by 70% ethanol. The final pellet was dried and proteins suspended in 8 M urea-7 M thiourea buffer. Protein pooled from roots of several plants growing in polluted and control soils were performed independently and each sample (n = 4) was injected five times. Protein quantification was carried out with the Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA).

4.3.8 Proteome analysis

For each sample of mycorrhizal and non-mycorrhizal roots growing in both soils, 50 μg of proteins were dissolved in denaturing buffer containing 5 mM dithiothreitol (DTT; Sigma-Aldrich) at 56 °C for 25 minutes. Then 14mM iodoacetamide was added and incubated at room temperature for 30 minutes. Samples were digested 16 h with trypsin (V5280, Promega, Madison, WI) at 37 °C. Samples were desalted using 1CC Oasis Sep-Pak cartridges (Waters, Milford, MA) dried in a vacuum concentrator (Sigma-Aldrich) and re-suspended in 50 μL of 10 mM ammonium formate. Five μg of peptides were injected into a chromatograph (Nano ACQUITY UPLC, Waters, Milford, MA) with a 2D separation setup and five analytical replicates. The first dimension of separation used a 5 μm XBridge BEH130 C18 (300 μm × 50 mm) and a

Symmetry C18 5 μm (180 μm × 20mm) trapping column at a flow rate of 2000 μL min^{-1}. The second dimension used a 1.7 μm BEH130 C18 1.8 μm (100 μm ×100 mm) analytical column, at a flow rate of 400 μL min^{-1}. Samples were separated in five fractions with a gradient of 10.8, 14.0 16.7, 20.4, and 65.0% acetonitrile. This set up was coupled to a NanoLock ESI-Q-ToF SYNAPT G2-S (Waters) mass spectrometer. Acquisition ranged from 50–2,000 Da, in MS^E mode (data independent analysis) at a scan rate of 0.5 s and an interscan delay of 0.1 s. Raw data were processed with the Protein Lynx Global SERVER (PLGS) 3.0.2 software. Searches were performed against NCBI Orchidaceae protein database (107,556 sequences, downloaded 03/2016)..

Scaffold Proteome Software 4.4.6 (Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications, according to Keller et al. (2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and containing at least one identified peptide sequence. Proteins were considered up- or down-regulated if their Log_2Fold Change (FC) >1 or <–1 (which is the same as an FC greater than 2 times). Blast2GO software 3.3.5 (Conesa & Götz, 2008) was used to categorize proteins according to their biological process, molecular functions, and cellular location of proteins.

4.3.9 Statistical analyses

The main effects of variables were tested by ANOVA or Student's t-test. If the P value indicated significant differences between treatments ($p < 0.05$), post hoc pair-wise comparisons were performed, using the SD of means and Tukey's multiple range test. A linear regression analysis was conducted to explore the effect of OMF on exudation of organic acids and phenolic compounds, using the Pearson correlation coefficient (R).

Statistical significance was set at $p < 0.05$. All statistical tests were conducted using the R software (https://www.r-project.org) (Ihaka & Gentleman, 1996).

4.4 RESULTS

4.4.1 Identification of mycorrhizal fungi

Sixteen fungal groups out of 67 isolates were isolated. BLAST analyses showed that three were closely related to *T. calospora* (GenBank Accession EU218888) (**Figure 4.1 e**), five related to uncultured Tulasnellaceae (GenBank Accession KP306589), three to *Leptodontidium* sp. (GenBank Accession KP278160), two to *Phomopsis columnaris* (GenBank accession KP278157), and three were unidentified (Chen et al., 2011) (**Table 4.6**). The fungal sequences showed high similarity to other Tulasnellaceae fungi isolated from *B. fimbriata* (**Figure 4.2**). All *Tulasnella* spp. (**Figure 4.1 e**) can promote seed germination to stage 2 (**Figure 4.1 c**). Percentage of colonization in mature roots varied from 0–20% (natural soil) to 20–40% (contaminated soil) (**Figure 4.4**).

4.4.2 Chemical analyses

In the polluted ecosystem, metal(loid) content exceeds, by many times, the content in control soil, specifically copper, arsenic, and zinc (**Table 4.2**). Higher accumulation of phosphorus and heavy metals were found inside mycorrhizal roots growing under metal(loid) stress, specifically copper, aluminium, iron, and zinc (**Table 4.2**).

4.4.3 Establishing plantlets in contaminated soil

B. fimbriata plantlets produced root biomass even at high doses of available copper in soil, similar to plants growing in uncontaminated soil (**Table 4.2**). Concomitant with root development that was stimulated 1.4-fold more in contaminated

soil than in controls, the plant shoots biomass in contaminated soil was insignificantly different from those of plants growing in natural soil at the end of the cultivation period (**Table 4.2**; **4.5**).

4.4.4 Root exudates

We detected exudation of four organic acids from *B. fimbriata* plants in both soils: citrate, succinate, malate, and oxalate. *B. fimbriata* plantlets grown in the polluted soil had the highest overall exudation rate of citrate, succinate, and malate, but lower concentrations of oxalic acid (**Figure 4.3 a**, **b**, **c**, **d**). For phenolic compounds, our analysis only detected cinnamic acid, which was exuded in similar quantities from roots growing in natural or contaminated soils (**Figure 4.3 e**). Linear regression analysis showed that cinnamic acid, succinate, and oxalate were positively related to OMF colonization, while malate and citrate were negatively correlated with mycorrhizal fungi colonization (R cinnamic acid = 0.87; R succinate = 0.92; R oxalate = 0.90; R malate = –0.61; and R citrate = –0.93) (**Table 4.3**).

4.4.5 Proteome analyses

The proteome of roots was different between natural and contaminated soil and was strongly affected by mycorrhization. Higher protein diversity was detected in roots developed in control soils, including 119 protein clusters in mycorrhizal and 91 in non-mycorrhizal roots. Proteome of roots developed in the polluted soil was less complex, with 86 protein clusters in mycorrhizal and 55 in non-mycorrhizal roots. Description of gene ontology categories (biological processes, molecular function, and cellular locations) of proteins are listed in **Table 4.7**.

The proteomic mechanisms found in mycorrhizal and non-mycorrhizal roots are different in plants developed in the metal(loid)-polluted soil. Specifically, comparing

non-mycorrhizal roots from polluted and control soils, we observed 320 proteins, grouped in 108 protein clusters. Seven proteins were exclusively detected in roots from metal(loid)-polluted soil, 16 in roots from control soils, and 41 were differentially regulated. After filtering (proteins detected in four analytical replicates and $p < 0.1$), 22 proteins were selected; three exclusively present in roots from metal(loid)-polluted soil, 4 in roots from the controls, and 15 with differential accumulation. Up-regulated proteins (ln fold change > 2), were related to carbon metabolism (glyceraldehyde-3-phosphate dehydrogenase, sucrose synthase), membrane transport (copper transport 6), and carotenoid metabolism (epoxycarotenoid dioxygenase), translation (ribosomal protein), response to external stimulus (glutamate decarboxylase), cytoskeleton organization (actin) redox homeostasis (peroxidase), and stress response (HSP70); whereas down-regulated proteins were related to carbon metabolism (sucrose synthase), DNA binding (LFY-like protein OrcLFY), cytoskeleton organization (alpha tubulin beta-tubulin, profilin), flavonoid metabolism (isoflavone reductase), redox homeostasis (catalase, monodehydroascorbate reductase), ATP metabolism (ATP synthase alpha subunit), and defence (orcinol O-methyltransferase, lipoxygenase, allene oxidase, ubiquitin-like protein) (**Table 4.4**).

In mycorrhizal roots, we also found specific protein modifications (polluted/control soil), identifying 391 proteins, grouped in 138 protein clusters. We detected 24 proteins exclusively in mycorrhizal roots of metal(loid)-polluted soil, 53 in controls, and 61 with differential accumulation. After filtering, 19 proteins were selected; one protein was exclusively related to roots from the metal(loid)-polluted soil and four to control roots, whereas 14 proteins showed differential accumulation between treatments (**Table 4.4**). Up-regulated proteins in mycorrhizal roots from the metal(loid)-polluted soil were related to carbon metabolism(sucrose synthase), DNA binding

(knotted-like protein), flavonoid metabolism (phenylalanine ammonia lyase), cytoskeleton organization (alpha tubulin), response to oxidative stress (monodehydroascorbate reductase, peroxidase), membrane transporter (V-ATPase E subunit, copper transporter 6, ATP-binding cassette transporter), and defence (HSP70); whereas down-regulated proteins were related to energy (ribulose-1,5-bisphosphate carboxylase/oxygenase), flavonoid metabolism (chalcone synthase), defence (3-ketoacyl-CoA thiolase, orcinol O-methyltransferase), translation (ribosomal protein S3a), protein modification (peptidyl-prolyl cis-trans isomerase), and redox homeostasis (ascorbate peroxidase) (**Table 4.4**).

4.5 DISCUSSION

The Puchuncaví Valley received intense metal(loid) pollution since the installation of the Ventanas copper smelter in 1964, which has limited establishment of vegetation. In these soils, metal(loid)s tend to accumulate in the upper layers and reach levels as high as 2000 mg kg^{-1} for copper in the rhizospheric soil below some metallophyte species (Ginocchio, 2000; González et al., 2014). Earlier, we detected that the soil microsites, where the orchid *B. fimbriata* grow with roots developed in deep soil and commonly in association with the succulent plant *Carpobrotus chilensis* (Doca), had lower copper concentrations (150 to 300 mg kg^{-1}) (Ginocchio et al., 2004).

Orchids developed in terrestrial substrates depend on mycorrhizal interactions to obtain carbon and other nutrients especially under stress conditions, showing specialization with different OMF (McCormick et al., 2006; Shefferson et al., 2008). In this study, we identified OMF that are closely related to other *Rhizoctonia*-like fungi isolated from *B. fimbriata*, including members of the ascomycetes order Pezizales, which are key fungi promoting plant establishment in polluted environments, such as

mining activities, terrestrial oil spills, and soils contaminated with pesticides (Regvar et al., 2010). Additionally, the proteomic responses of roots under both soil conditions were affected by the presence of mycorrhizal fungi, increasing synthesis of membrane transports proteins and proteins having a role in controlling mycorrhizal fungi that spread and colonize vital tissues. Further analyses are necessary to clarify whether mycorrhizal fungi in this orchid are adapted to specific soil conditions, acting as heavy metal accumulators similar to what is known in AM-fungi, and in the orchids *Epipactis atropurpureum*, *E. helleborine*, and *Dactylorhiza majalis* (Aguilera et al., 2011; Jurkiewicz et al., 2001; Turnau et al., 2012).

Exudates in *B. fimbriata* roots is different, related to the soil where they grow, showing a different exudation pattern of organic acids and a phenolic compound that are essential mechanisms to promoting metal exclusion in metal(loid)-polluted soils (Hall, 2002). We detected greater exudation of malic, succinic, and citric acids in orchid roots in polluted soils. Higher organic acid exudation by plant roots induce changes in the rhizosphere, mainly by their active role as heavy-metal chelators, inorganic nutrient solubilizers, pH changers, and stimulation of native soil microorganism (Nigam et al., 2001; Schmalenberger et al., 2015). Therefore, *B. fimbriata* roots may respond like other plant species that modify their exudates as a response to heavy-metal stress (Mucha et al., 2010; Nian et al., 2002). In the case of phenolic compounds, we found only one compound, cinnamic acid and did not detect any significant differences in exudation. This may be explained by the rapid consumption of cinnamic acid, serving as building blocks for other compounds related to pathogen control, antioxidants, synthesis of cell wall components and may also act as heavy-metal chelators that are more effective than organic acids (Meier et al., 2012).

In orchids from terrestrial substrates, non-mycorrhizal roots are different from mycorrhizal ones, mainly by the presence of mycorrhizal fungi in pelotons. Non-mycorrhizal roots are expected to act as a starch sink, whereas mycorrhizal roots host the OMF (Cameron et al., 2008; Cameron et al., 2006). Our results suggest that, under either natural or metal-contaminated soil, the proteome was affected by the presence of the OMF (Chiapello et al., 2015). Interestingly, some of the molecular responses developed in symbiotically germinated protocorms were also found in our analyses, such as proteins with a key role in metabolizing fungal-origin carbon, promoting growth and control of OMF spread (Valadares et al., 2014; Valadares, 2014; Zhao et al., 2014).

Mycorrhizal activity in orchids is a dynamic process, in which non-mycorrhizal tissues may be colonized even at advanced developmental stages (Hou & Guo, 2009). Mycorrhizal tissues contain intact and degraded pelotons, both of which are potential sources of nutrients to the plant partner (Cameron et al., 2008; Kuga et al., 2014). Our results showed that in metal(loid)-polluted soils, non-mycorrhizal roots had lower protein content related to OMF control. In addition, the production of enzymes able to metabolize carotenoid-derived molecules also play a role in signalling and functionality of the mycorrhizal interaction (Valadares et al., 2014). This is the case of epoxycarotenoid dioxygenase detected in non-mycorrhizal roots, which together with phytohormones such as abscisic acid, are involved in strigolactone synthesis able to attract and induce anatomic modifications in mycorrhizal fungi (Herrera‐Medina et al., 2007).

Still under heavy-metal stress, in non-mycorrhizal roots we also detected a decrease in protein synthesis of defence-related mechanisms (flavonoid, phytoalexins, and ethylene) and production of enzymes related with strigolacton metabolism, which have been defined as root colonization promotors of mycorrhizal fungi in AM

symbioses by Yoneyama et al. (2008). Colonization of new cells requires considerable structural changes in the host, in which actin is crucial to the reorganization of the invaded cell (Gutjahr & Parniske, 2013). Furthermore, in mycorrhizal roots the presence of OMF in root tissues enhances accumulation of ATP synthase and reactive oxygen species, as a result of higher metabolic activity (Laparre et al., 2014; Nath et al., 2016). We also detected higher accumulation of proteins related to cell membranes, energy generation, and membrane polarization, such as clusters of ATPases, protein kinases, phospholipase D, and ATP-binding cassette, mainly involved in cross-membrane signalling and transport, as in the case of the orchid *Cymbidium hybridum* (Zhao et al., 2014). Under metal(loid) stress, we found enhanced accumulation of transmembrane ATPases involved in nutrient transport across the plasma membrane, similar to other mycorrhizal interactions (Wang et al., 2014; Yamaji et al., 2013).

We detected proteins related to copper transport that were over-expressed in non-mycorrhizal roots developed in the metal(loid)-polluted soil, which reflected an active across-membrane transit of copper in the root cell membranes; however, in mycorrhizal roots, protein synthesis related to copper transport was down-regulated. This may be explained by the presence of OMF, which may play a role in reducing the available concentrations of toxic compounds (such as copper), by the production of organic compounds or immobilization in the fungal mycelium (Jurkiewicz et al., 2001). We also expect that, under metal(loid) stress, the OMF *Tulasnella calospora* could supply nutrients to the host orchid (over-accumulation of ATPases), which is consistent with the enhanced accumulation of proteins related to lipid and carbon metabolism and membrane transporters (ATP binding cassette, copper transport). Valadares et al. (2014) found that the infection of OMF in orchids requires specific and efficient responses against infection of vital tissues, including biosynthesis of defence molecules,

biosynthesis, and perception of ethylene and jasmonic acid. In our experiments, mycorrhizal roots showed over-expression of proteins related to jasmonic acid biosynthesis, such as lipoxygenase and allene oxidase and ethylene, which trigger specific responses against pathogen attack (Song et al., 2014). The down-regulation of defence proteins in non-mycorrhizal roots from the metal(loid)-polluted soil is an indirect mechanism to promote mycorrhizal colonization to have nutritional benefits and reduce the negative effects of the metal(loid)s present in metal(loid)-contaminated soil.

4.6 CONCLUSIONS

Our results showed that establishment of *B. fimbriata* in metal(loid)-polluted soil induces specific changes in roots, such as an increase in exudation of specific organic acids, phenol compounds, and induced specific proteomic changes that allow establishment of orchid *Mycorrhizae* in the metal(loid)-polluted soil. This indicates that the presence of the orchid mycorrhizal fungi, adapted to the specific soil conditions, influences the metabolic responses of the roots.

4.7 AUTHOR CONTRIBUTIONS

H. H., R.V., and C.A. designed and supervised the experiments. H.H., R.V., and S.N. performed the experiment. H.H., G.O., R.V., and C.A. analysed the data. H.H., Y.B., and C.A. wrote the manuscript.

4.8 ACKNOWLEDGEMENTS

The authors thank the Instituto Tecnológico Vale (Belem, Brazil) for equipment supply and technical assistance and Ira Fogel for editorial and English improvement. This study was supported by the ‘Fondo Nacional de Desarrollo Cientifico y

Tecnologico' of Chile (grant 1170931) and the 'Comision Nacional de Investigacion Cientifica y Tecnologica' of Chile (grant 21130491).

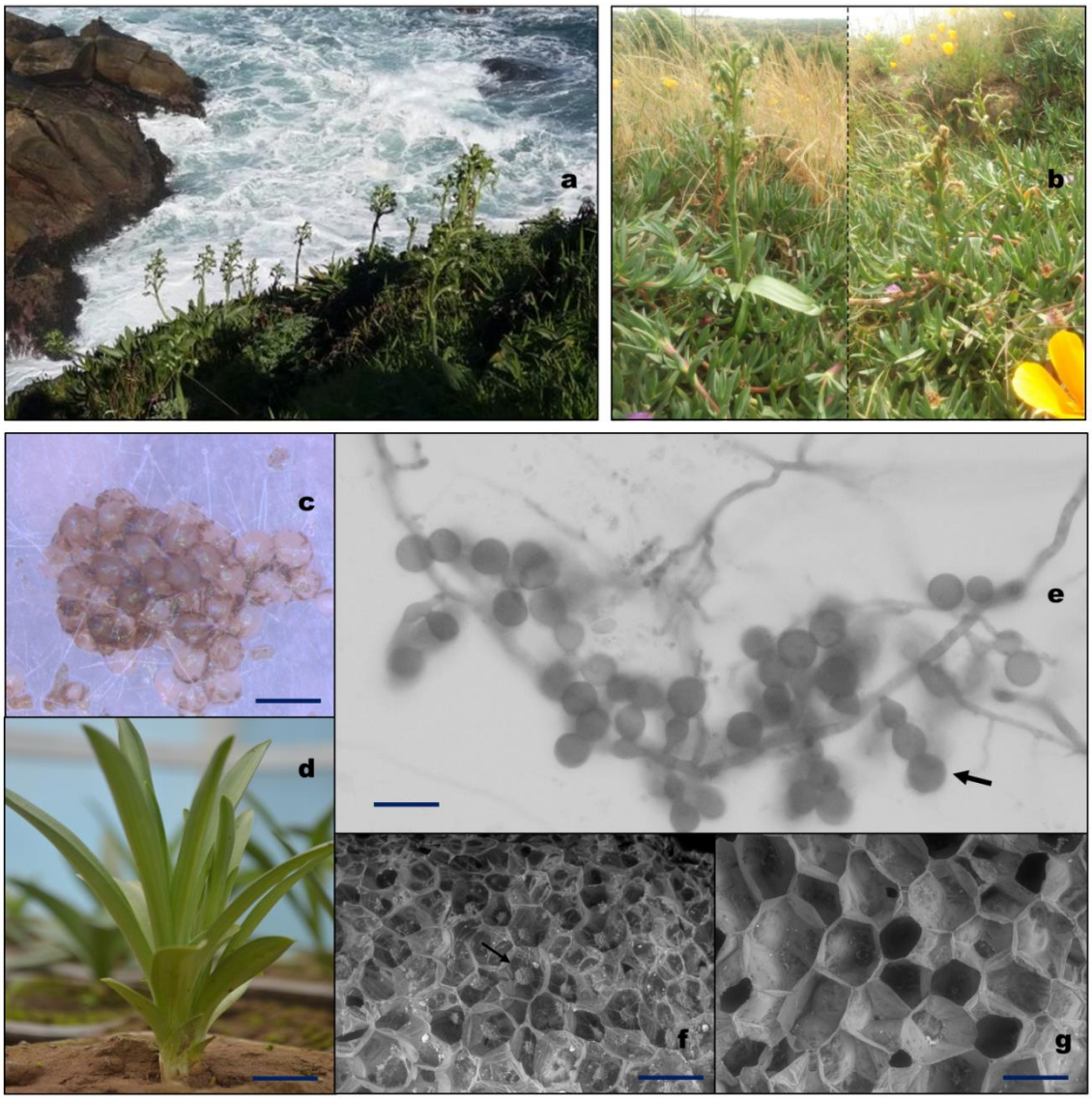


Figure 4.1 *Bipinnula fimbriata* populations growing at their natural environments in Concon (**a**) and near the Ventanas industrial complex (**b**) in the region of Valparaiso; (**c**) *Bipinnula fimbriata* protocorms obtained by symbiotic germination (scale bar = 500 µm); (**d**) *Bipinnula fimbriata* plantlets established in the metal(loid)-polluted soil (scale bar = 1 cm); (**e**) main mycorrhizal fungi isolated from orchids developed in the metal(loid)-polluted soil, showing moniliod cell (black arrow) (scale bar = 20 µm); and mycorrhizal (**f**) and non-mycorrhizal (**g**) root tissues showing pelotons (black arrow) (scale bars = 200 µm).

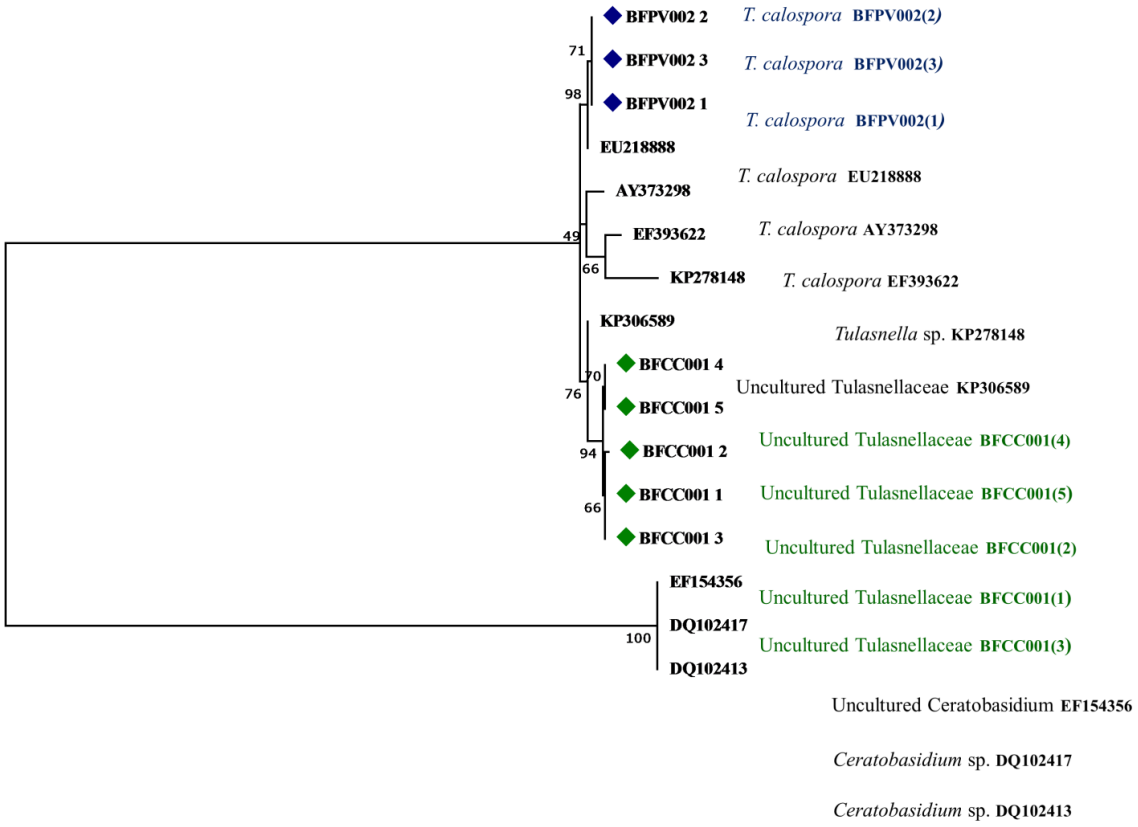


Figure 4.2 Maximum likelihood tree of ITS sequences of orchid mycorrhizal fungi isolated from *Bipinnula fimbriata* roots from Puchuncaví (blue) and Concon (green) soils. The tree was constructed using other fungal sequences isolated from *Bipinnula fimbriata* roots (black). Numbers near branches refer to bootstrap support values.

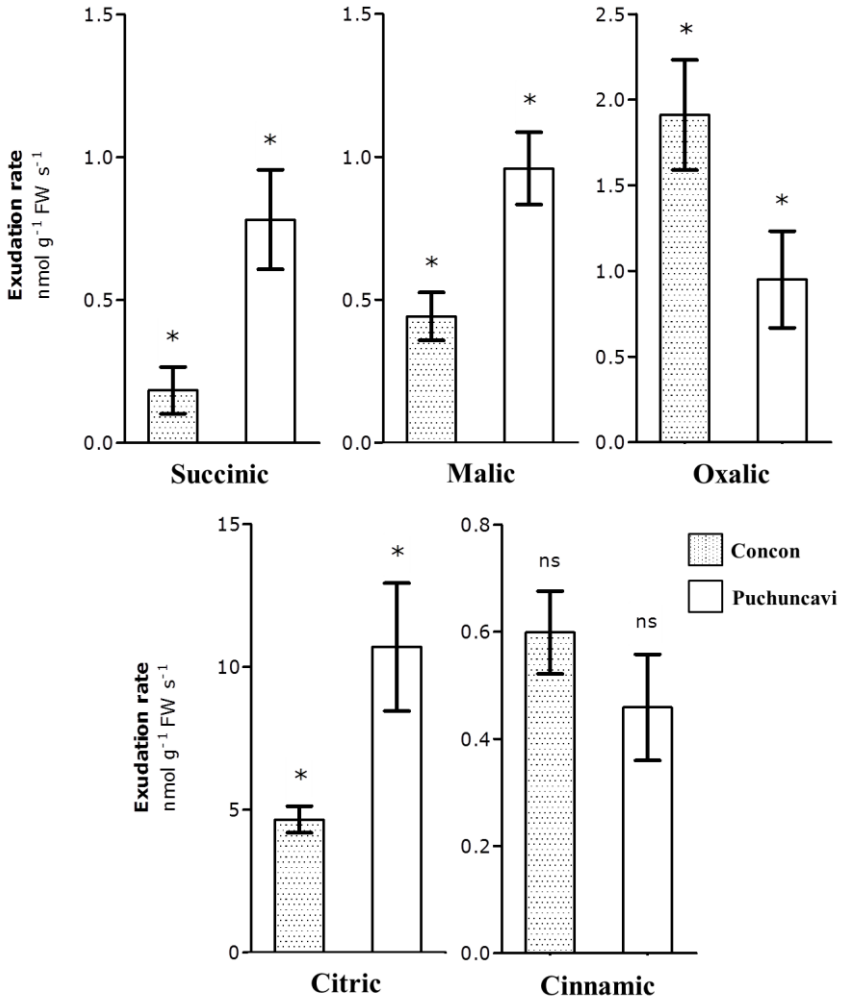


Figure 4.3 Exudation of low molecular weight organic acids (**a**, **b**, **c**, **d**) and phenolic compound (**e**) measured in *Bipinnula fimbriata* roots established in different soils (control and polluted soil (*p < 0.01 by student's t-test; ns = no significant differences between soils).

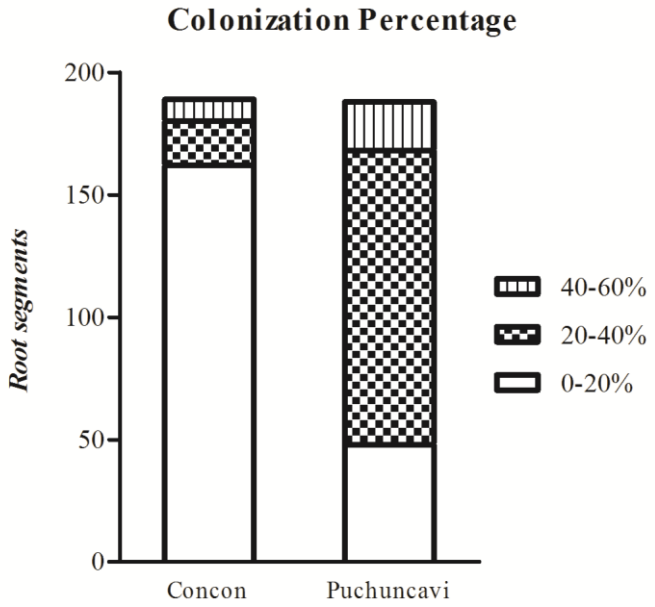


Figure 4.4 Mycorrhization percentage (range of root surface hosting pelotons) of orchid roots developed in control (Concon) and the metal(loid)-polluted (Puchuncaví) soil.

Table 4.1 Chemical characteristics from soils associated with *Bipinnula fimbriata* in Concon and Puchuncaví.

	Concón	Puchuncaví
N [a]	11	29
P [a]	26	39
K [a]	117	207
pH [b]	6,73	5.55
Organic matter [c]	3	3
K [d]	0.30	0.53
Na [d]	0.65	0.08
Ca [d]	6.40	3.9
Mg [d]	2.06	1.12
Al [d]	0.01	0.12
CEC [d]	9.42	5.75
Base saturation [d]	9.41	5.63
Cu [a]	3.45	197
As [a]	1.13	18.3
Zn [a]	80.3	169

[a] mg Kg^{-1} (total)
[b] in H_2O
[c] %
[d] (cmol+ Kg^{-1})

Table 4.2 Different variables measured in the orchid *Bipinnula fimbriata* developed in control (Concon) and the metal(loid)-polluted (Puchuncaví) soil. Results are mean ± SD.

Variable	Control soil	Metal(loid)-polluted soil
Root exudation [a]		
Oxalic acid	1.93 ± 0.31*	0.85 ± 0.28*
Malic acid	0.44 ± 0.12*	0.96 ± 0.13*
Succinic acid	0.18 ± 0.08**	0.78 ± 0.17**
Citric acid	4.66 ± 0.47**	15.7 ± 2.24**
Cinnamic acid	0.59 ± 0.06^{ns}	0.46 ± 0.10^{ns}
Root biomass [b]	48.6 ± 4.22 ^{ns}	64.5 ± 6.70 ^{ns}
Shoot biomass	6.5 ± 3.1 ^{ns}	4.4 ± 2.8 ^{ns}
Total protein content [c]		
Mycorrhizal root	337 ± 5*	303 ± 4*
Non-mycorrhizal root	316 ± 4**	224 ± 7**
Metal(loid)s in soil [d]		
Cu	3.45 ± 0.95**	197.5 ± 32.2**
As	1.13 ± 2.8*	18.25 ± 6.7*
Zn	80.29 ± 7.5**	168.9 ± 12.3**
Heavy metals in root [e]		
Cu	0.25 ± 0.03**	2.02 ± 0.54**
Al	736 ± 158*	1324 ± 345*
Fe	1268 ± 224^{ns}	1718 ± 612^{ns}
Zn	91.3 ± 12.4*	53.8 ± 5.3*
P in root [e]		
Mycorrhizal root	1328.0 ± 143*	1904.7 ± 305*
Non-mycorrhizal root	532.4 ± 27**	281.8 ± 45**
Colonization percentage [g]	17.8 ± 3.1*	24 ± 2.5*
Seed viability (%)	70 ± 15*	53 ± 8*

^{ns} = not significant

* = P<0.01

** = P<0.05

NA= Not applicable

[a] nmol g^{-1} FW s^{-1}

[b] mg

[c] total proteins identified on each sample

[d] mg kg^{-1} (total)

[e] µg g^{-1}

[f] µg L^{-1}

[g] mean of percentage of pelotons from five random mycorrhizal root segments

Table 4.3 Correlation matrix of some selected parameters in *Bipinnula fimbriata* plantlets growing in the metal(loid)-polluted soil.

Variables	Malic Acid [a]	Succinic Acid [a]	Citric Acid [a]	Cinnamic Acid [a]	Radical biomass [b]	Total protein [c]	Cu in soil [d]	Cu in root [e]	Available Cu [f]	% colonization
Oxalic acid [a]	-0.75	0.89	-0.87	-0.60	-0.41	0.69	-0.85	-0.64	-0.80	0.90
Malic acid [a]	-	0.88	0.90	-0.59	0.54	-0.56	0.88	0.81	0.90	-0.61
Succinic acid [a]		-	0.91	-0.69	0.51	-0.68	0.88	0.69	0.89	0.92
Citric acid [a]			-	-0.61	0.55	-0.72	0.91	0.76	0.89	-0.93
Cinnamic acid [a]				-	-0.36	0.59	-0.64	-0.41	-0.59	0.87
Radical biomass [b]					-	-0.35	0.62	0.54	0.62	0.25
Total protein [c]						-	-0.65	-0.21	-0.49	0.48
Cu in soil [d]							-	0.85	0.95	-0.38
Cu in root [e]								-	0.89	0.86
% Colonization									-	-

[ns] = non significant

* = $P<0.05$

NA= Not applicable

[a] nmol g^{-1} FW s^{-1}

[b] mg

[c] total proteins identified in each sample

[d] mg kg^{-1}

[e] µg g^{-1}

[f] µg L^{-1}

Table 4.4 Most representative proteins identified in *Bipinnula fimbriata* mycorrhizal and non-mycorrhizal roots developed in control and the metal(loid)-polluted soil.

Protein description	*Organism*	*Accession (protein)*	*Molecular weight*	p-value	*Log Fold change*
NON-MYCORRHIZAL ROOTS (metal(loid) polluted / control soil)					
Ribosomal protein	*Gymnadenia conopsea*	ABD66516	14 kDa	0.04	Polluted
Copper transporter 6	*Oncidium* hybrid cultivar	AID66717	20 kDa	0.04	Polluted
Epoxycarotenoid dioxygenase	*Oncidium* hybrid cultivar	AAX85471	27 kDa	0.03	Polluted
ATP synthase	*Bromheadia finlaysoniana*	CAW38501	76 kDa	0.08	4.55
Peroxidase	*Doritis pulcherrima*	ACN25040	37 kDa	0.09	3.83
HSP70	*Dendrobium catenatum*	AGR45355	71 kDa	0.06	3.76
Actin	*Vanda* hybrid cultivar	ADQ57816	42 kDa	< 0.01	3.62
Glyceraldehyde-3-phosphate dehydrogenase	*Dendrobium catenatum*	AKR76257	37 kDa	0.03	3.54
Sucrose synthase	*Phalaenopsis* hybrid cultivar	AFS60092	93 kDa	0.03	3.51
Glutamate decarboxylase	*Dendrobium catenatum*	AIU48022	56 kDa	0.04	1.21
Monodehydroascorbate reductase	*Oncidium* hybrid cultivar	ACJ38541	47 kDa	0.07	-1.14
ATP synthase alpha subunit	*Pogoniopsis schenkii*	AIZ66439	45 kDa	< 0.01	-2.27
Ubiquitin-like protein	*Vanilla planifolia*	AEI29169	11 kDa	0.04	-2.33
Beta-tubulin	*Vanilla planifolia*	AHA92091	26 kDa	0.04	-2.73
Isoflavone reductase	*Dendrobium catenatum*	AID53186	34 kDa	< 0.01	-3.00
Catalase 1	*Dendrobium catenatum*	AIU48021	57 kDa	0.02	-4.98
Profilin	*Phalaenopsis* hybrid cultivar	AAD21619	14 kDa	0.02	-5.29
Allene oxide synthase	*Cymbidium ensifolium*	AFH89624	55 kDa	0.06	-9.45
Alpha-tubulin	*Dendrobium candidum*	ABO37803	11 kDa	< 0.01	Control
Orcinol O-methyltransferase	*Vanda* hybrid cultivar	AIB06955	42 kDa	0.04	Control
LFY-like protein OrcLFY	*Serapias lingua*	BAC55082	51 kDa	0.04	Control
Lipoxygenase	*Cymbidium ensifolium*	AFH89626	49 kDa	0.01	Control
MYCORRHIZAL ROOTS (metal(loid) polluted / control soil)					
Knotted-like protein	*Orchis anthropophora*	AEX56221	14 kDa	0.08	Polluted
Phenylalanine ammonia lyase	*Bromheadia finlaysoniana*	AHA92089	77 kDa	0.06	19.00
ATPase	*Epifagus virginiana*	AID52224	284 kDa	< 0.01	8.84
HSP70	*Dendrobium catenatum*	AGR45355	71 kDa	0.06	7.21
Hypothetical protein (related to ATP binding cassette domain*)	*Oryza sativa*	Q9FLT8	68 kDa	0.02	6.27
Monodehydroascorbate reductase	*Oncidium* hybrid cultivar	ACJ38541	47 kDa	0.01	5.68
ATP synthase	*Bromheadia finlaysoniana*	CAW38501	76 kDa	0.08	5.13
S-adenosylmethionine synthetase	*Phalaenopsis* hybrid cultivar	ALB75300	43 kDa	0.01	4.45
V-ATPase E subunit	*Phalaenopsis* hybrid cultivar	AML60995	29 kDa	< 0.01	3.85
Peroxidase	*Doritis pulcherrima*	ACN25040	37 kDa	0.01	3.09
Copper transporter 6	*Oncidium* hybrid cultivar	AID66717	20 kDa	< 0.01	2.79
Alpha-tubulin	*Dendrobium candidum*	ABO37803	11 kDa	< 0.01	2.61
Sucrose synthase	*Phalaenopsis* hybrid cultivar	AFS60092	93 kDa	0.03	2.57
3-ketoacil-CoA thiolase	*Vanda* hybrid cultivar	AIB06952	47 kDa	0.04	-1.18
Orcinol O-methyltransferase	*Vanda* hybrid cultivar	AIB06955	42 kDa	0.06	-1.57
Chalcone synthase	*Bletilla striata*	AHH25569	43 kDa	0.02	-1.98
Ascorbate peroxidase	*Oncidium* hybrid cultivar	ACJ38537	27 kDa	0.03	-4.24
Ribulose-1,5-bisphosphate carboxylase/oxygenase	*Pterichis* sp.	AAR29775	49 kDa	0.04	Control
Peptidyl-prolyl cis-trans isomerase	*Dendrobium catenatum*	AKR76256	18 kDa	0.08	Control
Ribosomal protein S3a	*Cymbidium* hybrid cultivar	ABK56834	30 kDa	0.08	Control

* protein identified according their best BLAST match.

Table 4.5 *F*-values and probabilities of significance for the main effects of soils and colonization percentages in the variables analysed by mean of factorial ANOVA.

Variable	*F*-values	
	Soils	% colonization[g]
Oxalic acid[a]	17.4*	20.8*
Malic acid[a]	47.9*	3.8[ns]
Succinic acid[a]	29.3*	14.7[*]
Citric acid[a]	70.2*	15.7[ns]
Cinnamic acid[a]	5.2[ns]	15.5*
Radical biomass[b]	2.7[ns]	3.0[ns]
Total protein content[c]	512.9*	7.3**
Cu in soil[d]	127.1*	11.5*
Cu inside root[e]	165.3*	8.3*
Available Cu[f]	87.1*	19.2*

[ns] = non significant

* = $P<0.01$

** = $P<0.05$

NA= Not applicable

[a] nmol g^{-1} FW s^{-1}

[b] mg

[c] total proteins identified on each sample

[d] mg kg^{-1}

[e] µg g^{-1}

[f] µg L^{-1}

[g] mean of percentage of pelotons from five random mycorrhizal root segments

Table 4.6 Molecular identification of mycorrhizal fungi isolated from *Bipinnula fimbriata* roots, based on the closest match in the GenBank database.

Fungal isolate	% occurrence	Number of isolates	Best GenBank match	% identity	Source	Reference
BF-CC001	39	26	*KP306589* (Uncultured Tulasnellaceae)	98	Photosynthetic orchid	GenBank
BF-PV002	24	16	*EU218888* (*Tulasnella calospora*)	99	Not specified	Taylor and McCormick 2007
BF-PV003	9	6	*KP278160* (*Leptodontidium* sp.)	97	*Chloraea longipetala*	Herrera et al., 2017
BF-CC004	17	11	*KP278157* (*Phomopsis columnaris*)	99	*Chloraea gavilu*	Herrera et al., 2017
BF-CC005	11	7	Unidentified	< 95	- - -	- - -

Table 4.7 Gene ontology categories of identified proteins in *Bipinnula fimbriata* roots. Results are number of protein clusters assigned at biological processes, molecular functions and cellular components. Numbers between parentheses refer to percentage of total proteins. (Nat myc = mycorrhizal roots from control soil; Nat non-myc = non-mycorrhizal roots from control soil; HM myc = mycorrhizal roots from metal(loid)-polluted soil; HM non-myc = non-mycorrhizal roots from metal(loid)-polluted soil

		Nat myc	**Nat non-myc**	**HM Myc**	**HM non-myc**
Biological process	Oxidation reduction process	29(25)	18(17)	24(28)	13(24)
	Transcription, DNA template	19(17)	10(10)	14(16)	8(15)
	Signal transduction	15(13)	9(9)	13(15)	7(12)
	Response to abiotic stimulus	3(3)	3(3)	4(5)	3(5)
	Regulation of gene expression	2(2)	-	1(1)	1(1)
	Regulation of cellular macromolecule biosynthetic process	3(3)	7(7)	1(1)	2(4)
	Microtubule based process	3(3)	-	3(3)	-
	Organic acid metabolic process	2(2)	2(2)	-	3(5)
	Organo-nitrogen compound biosynthetic process	1(1)	11(10)	1(1)	2(3)
	Response to oxidative stress	3(3)	1(1)	3(4)	2(4)
	Cellular protein modification metabolic process	3(3)	9(9)	3(4)	-
	Glucose metabolic process	1(1)	7(7)	1(1)	2(4)
	Single-organism carbohydrates metabolic process	2(2)	2(2)	2(2)	-
	Phosphate-containing compound metabolic process	1(1)	2(2)	1(1)	-
	Single-organism metabolic process	1(1)	2(2)	-	-
	Regulation of primary metabolic process	1(1)	2(2)	-	-
	Catabolic process	-	-	1(1)	-
	Unknown	11(10)	8(8)	3(4)	4(8)
Molecular function	Oxidoreductase activity	36(32)	24(23)	25(29)	12(22)
	Metal ion binding	3(3)	10(10)	9(11)	9(16)
	DNA binding	16(14)	15(14)	11(13)	10(19)
	ATP binding	15(13)	9(9)	14(16)	8(14)
	GTP binding	10(9)	7(7)	8(9)	7(12)
	GTPase activity	-	9(9)	4(5)	5(9)
	Transferase activity, transferring phosphate-containing groups	2(2)	1(1)	3(3)	1(1)
	Nucleoside-triphosphate activity	5(4)	2(2)	-	-
	Cofactor and coenzyme binding	2(2)	2(2)	2(2)	1(1)
	Structural molecule activity	6(5)	8(8)	3(4)	1(1)
	Unknown	18(16)	13(13)	7(8)	3(5)
Cellular component	Integral component of membrane	19(17)	8(7)	13(15)	7(12)
	Chloroplast-thylakoid	7(6)	8(7)	3(4)	4(7)
	Cytoskeleton	14(12)	7(7)	11(13)	3(6)
	Nucleus	10(9)	11(11)	8(9)	3(6)
	Membrane protein complex	8(7)	4(4)	7(8)	2(3)
	Thylakoid membrane	1(1)	2(2)	1(1)	-
	Cell periphery	2(2)	4(4)	6(7)	3(5)
	Macromolecular complex	-	-	-	1(1)
	Unknown	52(46)	57(57)	32(37)	30(55)

CHAPTER V

Dynamic of mycorrhizal associations of endemic orchids under soil contaminated with metal(loid)s

Manuscript to be submitted to Plant and Soil.

Associated to specific objectives 1, 3 and 4

Dynamic of mycorrhizal associations of endemic orchids and mycorrhizal interactions under soil contaminated with metal(loid)s

5.1 ABSTRACT

The adaptation and performance of orchid mycorrhizas at soil contaminated with metal(loid)s have been scarcely explored. Furthermore, there is a lack of knowledge of gene expression and metabolic regulations of mycorrhizal orchids under natural and contaminated soils. This chapter summarize diverse experiments that were carried out to unravel different aspect of orchid mycorrhiza interactions. First, a morphometric analysis of the in-vitro interaction between *Bipinnula fimbriata* and the mycorrhizal fungi *Tulasnella calospora* under metal(loid)s stress was carried out. Furthermore, RT-PCR analyses were done to know *B. fimbriata* gene sequences for expression assays. For the interaction assay, *B. fimbriata* seeds from one mature capsule were sowed in oatmeal agar and inoculated with *T. calospora*. Besides, asymbiotic treatments were performed in Murashige and Skoog Media. Petri dishes were supplemented with Cu, As, and Fe at 5 mg L^{-1}; and their development was monitored at 7-day intervals, using software image analyses. For gene expression analyses, *B. fimbriata* plantlets were placed into individual pots, with natural or contaminated soil, and cultured in a greenhouse for 24 months. After that, roots were screened for the presence of mycorrhizal fungi, and total RNA and proteins were extracted from mycorrhizal and non-mycorrhizal roots. A set of mutualistic and antagonistic primers were tested in RT-PCR analyses. Cu showed a negative effect on seed differentiation and growth; whereas Fe did not show any significant effects on orchid protocorm growth, and under Arsenic supply seed growth promotion were not achieved. It was also detected improved mycorrhization in orchid plantlets developed in a metal(loid)s contaminated soil,

ranging from 7 to 24% of peloton-containing root cuts, with 20-40% of infected surface. RT-PCR confirms the presence of mutualistic genes with a well-defined PCR amplicon in mycorrhizal samples; specially nodulin, a bidirectional sugar transporter, and acid chitinases. For the antagonistic genes, the wound stress precursor showed no differences in amplicon intensity between treatments; however, pathogenesis-related protein and mannose-binding lectin, showed a bigger amplicon in orchid roots under stress. Proteome results suggested that under natural soil conditions mycorrhizal roots stimulate synthesis of proteins related to growth, membrane transport, pathogen control and oxidative stress, whereas non-mycorrhizal roots showed stimulation of proteins mainly related to stress, carbon metabolism and cell wall reorganization. The results showed embryo growth promotion by mycorrhizal fungi in the contaminated media, which denotes a positive role to promote growth and differentiation even under stress. In addition, a clear predisposition to promote mycorrhizal colonization was shown, in order to have more mycorrhizal benefits under the deficient soil conditions. Moreover, the gene expression of mutualistic genes and no-strong expression of antagonistic markers is a sign of orchid-fungi compatibility; and, the activated pathogenic mechanisms are related to control mycorrhizal fungi spread in root tissues.

Key words: Orchid mycorrhiza; *Bipinnula fimbriata*; Mycoheterotrophy; Gene expression; Proteome.

5.2 INTRODUCTION

Orchidaceae is one of the world's largest plant families, adding up to about 30% monocotyledons (Dressler, 1993; Lucksom & Lucksom, 2007). Orchids grow in various habitats and possess different trophic strategies ranging from epiphytic to terrestrial, and from autotrophic to fully mycoheterotrophic, comprising variable dependence degrees on specific fungal and bacterial endophytes (McCormick et al., 2004; Puente & Bashan, 1994). The orchid life cycle is characterized by the production of small thin and non-endospermic seeds, which make the "dust-like" seeds dependent on mycorrhizal fungi to obtain carbon and other nutrients, to achieve plant establishment on diverse altered ecosystems, including oil spills, fire forest and metal(loid) polluted soils (Shefferson et al., 2008; Turnau et al., 2012). Orchids establish a symbiosis with free-living fungi from the *Rhizoctonia*-like fungi complex, which are the mycorrhizal fungi commonly associated with orchids (Herrera et al., 2017). Orchid seeds are minuscule and produced in large amounts, each with minimal nutritional reserves (Rasmussen & Rasmussen, 2009; Smith & Read, 2010). This renders orchids dependent on mycorrhizal fungi for organic nutrient acquisition during the mycoheterotrophic stage, known as protocorm (transition from seed to plantlet) (Porras-Alfaro & Bayman, 2007; Sathiyadash et al., 2012). Under natural conditions, the orchid life cycle starts when dust-size seeds are dispersed into the environment by several mechanisms. During germination, compatible mycorrhizal fungus provides carbohydrates to promote embryo development (Bonnardeaux et al., 2007; Brundrett, 2002). These mycorrhizal fungi must be adapted to the particular soil conditions to sustain growth and functionality of the orchid mycorrhizal symbioses (Roberts & Dixon, 2008; Waterman & Bidartondo, 2008).

In terrestrial orchid mature roots, vestiges of the symbiotic interaction established in the seedling stage are present in mycorrhizal structures conserved in the underground

organs, which are so-called “pelotons” (Bidartondo et al., 2004). However, root tissue colonization degrees are mostly undetermined because of the huge diversity of the Orchidaceae family (Selosse & Roy, 2009). Pelotons are digested within plant cells, and lysis of the fungus by the plant has been proposed as the main route to bring carbon and other nutrients to the orchid (Perotto et al., 2014; Rasmussen & Rasmussen, 2009). By contrast, more recent experiments strongly suggest that nutrient transfer mainly takes place through intact membranes (Kuga et al., 2014; Roberts & Dixon, 2008). Bougoure et al. (2014) proposed three main ways for nutrient movement between orchid mycorrhiza symbionts: i) biotrophic nutrient transfer through an active interface formed between plant and fungus, which can be uni- or bi-directional; ii) necrotrophic nutrient transfer after fungal peloton lysis (from fungus to plant); or iii) partial mycoheterotrophy, which is a combination of both. Thus, the nature of the symbiotic orchid–fungus relationship in the adult stage can be rather complex depending on the host plant trophic strategy and on environmental conditions.

B. Fimbriata is an endemic orchid, which colonizes littoral ecosystems between Region de Coquimbo and Region del Maule (Mujica et al., 2016; Novoa et al., 2015). This orchid is characterized by production of highly viable seeds practical to be cultured in symbiotic and asymbiotic media (Herrera et al., 2017; Pereira et al., 2015; Steinfort et al., 2010). The performance of orchid mycorrhiza under stress have been poorly studied and there are few reports analyzing orchid performance under metal(loid) stress (Jurkiewicz et al., 2001; Shefferson et al., 2005).

Although orchid mycorrhiza molecular regulations are little known, recent studies have Identified genes and proteins having a key role at young developmental stages of the orchid-fungi interaction (Valadares et al., 2014; Valadares, 2014; Zhao et al., 2014). Since it is difficult to define the direction (beneficial or pathogenic) that may be taken

by the orchid to control the mycorrhizal interaction, more specific studies are necessary to better define the nature of the symbiosis. Thus, the specific objectives of this manuscript are: i) to evaluate the in-vitro interaction between *B. fimbriata* seeds and the mycorrhizal fungus *Tulasnella calospora*, under metal(loid) stress, using morphometric analyses; ii) to explore the orchid proteome developed to identify key proteins involved in both the preservation of the orchid-fungi association in orchid roots; and iii) to test a pool of reported antagonist and mutualistic genes to know how orchid mycorrhizal fungus (OMF) affects orchid root gene expression.

5.3 MATERIALS AND METHODS

5.3.1 Symbiotic germination

Mycorrhizal fungi from *B. fimbriata* roots sampled near Ventanas industrial complex (Puchuncaví Valley, Region de Valparaiso) were isolated according to Herrera et al. (2017). Molecular identification of mycorrhizal fungi was done by PCR using ITS1 and ITS4 primers, following sequencing and BLAST search as described in Steinfort et al. (2010). After mycorrhizal fungi identification, a tetrazolium test was conducted to assess seed viability (Lakon, 1949), and symbiotic germination trials were done to complete measuring the mycorrhizal fungi potential to promote growth under different metal(loid)s stress. 200 μg of seeds from one mature fruit capsule were superficially disinfected according to Dutra et al. (2009), with minor modifications. Seeds were immersed in an 8:1:1 solution of 8 mL of sterile deionized water, 1 mL of sodium hypochlorite (5% chlorine), and 1 mL of 100% alcohol for 3 min, followed by five washes in sterile deionized water. The seeds were then placed in a suspension of 50 mL of deionized water. Afterwards, 500 μL of this suspension were dispersed in Petri dishes containing 20 mL of modified oatmeal agar (4 g oats L^{-1} and 10 g agar at pH 5.6,

supplemented with 100 mg L^{-1} streptomycin) and a mycelia plug. A randomized design was used, with each fungal isolate as a treatment, with ten replicates per treatment (Cu, As and Fe at 5 ppm) and a control of ten un-inoculated plates. Also, an asymbiotic seed culture was performed on Murashige and Skoog media (at 50% salt concentration) to observe the effects on seed germination without mycorrhizal fungi. Plates were incubated in the dark at 25 ± 1 °C for 8 weeks, and transferred to a climatic chamber. All the cultures were incubated at 25 °C and 16-h photoperiod provided by cool white fluorescent lamp with a photon flux density PFD) of 40 µmol m^2s^{-1}. Each Petri was analysed at 7-days intervals under a microscope (×20–40) and germination stages of 100 seeds per plate were recorded (Stewart & Kane, 2006). The growth index (GI) was calculated as follows: $GI=(N_1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4)/(N_0 + N_1 + N_2 + N_3 + N_4)$, where N_0 is the number of seeds in stage 0, N_1 is the number of seeds in stage 1, and so on (Valadares et al., 2011).

5.3.2 Image analysis

Starting 7 days after seed sowing, the growth of symbiotic and asymbiotic controls, and the plates supplemented with metal(loid)s, were monitored at 7-day intervals for 84 days using a stereomicroscope (SteREO Discovery.V8; Carl Zeiss Microscopy, NY), capturing the images with a Zeiss axiocam icc 5 (Carl Zeiss) camera, according to López-Chávez et al. (2016), with modifications. The images were segmented in Microsoft Paint, the area (pixels) and roundness factor $RF = \frac{4\pi A}{P^2}$ (A=area, P= perimeter) were obtained using the MATLAB R2016a program (MathWorks, Inc.), and statistically analysed using ANOVA with R version 2.15.2 (R Core Team 2013), providing an indirect measure of protocorm growth and differentiation (RF values <1 indicated differentiated protocorms)

5.3.3 Growth conditions

Two month old *B. fimbriata* plantlets obtained as described by Jiang et al. (2015) (young rhizome replication) were transferred to individual plastic pots (10 per soil) with 800 g of sieved soil (2mm). The soil samples were collected from two semiarid Mediterranean ecosystems: (1) ultisol top soil (20 cm) affected by metal(loid)s pollution from Puchuncaví Valley in central Chile, 1.5 km southeast of the Ventanas copper smelter, which was exposed to atmospheric deposition of metal(loid)s enriched particles (HM) and (2) dunesand ultisol soil from Concon (Nat), which was defined as control, because its natural conditions promote development of naturally germinated plants. Soils were maintained at their 60% water holding capacity. Microcosms were maintained in the greenhouse for fourteen months. A pool of mycorrhizal and non-mycorrhizal root segments, under both soil conditions, was prepared in order to obtain total proteins. Each replicate consisted of a pool of root segments from five plants. After screening, root segments were placed in 1.5 ml microtubes, immediately frozen in liquid nitrogen and stored at –80°C until use. Treatments were defined as follows: Nat myc = mycorrhizal roots developed in natural soils; Nat non-myc = non-mycorrhizal roots developed in natural soils; HM myc = mycorrhizal roots developed in metal(loid)s contaminated soils; HM non-myc = non-mycorrhizal roots developed in metal(loid)s contaminated soils.

After the growth period, roots were screened for OMF presence (pelotons) (**Figure 5.1**) in natural and metal(loid)s contaminated soil (soil from Puchuncaví). Three complete plantlets were screened for pelotons, under both soil conditions, in order to know the mycorrhizal colonization percentage (number of root segments with pelotons) and the colonization percentage of each infected root segment.

5.3.4 Protein extraction, mass spectrometry and database search for protein identification.

Proteins were extracted following the procedure described in **section 4.3.7** and **4.3.8**, comparing mycorrhizal and non mycorrhizal roots of individual plants developed in natural soil.

5.3.5 RNA extraction and processing

Roots were quickly cut (non-mycorrhizal roots), screened for pelotons (mycorrhizal roots) and immediately frozen in liquid nitrogen. Total RNA was obtained from 100 mg of mycorrhizal and non-mycorrhizal roots after pulverization in liquid nitrogen, according to Fuentes et al. (2016). Total RNA was isolated using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) and then re-suspended in 40 mL of RNase-free water. RNA integrity was verified, and concentration and purity were measured by UV spectrophotometry at 260/280 nm (MaestroNano spectrophotometer, Maestrogen®). RNA samples (10 ng) were treated with an RNase-free DNase I Set (E.Z.N.A). Finally, RNA was purified using clean-up Hi-Bind RNA Mini Columns from a Total RNA Kit I (E.Z.N.A)

5.3.6 cDNA synthesis and RT-PCR

The cDNA synthesis was performed using the AffinityScript RT-PCR kit (Stratagene, Cedar Creek, TX) according to manufacturer's recommendations. First, 1 ng of total RNA was added to a mixture containing 170 ng of oligo (dT) and 30 ng of random primers in a final volume of 20 mL. The mixture was incubated for 5 min at 25° C, 45 min at 42° C, and 5 min at 95° C. Primer design for gene expression in root tissues was performed according to gene sequences from mycorrhizal *Serapias vomeracea* protocorms, taking into consideration a pool of mutualistic and antagonistic

genes considered as key to define the nature of plant-fungus interactions (mutualistic or antagonistic); thus including elongation factor 1 α (EF-1 α) and ubiquitin (UBI) as housekeeping genes; nodulin (NOD), remorin (REM), sugar transport (MTN) and acid endochitinase (END) as mutualistic markers; as well as wound stress protein (WSP), mannose binding lectin (MAN) and pathogenesis related protein (PRP) as antagonistic markers (**Table 5.2**). Because there is no available information about *B. fimbriata* genome, degenerate primers were designed considering RNA sequences from other available orchids, retrieved from the GenBank database. Multiple alignments were carried out using Clustal W and primers were designed in conserved domains using primer BLAST. The product size and specificity for each primer pair were verified by RT-PCR, and visualized using a 2% agarose gel according to Fuentes et al. (2016). Amplicons of the selected size were sent to sequencing to Macrogen (Seoul, Korea), to obtain definitive sequences and to finally quantify the expression level of the selected genes.

5.3.7 Mycorrhizal fungi and seed preservation

Identifying mycorrhizal fungi associated with endemic orchids is the start point to design strategies to reintroduce orchids with conservation problems. During all analyses developed, a set of fungi associated with ten endemic orchids was identified. Seven mycorrhizal fungi was stored as actively growing cultures on 2% Malt Extract Agar (MEA) at 4 °C, and also cryopreserved at −80 °C for 1 to 2 years, following Voyron et al. (2009). Seeds of the endemic orchids *B. fimbriata*, *Chloraea bletioides, C. maguellanica, C. longuipetala, C. gavilu, C. crispa, C. grandiflora, C. chrysantha, C.* spp. hybrids, *Gavilea lutea*, *G. araucana*, and *Codonorchis lessonii* were collected at their natural distribution areas, and were dried at room temperature in silica gel for 2

weeks, and stored in glass vials at 4°C. Seed germination trials were performed according to Herrera et al., 2017 at one year after the preservation experiments.

5.4 RESULTS

5.4.1 Mycorrhizal protocorm growth in contaminated media

Mycorrhizal fungi identification showed *T. calospora* as the main mycorrhizal fungi inhabiting *B. fimbriata* roots from the degraded soil. This isolate was tested in symbiotic germination trials, using seeds with ≈50 viability, showing contrasting results in protocorm development. Seed development was negatively affected by As treatment, which showed lower germination index, with little seeds reaching stage II. Significant differences in growth were obtained for Cu after 49 days culture (GI 0 1.955; GI As: 0.478; GI Cu 2.674; GI Fe 1.786). Despite the fact that the data obtained was very variable, we observed a development decrease in *B. Fimbriata* seeds under metal(loid) stress, inoculated with mycorrhizal fungi or asymbiotic. However, after 84-day culture, the presence of *T. calospora* improves protocorm development, reaching higher rhizoid production rates than asymbiotic media. At the beginning of the experiment, seed development was similar, showing negatively significant differences after 28 days of culture. Compared to the asymbiotic media, mycorrhizal protocorms showed better growth after 49 days, with an estimated area 20% higher than symbiotic culture under Cu supply. Under metal(loid) stress, a lower seed development was observed, with estimated areas at least 37% smaller than asymbiotic controls during 84 days culture. The RF, defined as an indirect measure of protocorm differentiation, was close to 0.3 after 60 days of symbiotic culture. On the other side, the copper and arsenic supplied to the media induces low protocorm differentiation, with RF close to 0.5; however, protocorm showed greater rhizoid presence under copper stress. After experimental

time, we were able to produce protocorms at stage IV of development, which continues growing in-vitro media. The inoculation of mycorrhizal fungi in seed plates submitted to heavy metal stress results in greater biomass and better differentiation compared to asymbiotic control, suggesting a beneficial effect of the fungus. In contrast, asymbiotic protocorm growth and differentiation was much less evident under metal(loid) stress.

5.4.2 Root fungal colonization in *B. fimbriata* plantlets

The mycorrhization in orchid roots was different according to the soil from which plants were harvested. Roots from control soils showed pelotons in at least 7% of the cuts (3 mm segments) (>2% of infected surface) (**Figure 5.2**). Root cuts from metal(loid)s contaminated soils showed an increase in peloton amount, with approximately 24% of mycorrhizal segments. Although roots from control soil were longer, root biomass was higher in plants from the metal(loid)s contaminated soil, showing higher number of short roots and better mycorrhization percentages (**Figure 5.2**). In mycorrhizal root segments from the metal(loid)s contaminated soil, the surface occupied by mycorrhizal fungi ranged from 30 to 65%, which denotes an increase on mycorrhizal storage organs (pelotons). On the other side, control soil showed less peloton abundance with vales ranging from 5 to 20% of infected surface; however, no significant differences were obtained due to data variability. This obtained data is critical to understand the dynamics of orchid mycorrhizas under metal(loid) stress.

5.4.3 Gene expression in mycorrhizal and non-mycorrhizal root segments

The biggest challenge of this report was to successfully design degenerate primers to assess gene expression in mycorrhizal and non-mycorrhizal roots. As no reference genome was available, GenBank database search of the selected genes was limited to Orchidaceae. Available sequences were mainly related to *Phalaenopsis* and *Cymbidium* orchid ornamental plants, two of the main ornamental genera of the Orchidaceae family. After search and multiple alignments, the degenerate primers described in **Table 5.2** were designed. Two candidate reference genes (EF-1 α and UBI), four mutualistic markers (NOD, REM, MTN and END), and three antagonistic genes (WSP, SER and PRP) were evaluated by RT-PCR to detect their presence in mycorrhizal and non-mycorrhizal roots. These genes were selected because predicted proteins or conserved protein functional domains showed high similarities with proteins involved in plant–microbe interactions and induced in mutualistic or pathogenic interactions (Perotto et al., 2014). Orchid gene expression was assessed in mycorrhizal and non-mycorrhizal roots from both soil treatments, in which *T. calospora* hyphae formed intracellular pelotons in mycorrhizal roots mostly in the metal(loid)s contaminated soil, and non-mycorrhizal roots showed a cortex that was never colonized by the fungus (**Figure 5.2**).

Primers designed for expression analyses were used for one-step RT-PCR to assess RNA quality and possible contamination of root RNA samples with genomic DNA. The EF-1 α and UBI primers amplified *B. fimbriata* transcripts in mycorrhizal and non-mycorrhizal roots from both soil conditions (**Figure 5.3**). However, amplicon integrity was better in EF-1 α . For this reason, only EF-1 α was selected as housekeeping gen for the quantitative PCR experiments. The RT-PCR reactions, with the selected orchid genes listed in **Table 5.2**, showed that some of the genes were

uniquely expressed in mycorrhizal segments. Specifically, mycorrhizal roots showed the presence of an amplicon related to Nodulin-like gene and for a putative remorin gene in metal(loid)s contaminated treatments (**Figure 5.3**). Under stress we showed presence of amplicon in all genes related to pathogen control, such as wound stress precursor and pathogenesis related protein. The tested genes were also expressed to different degrees (amplicon intensity) in the mycorrhizal and non-mycorrhizal roots from both soils (**Figure 5.3**). As RT-PCR is not a quantitative technique, and only detected the presence and absence of amplicons, real-time quantitative PCR (qRT-PCR) experiments are necessary to better confirm the results of gene expression in roots. RT-PCR confirmed expression of antagonistic markers related protein in mycorrhizal and non-mycorrhizal roots from both soils. The expression of these antagonistic markers is better defined under stress which denotes an enhancement of control mechanisms of mycorrhizal fungi spread. Signs of OMF compatibility are confirmed with specific amplicon of nodulin genes, which are present in mycorrhizal tissues, especially under metal(loid) contaminated soil (**Figure 5.3**).

5.4.4 Proteome analysis

Proteome profile was affected by the presence of mycorrhizal fungi, identifying 119 protein clusters in treatment mycorrhizal and 91 in non-mycorrhizal roots. A total of 373 proteins with differential accumulation were detected, grouped in 147 protein clusters. The proteome profiles revealed 46 proteins exclusively found in mycorrhizal roots and 33 in non-mycorrhizal roots, and 68 differentially expressed. For the analyses we only considered proteins that were detected in four analytical samples and with p value<0.1. After filtering, 29 proteins were selected (**Table 5.1**), nine of which were found in mycorrhizal, two exclusively in non-mycorrhizal roots and 19 with differential accumulation. Up-regulated proteins (myc/non-myc) were related to carbon metabolism

(glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, sucrose synthase, phosphoenolpyruvate carboxylase), synthesis of anthocyanins (UDP-flavonoid glucosyltransferase), transport (ATP binding cassette transporter), flavonoid synthesis (phenylalanine ammonia lyase) responses to external stimulus (glutamate decarboxylase) defense (phospholipase D, allene oxidase, orcinol O-methyltransferase, 3-ketoacyl-CoA thiolase), phenylalanine metabolism (acetyl-CoA-C-acetyltransferase), signaling (calcium-dependent protein kinase 2), ATP metabolic process (ATP synthase CF1 beta chain), cytoskeleton reorganization (beta tubulin, COBRA-like protein), redox homeostasis (peroxidase, monodehydroascorbate reductase, flavonoid 3'-hydroxylase, quinone oxidoreductase) and photorespiration (ribulose-1,5-bisphosphate carboxylase). Whereas down-regulated proteins were related to photorespiration (chlororespiratory reduction) and defense (lipoxygenase), ethylene biosynthesis (aminocyclopropane-1-carboxylate oxidase), cell wall modifications (pectin methylesterase) and translation (40S ribosomal protein).

5.4.5 Seed and mycorrhizal fungi storage

After one year of preservation, all mycorrhizal fungi promoted germination of their associated seeds up to stage 2 (Herrera et al., 2017) (**Table 5.3**); however, seed viability was diminished. Germination indexes were lower than fresh sowed seeds. Significant differences between protocorm growth promotion were observed for *Tulasnella* sp., isolated from *C. gavilu* (GI: 1.374), which was previously classified (**Chapter III**) as the most effective mycorrhizal fungi to promote seed development without specificity, in despite of germination being negatively affected.

5.5 DISCUSSION

5.5.1 Effect of mycorrhizal fungi on seed germination under metal(loid)s stress

The performance of orchid mycorrhiza under metal(loid)s stress has been scarcely explored. Currently, only few studies have been done exploring some adaptations of mycorrhizal orchids in metal(loid) polluted environments (Jurkiewicz et al., 2001; Shefferson et al., 2008). In this chapter, we have characterized the effect of mycorrhizal fungi on seed germination in a media supplied with metal(loid)s, and better characterized the colonization of mycorrhizal fungi in root tissues of young plantlets at both soil conditions, exploring some of the genetic regulations at root level. The results showed that *T. calospora* promoted *B. fimbriata* protocorms growth and differentiation, which is indicative of a symbiotic interaction promoting germination even under stress. This conclusion agrees with previous studies showing that mycorrhizal fungi isolated from roots was beneficial to promote germination, protocorm development, and plantlet establishment at degraded areas (Shefferson et al., 2008). According to our results, the protocorm development promotion, under metal(loid) stress, by the fungus was evident (comparing to asymbiotic controls) (**Figure 5.1**), in despite of the large data variability for area and roundness factor. This variability could derive from the intrinsic genetic variation between orchid seeds and the particular developmental speed of each embryo to form a viable protocorm (Weber & Webster, 2001). López-Chávez et al. (2016) and Verdugo et al. (2007) have found that measurement of protocorm development by software image analyses was a better estimation of growth than random evaluations of developmental stages of protocorms in multiple Petry dishes. The presence of metal(loid) in the contaminated media show that mycorrhizal fungi were able to promote seed germination and development, in despite of lower growth and differentiation speed. This seed growth promotion was lower than non-contaminated

media, because seeds are under direct contact with the metal(loid)s, showing lower germination and growth rates. Estimates of protocorm development were higher in mycorrhizal treatment because the mycorrhizal fungi had the ability to improve protocorm development to different degrees (Otero et al., 2002). Plant growth promotion was achieved in almost treatments (except As). However, the roundness factor is near 0.4 because the protocorm developed foliar primordia pointing upwards, while others developed lateral positions (and thus, it was integrated into total area determinations).

5.5.2 *B. fimbriata* root colonization

The peloton abundance in roots is variable, and must change according to different environmental condition and orchid-fungus relationships (Smith & Read, 2010). Currently, there is no a defined nutritional flux between orchid mycorrhiza in adult orchid plants, except for fully mycoheterotrophic orchids, in which peloton containing roots are source of carbon and nutrient for associated orchids (Lee et al., 2015). Measurements of pelotons density in terrestrial orchids are a common technique to characterize the mycorrhization process, and have been commonly used to characterize symbiotic plants under different environmental conditions (Schatz et al., 2010). Terrestrial orchids are able to form distinct symbiotic associations with different fungal endophytes (Athipunyakom et al., 2004); however, mycorrhizal structures (peloton-containing roots) are an obligate characteristic of the commonly accepted mycorrhizal fungi: the polyphyletic *Rhizoctonia*-like complex (McKendrick et al., 2000). In despite of the broad number of studies related to the isolation and identification of mycorrhizal fungi from terrestrial orchids, there is no standard methodology to estimate root mycorrhizal percentage (Dearnaley, 2007). Thus, to better characterize orchid root colonization we analyzed three complete plants in order to

understand how the mycorrhization process dynamics is altered under the deficient soil conditions of the Puchuncaví soil. Based on the observations, it was detected an improved mycorrhizal colonization within root parenchyma cells of roots from metal(loid)s contaminated soils, in which the mycorrhizal fungi colonize radical tissues and form pelotons, the structures that are characteristic of the mycorrhizal infection of orchids (Smith & Read, 2010). Brundrett (2002) points out that fungi having a mycorrhizal function develop specialized hyphae that help the colonization of specialized plant organs and establish a bidirectional, synchronized transfer of nutrients that may strongly or weakly benefit plant development. However, this bidirectional nutrient flux may be rather complex in orchid roots, in which orchid mycorrhiza transit for the parasitism-mutualism continuum (Johnson et al., 1997). The improved mycorrhization detected in metal(loid)s contaminated soil is a sign of nutrient accessibility for the orchid, because orchids may aggressively take nutrients directly from mycorrhizal fungi held in pelotons (Kuga et al., 2014). This agrees with previous studies showing that mycorrhizal fungi play a role in improving orchid establishment in metal(loid)s contaminated areas, mainly by limiting metal(loid) availability for the plant, sequestering metal(loid) inside fungal hyphae kept in pelotons (Jurkiewicz et al., 2001).

5.5.3 Presence of mutualistic and antagonistic genes in orchid roots

On respect to gene expression assessed through RT-PCR, the housekeeping primers developed for gene expression analyses (EF-1 α) showed good amplicon intensity, which were excised and sent to sequence, as well as one mutualistic (NOD) and antagonistic markers (WSP). To better discuss the results of these experiments, we need to refer to **Chapter IV**, in which we showed that under metal(loid)s stress root tissues presented an enhanced accumulation of proteins related to the fungal attraction

and alleviation of defense anti-pathogen mechanisms, even though we cannot relate all proteins with the genes described in **Table 5.2**. However, the main nature of mutualistic and antagonistic genes is well described. Our results agree with previous studies analyzing gene expression in *Serapias vomeracea* mycorrhizal protocorms, in which the nature of the mycorrhizal interaction was defined as mutualistic, rather than an antagonistic interaction in favor of the plant (Perotto et al., 2014). At adult stages we have a lot of environmental processes that interfered with expression analyses, so this chapter was focused in better discussing some genes with a clear role in mutualistic and antagonistic interactions, rather than stress related genes.

In spite of the small panel of orchid genes used to assess the nature of the plant–fungus interaction (mutualistic or antagonistic), established under metal(loid)s contaminated soils, it was detected that mycorrhizal tissues do not develop strong defenses in the colonized tissues. RT-PCR indicated that *B. fimbriata* have expression for a putative pathogenesis related protein, known in other biological systems to be part of defense mechanisms (Alexander et al., 1993), which was not extremely up-regulated in mycorrhizal orchid tissues (showed by amplicon intensity in **Figure 5.3**). It was also tested a marker of wound-induced responses, which were present in similar intensity in the four treatments. The similar amplicon intensity in the mycorrhizal and non-mycorrhizal tissues would indicate high plant-fungus compatibility, because this gene family belongs to a large family of hydrolyzing enzymes that plays several roles in cellular processes, including defense responses against pathogen infection (Ponstein et al., 1994). The high amplicon intensity obtained for MAN and PRP suggested that this protein family is part of the system to avoid infection of vital tissues in mycorrhizal roots, as these proteins have a defined function to control pathogen spread inside root tissues (Peumans & Van Damme, 1995; van Loon, 1985).

Regarding to mutualistic markers, all tested genes showed a good amplicon intensity. END coding for a class III acidic chitinase, showed an amplicon in the four treatments. Although chitinases have been defined as part of pathogenic interactions, specific class III chitinases have been found to be up-regulated in arbuscular mycorrhizal roots these chitinases was also been found in the arbuscule-containing cells, where they may play a role in suppressing plant defense in symbiotic interfaces (Perotto et al., 2014). Similar function is predicted for NOD and MTN which codes for putative bidirectional sugar transporters of the SWEET family, and have a good defined amplicon in both mycorrhizal treatments. These transporters are up-regulated in fungal endophytes interactions to obtain carbohydrates from the associated plant (Allen, 1991).

5.5.4 Proteomic regulations in *B. fimbriata* roots

Proteomic changes of mycorrhizal roots under natural conditions. The OMF plays a vital role in orchid life cycle, not only to provide carbon during embryo development to protocorm, but also to ensure a correct development at further stages (Dearnaley et al., 2012). Growth of orchids in a particular environment involves mutual recognition and compatibility of host and fungi, hypha penetrating the cell wall of root hairs or epidermis and then entering the cells of the cortical parenchyma, forming characteristic and complex hyphal coils known as pelotons (Zhao et al., 2014). Such processes require a complex molecular mechanism to control the colonization process, especially at advanced developmental stages (Bonnardeaux et al., 2007; Collins et al., 2013; Dearnaley, 2007; Valadares, 2014). To date, the molecular regulations and changes taking place in orchid mycorrhiza are under study (Collins et al., 2013; Perotto et al., 2014; Selosse et al., 2004) and these are mainly focused on orchid protocorms more than on adult plants (López-Chávez et al., 2016; Valadares, 2014). This is unquestionably an essential issue due to the importance of the symbiosis at first

developmental stages (Smith & Read, 2010), but there are evidences that in fully-grown orchids and under some condition (stress) the orchid may improve benefits due to their mycorrhizal interaction (Shefferson et al., 2008).

In terrestrial orchids, non-mycorrhizal roots are anatomically different from the mycorrhizal ones, which are characterized by lower diameter and presence of brownish zones, in which the OMF is restricted. Non-mycorrhizal roots are expected to act as sink of starch, whereas mycorrhizal roots host the mycorrhizal fungi and their mycorrhizal structures, from which obtain mycorrhizal derived nutrients (Cameron et al., 2008; Cameron et al., 2006). For this reason, it is expected that particular physiological process may control mycorrhizal and non-mycorrhizal roots (Zhao et al., 2014). To understand the physiological responses of *B. fimbriata* related with the presence of mycorrhizal fungi, the proteome profile was analyzed in mycorrhizal and non-mycorrhizal roots. Our results suggest that, under natural conditions, the proteome profile was affected by the presence of the mycorrhizal fungi. Down-regulation of proteins related to stress, carbon metabolism and cell wall reorganization was detected. The higher abundance of proteins in mycorrhizal roots, the higher metabolic activity, which may be related to the enhancement of essential cellular processes such as growth, membrane transport, pathogen control and oxidative stress (Chiapello et al., 2015; López-Chávez et al., 2016; Valadares, 2014). Interestingly, some of the molecular responses developed in symbiotically germinated protocorms are found in mycorrhizal roots, such as proteins with a key role to metabolize fungal origin carbon, growth and to control fungi inside young *Oncidium sphacelatum* protocorms (Valadares et al., 2014; Zhao et al., 2014). Primary responses of plants against mycorrhizal events (either compatible or incompatible fungi) are processes related to oxidative stress (Schützendübel & Polle, 2002). In this experiment it was detected up-regulation of

proteins related to scavenging of reactive oxygen species, such as glutathione S-transferase, peroxidase, xanthine dehydrogenase, quinone oxidoreductase and phenolic compounds. Alongside, mycorrhizal roots showed enhanced accumulation of proteins involved in metabolic pathways toward control and response against pathogen attack, including synthesis of ethylene and phenolic compounds. Certainly, mycorrhizal tissues in orchids must have an efficient mechanism to control fungi host in pelotons, a process that was detected in *B. fimbriata* roots. The enhanced accumulation of proteins related to orcinol biosynthesis and allene oxidase in mycorrhizal roots is the main mechanism to avoid colonization and spread of mycorrhizal fungi across plant plasmalemma (Sneh, 1996). Orcinol has been described as a key biomolecule in orchid mycorrhiza interactions, in which control of fungal growth in root cortex may be affected by its synthesis, although other processes such as phenolic deposition and wall thickenings, possibly together with the accumulation of other compounds, are crucial to sustain mycorrhizal activity (Beyrle et al., 1995).

5.5.5 Mycorrhizal fungi and seed conservation

Mycorrhizal fungi and orchid seed conservation is an effective measure to improve population number of endangered species. In this Doctoral Thesis, mycorrhizal fungi and seeds of endemic orchids were effectively preserved in the Laboratory of Bioremediation at the Universidad de La Frontera, which may act as germplasm bank of mycorrhizal fungi and endemic orchids. Germplasm conservation has been used as a measure for safeguarding orchids with reproduction problems (Seaton et al., 2010). More effective preservation methods have been described in Batty et al. (2001), which showed that dried seed stored in liquid nitrogen germinated better than seeds freshly collected or stored at 4, 18, and 22°C for 1 year. Additionally, the mycorrhizal fungi that promoted germination and growth of orchids could also be successfully stored in

liquid nitrogen, although fungi are readily stored using a variety of other methods including lyophilization and freezing (Swarts & Dixon, 2009).

5.6 CONCLUSIONS

This study have better describe the interaction between *T. calospora* and *B. fimbriata* seeds under metal(loid) stress, demonstrating a positive role of mycorrhizal fungi to promote protocorm growth and differentiation. It is clear that the restrictive conditions of the Puchuncaví soil stimulate mycorrhizal fungi colonization, forming condensed infected areas, with high rates of degraded pelotons, which are potential source of mycorrhizal fungi-derived nutrients for the orchid, as well as a potential physical place to store metal(loid)s, limiting availability to plant. It was also identified key proteins involved in mycorrhizal symbiosis regulation. Critical proteomic changes were detected even in the same plant, which denotes a different metabolism for mycorrhizal and non-mycorrhizal roots. Finally, gene expression evaluated by RT-PCR suggests that some of the mutualistic markers were significantly over-expressed in mycorrhizal roots, similarly to other mutualistic interactions, and the amplicon intensity stimulation of pathogenic markers may derive from the enhancement of mycorrhizal fungi colonization, to avoid infection of vital plant tissues. All tested methods to store mycorrhizal fungi and seeds for future orchid reintroduction strategies were analysed and performed to preserve both, mycorrhizal fungi and orchid seeds.

5.7 ACKNOWLEDGMENTS

Thanks to Bioremediation laboratory and Vale Institute of Technology for equipment supply. Funding was provided by FONDECYT project 1170931 to C. Arriagada and CONICYT fellowship 21130491 to H. Herrera.

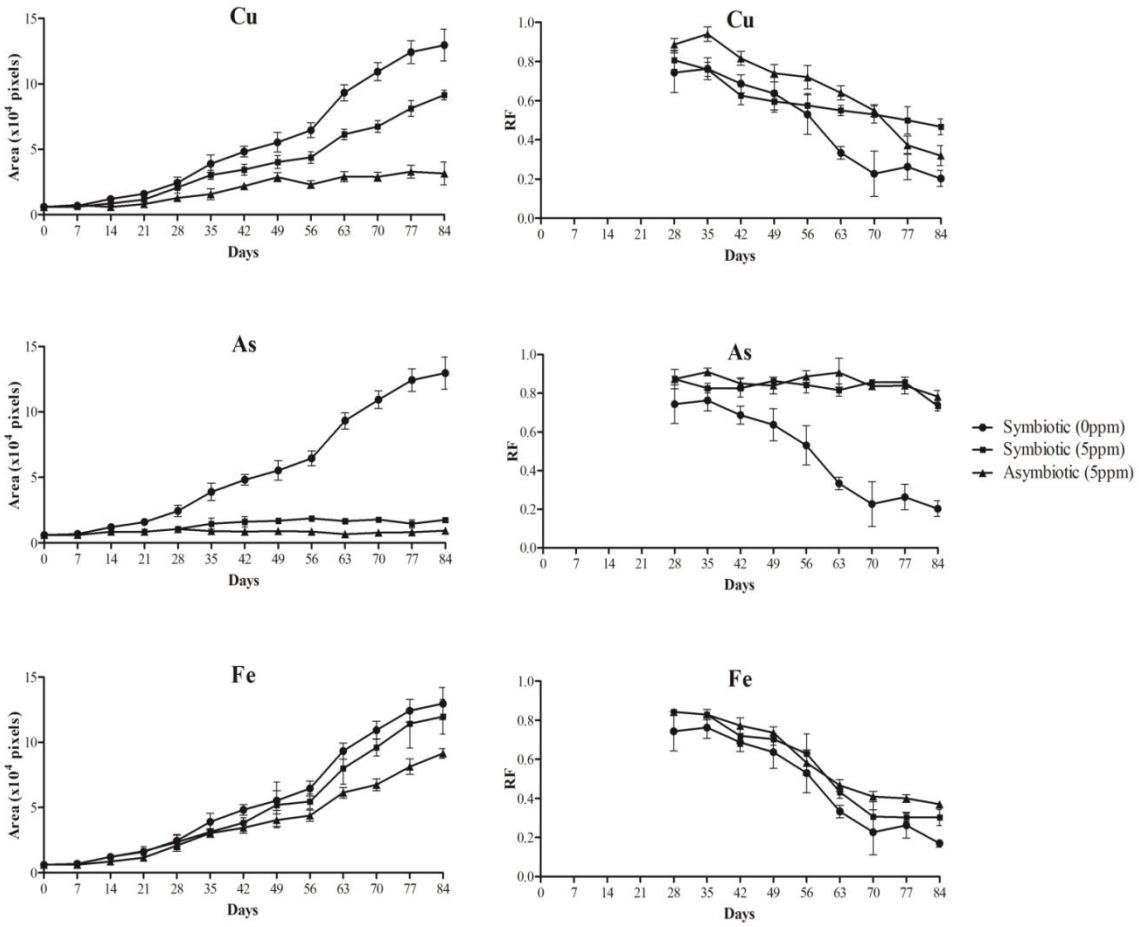
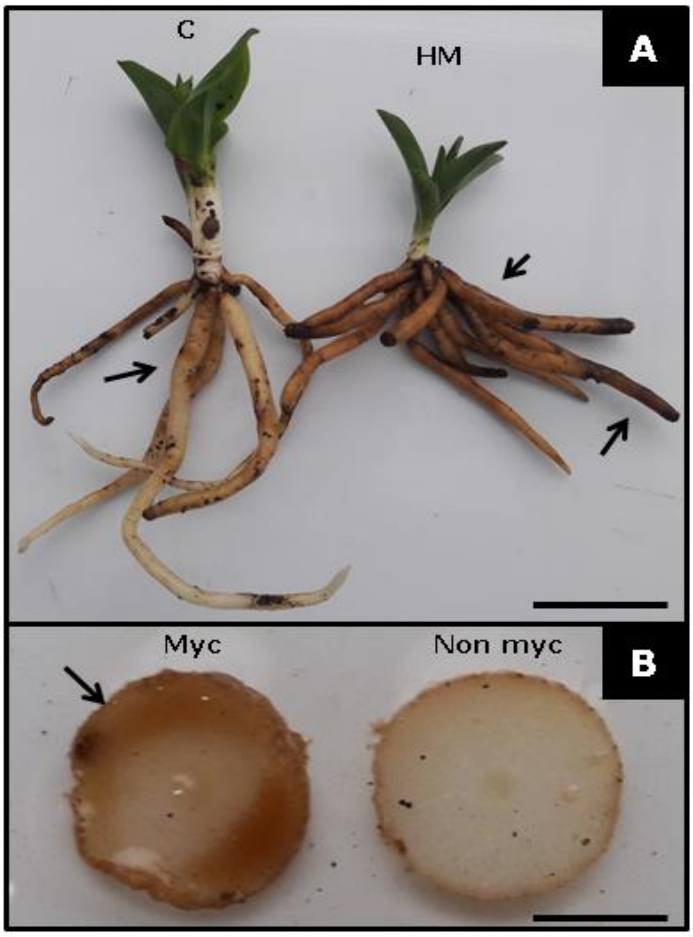


Figure 5.1 Development of *B. fimbriata* protocorms grown in Oatmeal Agar plus Cu, As and Fe in the symbiotic treatments (inoculated with *Tulasnella calospora*) at 0 and 5 ppm and the asymbiotic treatment (Murashige and Skoog) at 5 ppm. Values are mean ± SD, for n=10



Mycorrhizal segments

Control soil

Mycorrhizal	7 %
Non-mycorrhizal	93 %

Puchuncavi soil

Mycorrhizal	24 %
Non-mycorrhizal	76 %

Colonization percentage

Control soil	12 %
Puchuncavi soil	37 %

Figure 5.2 (**A**) *Bipinnula fimbriata* plantlets established in soil from Concon (C) and Puchuncaví (HM). Black arrows show mycorrhizal root segments. Scale bar = 3 cm; (**B**) mycorrhizal (Myc) and non-mycorrhizal (Non myc) roots. Black arrow shows the infected surface (degraded pelotons). Scale bar = 5 mm

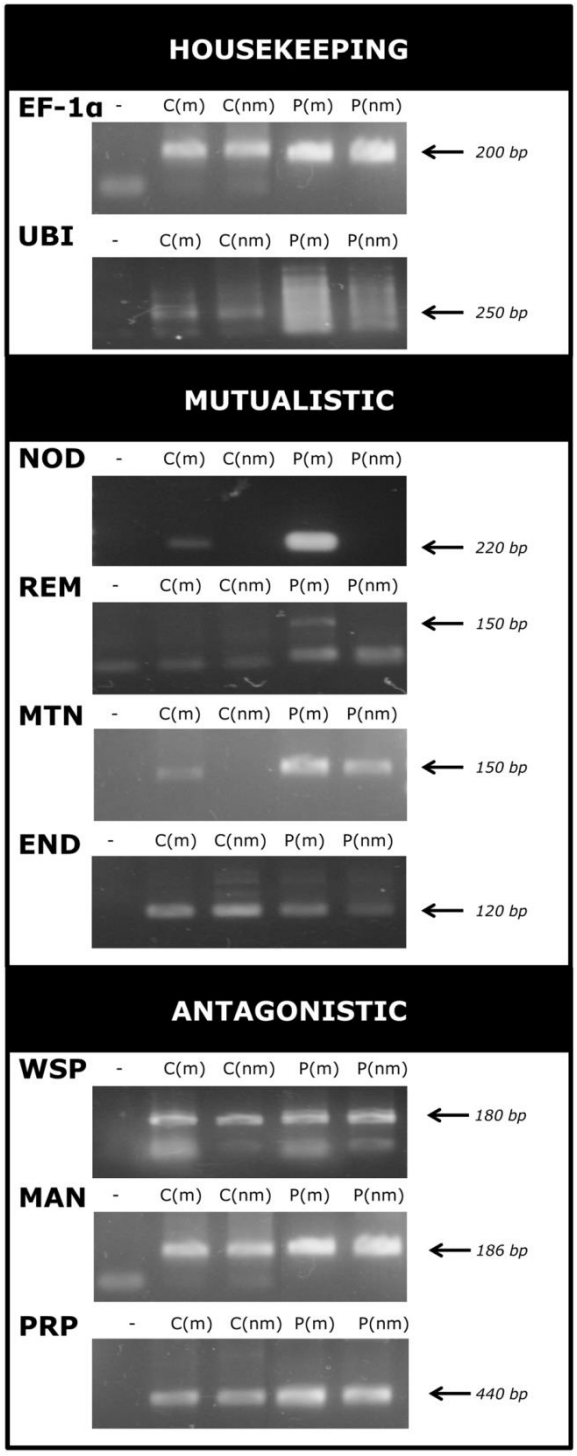


Figure 5.3 RT-PCR gene expression of mycorrhizal (M) and non-mycorrhizal roots (NM) from Concon "C" and Puchuncaví "P" soils. Gene expression was assessed for: (i) housekeeping genes elongation factor-1α (EF-1α) and ubiquitin related protein (UBI); (ii) mutualistic markers nodulin like protein (NOD); remorin family protein (REM); bidirectional sugar transporter (MTN); and class III acidic chitinase (END); and (iii) the antagonistic markers wound stress protein (WSP); mannose binding lectin (MAN); and pathogenesis related protein (PRP). All degenerate primers were testes in the four treatments plus a CDNA minus reaction (-). Black arrows show size of the expected amplicon.

Table 5.1 Most representative proteins identified in *Bipinnula fimbriata* mycorrhizal and non-mycorrhizal roots, from natural soils (CC myc = mycorrhizal roots developed in natural soil; CC non-myc = non-mycorrhizal roots developed in natural soil)

Protein description	*Organism*	*Accession (protein)*	*Molecular weight (kDa)*	p-value	*Log Fold change*
MYCORRHIZAL YS NON-MYCORHIZAL (Control soil)					
Acetyl-CoA-C-acetyltransferase	*Vanda* hybrid cultivar	AEI27246	39 kDa	< 0.01	CC myc
3-ketoacyl-CoA thiolase	*Vanda* hybrid cultivar	AIB06952	47 kDa	< 0.01	CC myc
Calcium-dependent protein kinase 2	*Dendrobium catenatum*	AGI21022	60 kDa	0.02	CC myc
UDP-flavonoid glucosyltransferase 2	*Phalaenopsis equestris*	ACB88211	53 kDa	0.09	CC myc
COBRA-like protein	*Oncidium* hybrid cultivar	AHJ14778	52 kDa	0.05	CC myc
Flavonoid 3'-hydroxylase	*Phalaenopsis equestris*	AIS35917	50 kDa	0.04	CC myc
Ribulose bisphosphate carboxylase large chain	*Aerides krabiensis*	AFP45761	9 kDa	0.04	CC myc
Quinone oxidoreductase	*Gymnadenia conopsea*	ABD66515	22 kDa	0.02	CC myc
ATP synthase CF1 beta chain	*Phalaenopsis Aphrodite*	AAW82507	54 kDa	< 0.01	CC myc
Xanthine dehydrogenase	*Wullschlaegelia calcarata*	ADQ00831	34 kDa	< 0.01	3.16
Phenylalanine ammonia lyase	*Bromheadia finlaysoniana*	AHA92089	77 kDa	0.06	2.94
RNA polymerase beta chain	*Phalaenopsis Aphrodite*	AAW82495	123 kDa	< 0.01	2.25
Monodehydroascorbate reductase	*Oncidium* hybrid cultivar	ACJ38541	47 kDa	< 0.01	2.08
Orcinol O-methyltransferase	*Vanda* hybrid cultivar	AIB06955	42 kDa	0.04	2.03
Allene oxide synthase	*Cymbidium ensifolium*	AFH89624	55 kDa	0.04	1.61
Phosphoenolpyruvate carboxylase	*Microcoelia exilis*	CAA62829	41 kDa	0.01	1.39
Glutamate decarboxylase	*Dendrobium catenatum*	AIU48022	56 kDa	< 0.01	1.22
Sucrose synthase	*Dendrobium catenatum*	ALS55905	92 kDa	< 0.01	1.06
Peroxidase	*Doritis pulcherrima*	ACN25040	37 kDa	< 0.01	0.71
Pyruvate kinase	*Dendrobium catenatum*	AGI60277	55 kDa	< 0.01	0.74
Hypothetical protein (related to ATP binding cassette domain)	*Oryza sativa*	Q9FLT8	68 kDa	0.02	0.67
Phospholipase D	*Dendrobium* hybrid cultivar	AIR77173	47 kDa	< 0.01	0.55
Adenosylhomocysteinase	*Phalaenopsis* sp.	P50249	53 kDa	0.06	0.52
Tubulin alpha chain	*Doritaenopsis* hybrid cultivar	AET80381	29 kDa	< 0.01	0.34
Cytosolic glyceraldehyde-3-phosphate dehydrogenase	*Phalaenopsis* hybrid cultivar	AFH88993	21 kDa	0.01	0.06
40S ribosomal protein S3a	*Cymbidium* hybrid cultivar	ABK56834	30 kDa	< 0.01	-0.45
Aminocyclopropane-1-carboxylate oxidase	*Oncidium* hybrid cultivar	AGB13770	37 kDa	< 0.01	-1.11
Pectin methylesterase	*Oncidium* hybrid cultivar	A CJ02103	58 kDa	0.05	-1.47
Chlororespiratory reduction 4	*Cypripedium formosanum*	AKH05175	61 kDa	0.06	CC non-myc
Lipoxygenase	*Cymbidium ensifolium*	AFH89626	49 kDa	0.01	CC non-myc

Table 5.2 List of degenerate primers designed according conserved domains defined by bioinformatic analyses and database search (EF-1α = elongation factor 1α; UBI = Ubiquitin-related protein; NOD = Nodulin-like protein; REM = Remorin family protein; MTN = Bidirectional sugar transport; END = Class III acidic endochitinase; WSP = Wound stress protein; MAN = Mannose binding lectin; PRP = Pathogenesis related protein.

Primer name	Primer sequence (5´ - 3')	Best identified match (Blastx)	Expected amplicon size (bp)	Obtained amplicon Quality
HOUSEKEEPING				
EF-1α	**Forward:** GTCTGGCAAGGAGCTTGAGA **Reverse:** AGGCCTCASCTGSTTGCTGT	Elongation factor 1 alpha	193 bp	Good
UBI	**Forward:** AGGCCTCASCTGSTTGCTGT **Reverse:** AGGCCTCASCTGSTTGCTGT	Ubiquitin 60S ribosomal protein	254 bp	Bad
MUTUALISTIC				
NOD	**Forward:** CKATGMTYGCCSCTGCAGTT **Reverse:** GATGGCCGGAACGGAGTTCT	Auxin-induced protein 5NG4-like (nodulinMtN21-like protein)	220 bp	Good
REM	**Forward:** GGAAGATGATGASAGGRCCA **Reverse:** TCASCTTCTCTTCTAARTTYGATCT	Remorin family protein	158 bp	Bad
MTN	**Forward:** TTGCTTTCTCCGTCAGCGTY **Reverse:** TGTCTTTGGAGAARAGGCCR	MtN3-like protein (bidirectional sugar transporter SWEET, putative)	153 bp	Good
END	**Forward:** ATMGCMGTCTACTGGGGTCA **Reverse:** GTGGCCGGCGAGGTTG	Class III acidic chitinase	110 bp	Good
ANTAGONISTIC				
WSP	**Forward:** YCTCGACATCTTCTCYGGC **Reverse:** CCACTGYTCRATGGTGAACT	Wound/stress protein precursor	172 bp	Good
MAN	**Forward:** TCAAGGGYTACATGGTTGGAA **Reverse:** GGCCYGAACCCTTTAYTGTGA	Mannose binding lectin	186 bp	Good
PRP	**Forward:** GCCAAMGAGGGCWACATCCT **Reverse:** GTARTCTCTGCTCWCCAGCTT	Pathogenesis related protein	447 bp	Good

Table 5.3 Mycorrhizal fungi isolates tested in this preservation experiments. All mycorrhizal fungi were isolated from Chilean orchids, mainly performing this Doctoral Thesis analyses (n = 10)

Fungal code	Molecular identification	Main orchid symbiont	Seed viability		Germination index	
			Year 0	Year 1	Year 0	Year 1
ORK1	*Tulasnella calospora*	*Bipinnula fimbriata*	70	48	2.144	1.275
ORK2	Uncultured Tulasnellaceae	*B. fimbriata*	73	51	0.944	0.428
ORK3	*Tulasnella* sp.	*Chloraea chrysantha*	56	32	1.255	0.355
ORK4	*Ceratobasidium* sp.	*Chloraea longipetala*	64	22	0.622	0.369
ORK5	*Tulasnella* sp.	*Chloraea gavilu*	57	42	1.625	1.374
ORK6	*Thanatephorus cucumeris*	*Chloraea bletioides*	62	46	0.573	0.477
ORK7	unidentified	*C. gavilu*	43	34	0.635	0.255

CHAPTER VI

General discussion, concluding remarks and future directions

6.1 GENERAL DISCUSSIONS

The broad orchid distribution range is an intrinsic characteristic of the Orchidaceae family (Roberts & Dixon 2008). This characteristic is conserved in Chilean Orchidaceae, which display orchid species in all national territory comprised between 'Region de Arica y Parinacota' and the continental territory of 'Region de Magallanes y la Antartica Chilena', except in extreme cold and hot environments such as volcanoes, the desert, and the Antartica (Herrera et al., 2017; Novoa et al 2015). In each natural distribution environment, orchids have developed particular characteristics to allow fitness to particular ecosystemic conditions, such as limited light exposure, seasonal cold and hot climatic conditions, and extremely human-altered ecosystems (Kottke 2010; McCormick et al., 2006). These adaptations are often related to symbiotic associations with appropriate mycorrhizal fungi adapted to particular ecosystemic characteristics, such as nutritional and climatic conditions. Mycorrhizal fungi isolated during this Doctoral Thesis allowed several orchids to complete their life cycle, promoting the transition from seed to protocorm, and finally to a partially mycoheterotrophic plantlet, which will be finally able to colonize metal(loid) contaminated soil. During this Doctoral Thesis, diverse mycorrhizal fungi associated with endemic orchids were identified, showing a limited orchid-mycorrhizal fungi specificity, which is the main reason for finding several orchids growing in diverse orchid hot-spots, such as the Parque Nacional Nahuelbuta (40% of Chilean Orchidaceae) (Novoa et al., 2015). In this Doctoral Thesis, members of the Tulasnellales order were shown as the main mycorrhizal fungi inhabiting roots of mature orchids, and they were classified as potential mycorrhizal fungi, because they were directly isolated from infected roots and were effectively promoting seed development of diverse orchids with low specificity (Herrera et al., 2017). This agrees

with previous studies classifying *Tulasnella* genus as main mycorrhizal fungi isolated from terrestrial orchids, and also as some related orchid species of the *Chloraea* genus, endemic from Argentina and Chile (Ogura-Tsujita & Yukawa, 2008; Fracchia et al., 2014; Steinfort et al., 2010).

In this Thesis, the identification of root fungal endophytes in the Chilean orchid roots was not limited to mycorrhizal fungi from the *Rhizoctonia*-like complex. A broad endophytic non-mycorrhizal fungi spectrum was also detected on the analysed orchid roots, including Dark Septate Endophytes (*Leptodontidium, Phialocephala* and *Cadophora* genera) as well as other endophytes (*Phomopsis*, *Chaetomium* and *Catenulostroma* genera) (Herrera et al., 2017). These fungi do not have a defined role in orchid metabolism (Bayman & Otero, 2006), but their widespread isolation from orchid roots still deserves more attention, in order to identify the potential ecological advantage for orchid to host a broad spectrum of non-mycorrhizal endophytic fungi (Herrera et al., 2017). As example, *Thanetophorus* genus was isolated directly from pelotons of *Chloraea longipetala* and *C. bletioides* growing in rocks from littoral ecosystems from Region del Maule. This genus includes one of the most relevant plant pathogens affecting diverse crops (Andersen & Rasmussen, 1996). The Chilean Orchidaceae seems to agree with the ability to host pathogenic strains in their mycorrhizal structures especially in an adverse environment such as rock surface, where plant communities, beneficial microorganisms and nutrients are very scarce (Herrera et al., 2017).

Chilean Orchidaceae has a broad distribution scope within the contrasting environmental conditions of the country. In extremely degraded areas, orchid mycorrhizal association are essential to achieve plant establishment, including metal(loid) contaminated ecosystems (Shefferson et al., 2008). According to our results presented in **Chapter IV**, *Bipinnula fimbriata* has the ability to grow in soils

contaminated with metal(loid)s, mainly promoted by anatomic, symbiotic, and symbiotic-induced molecular mechanisms. Anatomic modifications detected in *B. fimbriata* were related to root modifications, hence improving mycorrhization and root exudation. These mechanisms have been reported as adaptation strategies developed by several plants to achieve plant establishment in soils contaminated with metal(loid)s. Crucial molecular changes and regulation of mycorrhizal interactions were also detected in this soil, which allow orchids to improve the mycorrhizal benefits under the altered ecological conditions. Proteomic analyses agree with previous studies exploring molecular regulation of orchid mycorrhizas, in which a transitory decrease in defence mechanisms are key to promote the symbiotic association between orchid and fungi, which was also confirmed with the expression analyses. In this thesis, it was detected that mycorrhizal fungi presence changes root molecular responses, a process that was also detected under metal(loid) stress. Under this limited conditions, roots tend to facilitate mycorrhizal colonization as a measure to promote fungi-derived benefits that may relate with metal(loid)s exclusion or improve nutritional compounds intake. Consequently, allowing the orchid to be established in hot-spots for orchid development in this soil contaminated with metal(loid)s, as similar to detected in other mycorrhizal interactions (Aguilera et al., 2011; Shefferson et al., 2008), including root exudation of low molecular weight organic acids and phenols, which are metal(loid) chelating agents at diverse environments. The higher citric acid exudation rates in roots submitted to metal(loid) stress is an adaptive strategy and may play a key role to exclude metal(loid)s to achieve plant establishment in diverse heavy-metal-containing soils, such as Andisoils from Araucania region (high aluminium saturation), soils that have the conditions to promote establishment and development of 40% of the orchid endemic flora.

Briefly, the results obtained in this Doctoral Thesis have contributed to; i) understand the dynamics of mycorrhizal associations with endemic orchids, as well as the mechanisms involved in their symbiotic germination, which allow designing reintroduction strategies for endangered endemic orchids; ii) understand the molecular regulations of orchid mycorrhizal symbioses under metal(loid) stress, which contributes to improve the knowledge of molecular mechanisms developed at advanced orchid developmental stages; and iii) report, for the first time, a complete analysis of orchid mycorrhizas adaptation to metal(loid) contaminated areas, addressing both ecological and molecular mechanisms.

6.2 CONCLUDING REMARKS

According to our results we can conclude that:

- Mycorrhizal fungi isolated from endemic orchids were mainly related to *Tulasnella* genera and were effective to promote seed germination without specificity, and with different germination rates.

- Endophytic non-mycorrhizal fungi were widely present in different orchid roots, but their putative role as beneficial endophytes was not corroborated.

- Higher exudation rates of citric, succinic, and malic acids were detected in orchids developed in the metal(loid) contaminated soil.

- In metal(loid) contaminated soils, orchid roots showed higher root biomass and improved mycorrhization, in which *T. calospora* formed condensed infected areas, with high rates of degraded pelotons, which are a potential source of mycorrhizal fungi-derived nutrients, as well as a potential physical place to store metal(loid)s.

- *B. fimbriata* establishment in metal(loid) contaminated soil induced specific molecular changes in roots. Gene expression and proteomic analyses suggested that under metal(loid) stress, symbiotic mechanisms were similar to mutualism. Under these conditions, orchid roots promoted mycorrhization through the alleviation of defence mechanisms and the synthesis of carotenoid-derived molecules in non mycorrhizal roots. On the other hand, they improved membrane transporter synthesis in mycorrhizal roots.

- Mycorrhizal fungi isolated from orchids naturally grown in metal(loid) contaminated soil were effective to promote seed germination and differentiation orchid embryo under these stress.

- Mycorrhizal fungi and seeds from endemic orchids were effectively stored using preservation methods and will be useful to design reintroduction strategies of endangered orchids.

6.3 FUTURE DIRECTIONS

Our results indicated that mycorrhizal fungi and orchids form an extremely specific symbiotic interaction that is relevant for preserving the diversity of these beautiful flowering plants. We suggest the generation of an orchid seed and mycorrhizal fungi bank, and their consequent reintroduction in the field by using encapsulation technologies, focusing in extremely altered environments such as wildfires that have detrimental effects on millennial forest. These events cause extremely harmful effects on specific ecological interactions such as the ones between orchids and their mycorrhizal fungi. Accordingly, designing symbiotic reintroduction strategies for orchids in their natural distribution environments is mandatory.

Chile has 70 orchid species, many of which have the potential of being cultured and commercialized to prospective international markets, which cherish endemic patrimony and its sustainable production. In Chile, the internal market of ornamental flowers (according to Trade Map) considers imports of 19.5 million dollars in different ornamental flowers, with an annual growth rate of 9% from 2012 to date. Currently, there is no information on the participation of orchids in the internal flower market, but according to the National Custom Service (Servicio Nacional de Aduanas) and the Office of Agrarian Policies and Research (Oficina de Estudios y Políticas Agrarias, ODEPA), and considering only the tariff code position 06029015 ("Imports of plants and parts of orchid plants"), since 2012 Chile averages an annual import of 114.3 thousand dollars. Nonetheless, according to the biological characteristic of the orchid life cycle, it is expected that the internal market is much greater than those 114 MM. The internal market of orchids is under expansion and throug the knowledge of appropriate mycorrhizal fungi and some ecological aspects of the orchid life cycle, it would be very interesting to exploit the economic potential of endemic and exotic orchid species, for internal and external markets

Further studies in orchid mycorrhizal symbioses should address orchid potential to control the pathogenesis of certain fungal genera, such as *Thanetophorus cucumeris* detected in *B. fimbriata*, which have the ability to induce *C. bletioides* seed growth. Therefore, studying these mechanisms will allow designing eco-friendly technologies for controlling phytopathogens in diverse agronomic crops.

References

Abadie, J., Püttsepp, Ü., Gebauer, G., Faccio, A., Bonfante, P., Selosse, M. 2006. *Cephalanthera longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and nonphotosynthetic individuals. *Botany*, **84**, 1462-1477.

Adam, G., Duncan, H. 2001. Development of a sensitive and rapid method for the measurement of total microbial activity using fluorescein diacetate (FDA) in a range of soils. *Soil Biology and Biochemistry*, **33**, 943-951.

Aguilera, P., Borie, F., Seguel, A., Cornejo, P. 2011. Fluorescence detection of aluminum in arbuscular mycorrhizal fungal structures and glomalin using confocal laser scanning microscopy. *Soil Biology and Biochemistry*, **43**, 2427-2431.

Alexander, C., Hadley, G. 1985. Carbon movement between host and mycorrhizal endophyte during the development of the orchid *Goodyera repens* Br. *New Phytologist*, **101**, 657-665.

Alexander, D., Goodman, R., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E. 1993. Increased tolerance to two *oomycete* pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proceedings of the National Academy of Sciences*, **90**, 7327-7331.

Allen, M. 1991. *The ecology of Mycorrhizae*. Cambridge University Press. 184p.

Andersen, T., Rasmussen, H. 1996. The mycorrhizal species of *Rhizoctonia*. in: *Rhizoctonia species: taxonomy, molecular biology, ecology, pathology and disease control*, Springer Netherlands, pp. 379-390.

Arenson, L.U., Jakob, M., Wainstein, P. 2015. Effects of Dust Deposition on Glacier Ablation and Runoff at the Pascua-Lama Mining Project, Chile and Argentina. in: *Engineering Geology for Society and Territory-Volume 1*, Springer Cham, pp. 27-32.

Arias, J., Peralta-Videa, J., Ellzey, J., Ren, M., Viveros, M., Gardea-Torresdey, J., 2010. Effects of *Glomus deserticola* inoculation on Prosopis: enhancing chromium and lead uptake and translocation as confirmed by X-ray mapping, ICP-OES and TEM techniques. *Environmental and Experimental Botany*, **68**, 139-148.

Athipunyakom, P., Manoch, L., Piluek, C. 2004. Isolation and identification of mycorrhizal fungi from eleven terrestrial orchids. *Kasetsart Journal (Natural Science)*, **38**, 216-228.

Atwood, J. 1986. The size of the Orchidaceae and the systematic distribution of epiphytic orchids. *Selbyana*, **9**, 171-186.

Bailarote, B., Lievens, B., Jacquemyn, H. 2012. Does mycorrhizal specificity affect orchid decline and rarity? *American Journal of Botany*, **99**, 1655-1665.

Barrett, C., Freudenstein, J., Taylor, D., Kõljalg, U. 2010. Rangewide analysis of fungal associations in the fully mycoheterotrophic *Corallorhiza striata* complex (Orchidaceae) reveals extreme specificity on ectomycorrhizal *Tomentella* (Thelephoraceae) across North America. *American Journal of Botany*, **97**, 628-643.

Batty, A., Brundrett, M., Dixon, K., Sivasithamparam, K. 2006. In situ symbiotic seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. *Australian Journal of Botany*, **54**, 375-381.

Bayman, P., Otero, J. 2006. Microbial endophytes of orchid roots. in: *Microbial Root Endophytes*, Springer Netherlands, pp. 153-177.

Belimov, A., Puhalsky, I., Safronova, V., Shaposhnikov, A., Vishnyakova, M., Semenova, E., Zinovkina, N., Makarova, N., Wenzel, W., Tikhonovich, I. 2015. Role of Plant Genotype and Soil Conditions in Symbiotic Plant-Microbe Interactions for Adaptation of Plants to Cadmium-Polluted Soils. *Water, Air, and Soil Pollution*, **226**, 1-15.

Bender, S., Valadares, R., Taudiere, A. 2014. Mycorrhizas: dynamic and complex networks of power and influence. *New Phytologist*, **204**, 15-18.

Bending, G., Aspray, T., Whipps, J. 2006. Significance of microbial interactions in the mycorrhizosphere. *Advances in Applied Microbiology*, **60**, 97-132.

Beyrle, H., Smith, S., Franco, C., Peterson, R. 1995. Colonization of *Orchis morio* protocorms by a mycorrhizal fungus: effects of nitrogen nutrition and glyphosate in modifying the responses. *Canadian Journal of Botany*, **73**, 1128-1140.

Bidartondo, M., Read, D. 2008. Fungal specificity bottlenecks during orchid germination and development. *Molecular Ecology*, **17**, 3707-3716.

Bidartondo, M., Burghardt, B., Gebauer, G., Bruns, T., Read, D. 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London B: Biological Sciences*, **271**, 1799-1806.

Blakeman, J., Mokahel, M., Hadley, G. 1976. Effect of mycorrhizal infection on respiration and activity of some oxidase enzymes of orchid protocorms. *New Phytologist*, **77**, 697-704.

Bonnardeaux, Y., Brundrett, M., Batty, A., Dixon, K., Koch, J., Sivasithamparam, K. 2007. Diversity of mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships and alien invasions. *Mycological Research*, **111**, 51-61.

Bougoure, J., Ludwig, M., Brundrett, M., Cliff, J., Clode, P., Kilburn, M., Grierson, P. 2014. High-resolution secondary ion mass spectrometry analysis of carbon dynamics in mycorrhizas formed by an obligately myco-heterotrophic orchid. *Plant, Cell, and Environment*, **37**, 1223-1230.

Bougoure, J., Ludwig, M., Brundrett, M., Grierson, P. 2009. Identity and specificity of the fungi forming mycorrhizas with the rare mycoheterotrophic orchid *Rhizanthella gardneri*. *Mycological Research*, **113**, 1097-1106.

Bougoure, J., Bougoure, D., Cairney, J., Dearnaley, J. 2005. ITS-RFLP and sequence analysis of endophytes from *Acianthus*, *Caladenia* and *Pterostylis* (Orchidaceae) in southeastern Queensland. *Mycological Research*, **109**, 452-460.

Brundrett, M. 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytologist*, **154**, 275-304.

Brundrett, M.C. 2007. Scientific approaches to Australian temperate terrestrial orchid conservation. *Australian Journal of Botany*, **55**, 293-307.

Cameron, D., Johnson, I., Leake, J., Read, D. 2007. Mycorrhizal acquisition of inorganic phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. *Annals of Botany*, **99**, 831-834.

Cameron, D., Johnson, I., Read, D., Leake, J. 2008. Giving and receiving: measuring the carbon cost of mycorrhizas in the green orchid *Goodyera repens*. *New Phytologist*, **180**, 176-184.

Cameron, D., Leake, J., Read, D. 2006. Mutualistic mycorrhiza in orchids: evidence from plant–fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New Phytologist*, **171**, 405-416.

Cameron, D., Preiss, K., Gebauer, G., Read, D. 2009. The chlorophyll-containing orchid *Corallorhiza trifida* derives little carbon through photosynthesis. *New Phytologist*, **183**, 358-364.

Cameron, K., Chase, M. 2000. Nuclear 18S rDNA sequences of Orchidaceae confirm the subfamilial status and circumscription of Vanilloideae. *Wilson, K, L,, Morrison, D, A ed (s). Monocots: Systematics and Evolution.. CSIRO: Melbourne, Australia*, 457-464.

Capoen, W., Oldroyd, G., Goormachtig, S., Holsters, M. 2010. *Sesbania rostrata*: a case study of natural variation in legume nodulation. *New Phytologist*, **186**, 340-345.

Cawthray, G. 2003. An improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates. *Journal of Chromatography A*, **1011**, 233-240.

Cázares, E., Trappe, J., Jumpponen, A. 2005. Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. *Mycorrhiza*, **15**, 405-416.

Collins, C., Keane, T., Turner, D., O'Keeffe, G., Fitzpatrick, D., Doyle, S. 2013. Genomic and proteomic dissection of the ubiquitous plant pathogen, *Armillaria mellea*: toward a new infection model system. *Journal of Proteome Research*, **12**, 2552-2570.

Conesa, A., Götz, S. 2008. Blast2GO: A comprehensive suite for functional analysis in plant genomics. *International Journal of Plant Genomics*, **2008**, 619832.

Cornejo, P., Meier, S., Borie, G., Rillig, M., Borie, F. 2008. Glomalin-related soil protein in a Mediterranean ecosystem affected by a copper smelter and its contribution to Cu and Zn sequestration. *Science of the Total Environment*, **406**, 154-160.

Cowden, C., Shefferson, R. 2013. Diversity of root-associated fungi of mature *Habenaria radiata* and *Epipactis thunbergii* colonizing manmade wetlands in Hiroshima Prefecture, Japan. *Mycoscience*, **54**, 327-334.

Cozzolino, S., Nardella, A., Impagliazzo, S., Widmer, A., Lexer, C. 2006. Hybridization and conservation of Mediterranean orchids: Should we protect the orchid hybrids or the orchid hybrid zones? *Biological Conservation*, **129**, 14-23.

Crous, P., Braun, U., Groenewald, J. 2007. *Mycosphaerella* is polyphyletic. *Studies in Mycology*, **58**, 1-32.

Chemisquy, M., Morrone, O. 2012. Molecular phylogeny of *Gavilea* (Chloraeinae: Orchidaceae) using plastid and nuclear markers. *Molecular Phylogenetics and Evolution*, **62**, 889-897.

Chen, J., Hu, K., Hou, X., Guo, S. 2011. Endophytic fungi assemblages from 10 *Dendrobium* medicinal plants (Orchidaceae). *World Journal of Microbiology and Biotechnology*, **27**, 1009-1016.

Chen, J., Wang, H., Guo, S. 2012. Isolation and identification of endophytic and mycorrhizal fungi from seeds and roots of *Dendrobium* (Orchidaceae). *Mycorrhiza*, **22**, 297-307.

Chiapello, M., Martino, E., Perotto, S. 2015. Common and metal-specific proteomic responses to cadmium and zinc in the metal tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn. *Metallomics*, **7**, 805-815.

Chomicki, G., Bidel, L., Jay-Allemand, C. 2014. Exodermis structure controls fungal invasion in the leafless epiphytic orchid *Dendrophylax lindenii* (Lindl.) Benth. ex Rolfe. *Flora*, **209**, 88-94.

da Silva, J. 2013. Orchids: advances in tissue culture, genetics, phytochemistry and transgenic biotechnology. *Floriculture and Ornamental Biotechnology*, **7**, 1-52.

Dearnaley, J. 2007. Further advances in orchid mycorrhizal research. *Mycorrhiza*, **17**, 475-486.

Dearnaley, J., Martos, F., Selosse, M. 2012. Orchid Mycorrhizas: Molecular Ecology, Physiology, Evolution and Conservation Aspects. in: *Fungal Associations*, Springer Berlin Heidelberg, pp. 207-230.

Douds Jr, D., Pfeffer, P., Shachar-Hill, Y. 2000. Carbon partitioning, cost, and metabolism of arbuscular mycorrhizas. in: *Arbuscular Mycorrhizas: Physiology and Function*, Springer Netherlands, pp. 107-129.

Dressler, R. 1993. *Phylogeny and classification of the orchid family*. Cambridge University Press. 321p.

Dressler, R. 2005. How many orchid species? *Selbyana*, **26,** 155-158.

Dumas-Gaudot, E., Valot, B., Bestel-Corre, G., Recorbet, G., St-Arnaud, M., Fontaine, B., Dieu, M., Raes, M., Saravanan, R., Gianinazzi, S. 2004. Proteomics as a way to identify extra-radicular fungal proteins from *Glomus intraradices*–RiT-DNA carrot root mycorrhizas. *FEMS Microbiology Ecology*, **48**, 401-411.

Dunbar, J., Barns, S., Ticknor, L., Kuske, C. 2002. Empirical and theoretical bacterial diversity in four Arizona soils. *Applied and Environmental Microbiology*, **68**, 3035-3045.

Dutra, D., Kane, M., Richardson, L. 2009. Asymbiotic seed germination and in vitro seedling development of *Cyrtopodium punctatum*: a propagation protocol for an endangered Florida native orchid. *Plant Cell, Tissue and Organ Culture*, **96**, 235-243.

Esfeld, K., Hensen, I., Wesche, K., Jakob, S., Tischew, S., Blattner, F. 2008. Molecular data indicate multiple independent colonizations of former lignite mining areas in Eastern Germany by *Epipactis palustris* (Orchidaceae). *Biodiversity and Conservation*, **17**, 2441-2453.

Esitken, A., Ercisli, S., Eken, C. 2005. Effects of mycorrhiza isolates on symbiotic germination of terrestrial orchids (*Orchis palustris* Jacq. and *Serapias vomeracea* subsp. vomeracea (Burm. f.) Briq.) in Turkey. *Symbiosis*, **38**, 59-68.

Faeth, S., Saari, S. 2012. Fungal grass endophytes and arthropod communities: lessons from plant defence theory and multitrophic interactions. *Fungal Ecology*, **5**, 364-371.

Fan, L., Guo, S., Cao, W., Xiao, P., Xu, J. 1996. Isolation, culture, identification and biological activity of *Mycena orchidicola* sp. nov. in *Cymbidium sinense* (Orchidaceae). *Acta Mycologica Sinica*, **15**, 251-255.

Fang, W., Huang, Z., Wu, P. 2003. Contamination of the environmental ecosystems by trace elements from mining activities of Badao bone coal mine in China. *Environmental Geology*, **44**, 373-378.

Fernando, A., Currah, R. 1996. A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. *Canadian Journal of Botany*, **74**, 1071-1078.

Fisch, M., Flick, B., Arditti, J. 1973. Structure and antifungal activity of hircinol, loroglossol and orchinol. *Phytochemistry*, **12**, 437-441.

Fracchia, S., Aranda-Rickert, A., Flachsland, E., Terada, G., Sede, S. 2014. Mycorrhizal compatibility and symbiotic reproduction of *Gavilea australis*, an endangered terrestrial orchid from south Patagonia. *Mycorrhiza*, **24**, 627-634.

Freudenstein, J., Chase, M. 2001. Analysis of mitochondrial nad1 bc intron sequences in Orchidaceae: utility and coding of length-change characters. *Systematic Botany*, **26**, 643-657.

Freudenstein, J., Senyo, D., Chase, M. 2000. Mitochondrial DNA and relationships in the Orchidaceae. *Monocots: Systematics and Evolution: Systematics and Evolution*, 421.

Fuentes, A., Almonacid, L., Ocampo, J., Arriagada, C. 2016. Synergistic interactions between a saprophytic fungal consortium and *Rhizophagus irregularis* alleviate oxidative stress in plants grown in heavy metal contaminated soil. *Plant and Soil*, **407**, 355-366.

Gebauer, G., Meyer, M. 2003. ^{15}N and ^{13}C natural abundance of autotrophic and myco-heterotrophic orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist*, **160**, 209-223.

Ginocchio, R. 2000. Effects of a copper smelter on a grassland community in the Puchuncavı Valley, Chile. *Chemosphere*, **41**, 15-23.

Ginocchio, R., Carvallo, G., Toro, I., Bustamante, E., Silva, Y., Sepúlveda, N. 2004. Micro-spatial variation of soil metal pollution and plant recruitment near a copper smelter in Central Chile. *Environmental Pollution*, **127**, 343-352.

Girlanda, M., Segreto, R., Cafasso, D., Liebel, H., Rodda, M., Ercole, E., Cozzolino, S., Gebauer, G., Perotto, S. 2011. Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific mycorrhizal associations. *American Journal of Botany*, **98**, 1148-1163.

Girlanda, M., Selosse, M., Cafasso, D., Brilli, F., Delfine, S., Fabbian, R., Ghignone, S., Pinelli, P., Segreto, R., Loreto, F. 2006. Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Molecular Ecology*, **15**, 491-504.

González, I., Neaman, A., Rubio, P., Cortés, A. 2014. Spatial distribution of copper and pH in soils affected by intensive industrial activities in Puchuncaví and Quintero, central Chile. *Journal of Soil Science and Plant Nutrition*, **14**, 943-953.

Gowland, K., van der Merwe, M., Linde, C., Clements, M., Nicotra, A. 2013. The host bias of three epiphytic *Aeridinae* orchid species is reflected, but not explained, by mycorrhizal fungal associations. *American Journal of Botany*, **100**, 764-777.

Gregg, K., Kéry, M. 2006. Comparison of size vs. life-state classification in demographic models for the terrestrial orchid *Cleistes bifaria*. *Biological Conservation*, **129**, 50-58.

Guo, S., Fan, L., Cao, W., Xu, J., Xiao, P. 1997. *Mycena anoectochila* sp. nov. isolated from mycorrhizal roots of *Anoectochilus roxburghii* from Xishuangbanna, China. *Mycologia*, **89,** 952-954.

Gutjahr, C., Parniske, M. 2013. Cell and developmental biology of arbuscular mycorrhiza symbiosis. *Annual Review of Cell and Developmental Biology*, **29**, 593-617.

Hadley, G., Purves, S. 1974. Movement of 14carbon from host to fungus in orchid mycorrhiza. *New Phytologist*, **73**, 475-482.

Hall, J. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, **53**, 1-11.

Hall, T. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. pp. 95-98.

Hause, B., Fester, T. 2005. Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta*, **221**, 184-196.

Hendershot, W., Duquette, M. 1986. A simple barium chloride method for determining cation exchange capacity and exchangeable cations. *Soil Science Society of America Journal*, **50**, 605-608.

Herrera-Medina, M., Steinkellner, S., Vierheilig, H., Ocampo Bote, J., García Garrido, J. 2007. Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. *New Phytologist*, **175**, 554-564.

Herrera, H., Valadares, R., Contreras, D., Bashan, Y., Arriagada, C. 2017. Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in south central Chile. *Mycorrhiza*, **27**, 175-188.

Hopper, S., Brown, A. 2007. A revision of Australia's hammer orchids (*Drakaea*: Orchidaceae), with some field data on species-specific sexually deceived wasp pollinators. *Australian Systematic Botany*, **20**, 252-285.

Hou, X., Guo, S. 2009. Interaction between a dark septate endophytic isolate from *Dendrobium* sp. and roots of *D. nobile* seedlings. *Journal of Integrative Plant Biology*, **51**, 374-381.

Huber, F., Kaiser, R., Sauter, W., Schiestl, F. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia*, **142**, 564-575.

Ihaka, R., Gentleman, R. 1996. R: a language for data analysis and graphics. *Journal of Computational and Graphical Statistics*, **5**, 299-314.

Illyés, Z., Rudnóy, S., Bratek, Z. 2005. Aspects of in situ, in vitro germination and mycorrhizal partners of *Liparis loeselii*. *Acta Biologica Szegediensis*, **49**, 137-139.

Irwin, M., Dearnaley, J., Bougoure, J. 2007. *Pterostylis nutans* (Orchidaceae) has a specific association with two *Ceratobasidium* root-associated fungi across its range in eastern Australia. *Mycoscience*, **48**, 231-239.

Jacquemyn, H., Brys, R., Hermy, M., Willems, J. 2005. Does nectar reward affect rarity and extinction probabilities of orchid species? An assessment using historical records from Belgium and the Netherlands. *Biological Conservation*, **121**, 257-263.

Jacquemyn, H., Brys, R., Merckx, V., Waud, M., Lievens, B., Wiegand, T. 2014. Coexisting orchid species have distinct mycorrhizal communities and display strong spatial segregation. *New Phytologist*, **202**, 616-627.

Jacquemyn, H., Honnay, O., Cammue, B., Brys, R., Lievens, B. 2010. Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. *Molecular Ecology*, **19**, 4086-4095.

Jiang, J., Lee, Y., Cubeta, M., Chen, L 2015. Characterization and colonization of endomycorrhizal *Rhizoctonia* fungi in the medicinal herb *Anoectochilus formosanus* (Orchidaceae). *Mycorrhiza*, **25**, 431-445.

Johnson, N., Graham, J., Smith, F. 1997. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *New Phytologist*, **135**, 575-585.

Jones, R., Robeson, M., Lauber, C., Hamady, M., Knight, R., Fierer, N. 2009. A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *The ISME Journal*, **3**, 442.

Joynt, J., Bischoff, M., Turco, R., Konopka, A., Nakatsu, C. 2006. Microbial community analysis of soils contaminated with lead, chromium and petroleum hydrocarbons. *Microbial Ecology*, **51**, 209-219.

Julou, T., Burghardt, B., Gebauer, G., Berveiller, D., Damesin, C., Selosse, M. 2005. Mixotrophy in orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals of *Cephalanthera damasonium*. *New Phytologist*, **166**, 639-653.

Jumpponen, A. 2001. Dark septate endophytes–are they mycorrhizal? *Mycorrhiza*, **11**, 207-211.

Jumpponen, A., Mattson, K., Trappe, J. 1998. Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza*, **7**, 261-265.

Jumpponen, A., Trappe, J. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist*, 295-310.

Jurkiewicz, A., Turnau, K., Mesjasz-Przybyłowicz, J., Przybyłowicz, W., Godzik, B. 2001. Heavy metal localisation in mycorrhizas of *Epipactis atrorubens* (Hoffm.) Besser (Orchidaceae) from zinc mine tailings. *Protoplasma*, **218**, 117-124.

Kasiamdari, R., Smith, S., Scott, E., Smith, F. 2002. Identification of binucleate *Rhizoctonia* as a contaminant in pot cultures of arbuscular mycorrhizal fungi and development of a PCR-based method of detection. *Mycological Research*, **106**, 1417-1426.

Keller, A., Nesvizhskii, A., Kolker, E., Aebersold, R. 2002. An explanation of the Peptide Prophet algorithm developed. *Analytical Chemistry*, **74**, 5383-5392.

Kennedy, A., Taylor, D., Watson, L. 2011. Mycorrhizal specificity in the fully mycoheterotrophic *Hexalectris* Raf.(Orchidaceae: Epidendroideae). *Molecular Ecology*, **20**, 1303-1316.

Khadrani, A., Seigle-Murandi, F., Steiman, R., Vroumsia, T. 1999. Degradation of three phenylurea herbicides (chlortoluron, isoproturon and diuron) by micromycetes isolated from soil. *Chemosphere*, **38**, 3041-3050.

Klein, S., Heinzle, E. 2012. Isotope labeling experiments in metabolomics and fluxomics. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, **4**, 261-272.

Knapp, D., Pintye, A., Kovács, G. 2012. The dark side is not fastidious–dark septate endophytic fungi of native and invasive plants of semiarid sandy areas. *PLOS One*, **7**, E32570.

Kocyan, A., de Vogel, E., Conti, E., Gravendeel, B. 2008. Molecular phylogeny of *Aerides* (Orchidaceae) based on one nuclear and two plastid markers: A step forward in understanding the evolution of the *Aeridinae*. *Molecular Phylogenetics and Evolution*, **48**, 422-443.

Kottke, I., Suárez, J., Herrera, P., Cruz, D., Bauer, R., Haug, I., Garnica, S. 2010. *Atractiellomycetes* belonging to the 'rust'lineage (Pucciniomycotina) form *Mycorrhizae* with terrestrial and epiphytic neotropical orchids. *Proceedings of the Royal Society of London B: Biological Sciences*, **277**, 1289-1298.

Kristiansen, K., Freudenstein, J., Rasmussen, F., Rasmussen, H. 2004. Molecular identification of mycorrhizal fungi in *Neuwiedia veratrifolia* (Orchidaceae). *Molecular Phylogenetics and Evolution*, **33**, 251-258.

Kubartová, A., Ranger, J., Berthelin, J., Beguiristain, T. 2009. Diversity and decomposing ability of saprophytic fungi from temperate forest litter. *Microbial Ecology*, **58**, 98-107.

Kuga, Y., Sakamoto, N., Yurimoto, H. 2014. Stable isotope cellular imaging reveals that both live and degenerating fungal pelotons transfer carbon and nitrogen to orchid protocorms. *New Phytologist*, **202**, 594-605.

Kull, T., Hutchings, M. 2006. A comparative analysis of decline in the distribution ranges of orchid species in Estonia and the United Kingdom. *Biological Conservation*, **129**, 31-39.

Küster, H., Hohnjec, N., Krajinski, F., El Yahyaoui, F., Manthey, K., Gouzy, J., Dondrup, M., Meyer, F., Kalinowski, J., Brechenmacher, L. 2004. Construction and validation of cDNA-based Mt6k-RIT macro-and microarrays to explore root endosymbioses in the model legume *Medicago truncatula*. *Journal of Biotechnology*, **108**, 95-113.

Lakon, G. 1949. The topographical tetrazolium method for determining the germinating capacity of seeds. *Plant Physiology*, **24**, 389.

Laparre, J., Malbreil, M., Letisse, F., Portais, J., Roux, C., Bécard, G., Puech-Pagès, V. 2014. Combining metabolomics and gene expression analysis reveals that propionyl-and butyryl-carnitines are involved in late stages of arbuscular mycorrhizal symbiosis. *Molecular Plant*, **7**, 554-566.

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P., McWilliam, H., Valentin, F., Wallace, L., Wilm, A., Lopez, R. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**, 2947-2948.

Leake, J. 1994. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist*, **127**, 171-216.

Leake, J., Johnson, D., Donnelly, D., Muckle, G., Boddy, L., Read, D. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany*, **82**, 1016-1045.

Lee Taylor, D., Bruns, T. 1999. Population, habitat and genetic correlates of mycorrhizal specialization in the 'cheating'orchids *Corallorhiza maculata* and *C. mertensiana*. *Molecular Ecology*, **8**, 1719-1732.

Lee, Y., Yang, C., Gebauer, G. 2015. The importance of associations with saprotrophic non-*Rhizoctonia* fungi among fully mycoheterotrophic orchids is currently under-estimated: novel evidence from sub-tropical Asia. *Annals of Botany*, **116**, 423-435.

Liebel, H., Gebauer, G. 2011. Stable isotope signatures confirm carbon and nitrogen gain through ectomycorrhizas in the ghost orchid *Epipogium aphyllum* Swartz. *Plant Biology*, **13**, 270-275.

Liebel, H., Bidartondo, M., Preiss, K., Segreto, R., Stöckel, M., Rodda, M., Gebauer, G. 2010. C and N stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean and Macaronesia. *American Journal of Botany*, **97**, 903-912.

Lievens, B., Van Kerckhove, S., Justé, A., Cammue, B., Honnay, O., Jacquemyn, H. 2010. From extensive clone libraries to comprehensive DNA arrays for the efficient and simultaneous detection and identification of orchid mycorrhizal fungi. *Journal of Microbiological Methods*, **80**, 76-85.

López-Chávez, M., Guillén-Navarro, K., Bertolini, V., Encarnación, S., Hernández-Ortiz, M., Sánchez-Moreno, I., Damon, A. 2016. Proteomic and morphometric study of the in vitro interaction between *Oncidium sphacelatum* Lindl.(Orchidaceae) and *Thanatephorus* sp. RG26 (Ceratobasidiaceae). *Mycorrhiza*, **26**, 1-13.

Louarn, S., Nawrocki, A., Thorup-Kristensen, K., Lund, O., Jensen, O., Collinge, D., Jensen, B. 2013. Proteomic changes and endophytic micromycota during storage of organically and conventionally grown carrots. *Postharvest Biology and Technology*, **76**, 26-33.

Lucksom, S., Lucksom, S. 2007. *The Orchids of Sikkim and North East Himalaya*. Development Area, Jiwan Thing Marg, Gangtok, East Sikkim.

Martos, F., Dulormne, M., Pailler, T., Bonfante, P., Faccio, A., Fournel, J., Dubois, M., Selosse, M. 2009. Independent recruitment of saprotrophic fungi as mycorrhizal partners by tropical achlorophyllous orchids. *New Phytologist*, **184**, 668-681.

Massoumou, M., Van Tuinen, D., Chatagnier, O., Arnould, C., Brechenmacher, L., Sanchez, L., Selim, S., Gianinazzi, S., Gianinazzi-Pearson, V. 2007. *Medicago truncatula* gene responses specific to arbuscular mycorrhiza interactions with different species and genera of Glomeromycota. *Mycorrhiza*, **17**, 223-234.

Masuhara, G., Katsuya, K. 1994. In situ and in vitro specificity between *Rhizoctonia* spp. and *Spiranthes sinensis* (Persoon) Ames, var. amoena (M. Bieberstein) Hara (Orchidaceae). *New Phytologist*, **127**, 711-718.

McCormick, M., Whigham, D., O'Neill, J. 2004. Mycorrhizal diversity in photosynthetic terrestrial orchids. *New Phytologist*, **163**, 425-438.

McCormick, M., Whigham, D., Sloan, D., O'Malley, K., Hodkinson, B. 2006. Orchid-fungus fidelity: a marriage meant to last? *Ecology*, **87**, 903-911.

McKendrick, S., Leake, J., Read, D. 2000. Symbiotic germination and development of myco-heterotrophic plants in nature: transfer of carbon from ectomycorrhizal *Salix repens* and *Betula pendula* to the orchid *Corallorhiza trifida* through shared hyphal connections. *New Phytologist*, **145**, 539-548.

Meier, S., Borie, F., Curaqueo, G., Bolan, N., Cornejo, P. 2012. Effects of arbuscular mycorrhizal inoculation on metallophyte and agricultural plants growing at increasing copper levels. *Applied Soil Ecology*, **61**, 280-287.

Merckx, V., Bidartondo, M., Hynson, N. 2009. Myco-heterotrophy: when fungi host plants. *Annals of Botany*, **104**, 1255-1261.

Mingorance, M. 2002. Focused microwave-assisted digestion of vegetal materials for the determination of essential mineral nutrients. *Analytical and Bioanalytical Chemistry*, **373**, 153-158.

Mitchell, R. 1989. Growing hardy orchids from seeds at Kew. *Plantsman*, **11**, 152-169.

Motomura, H., Selosse, M., Martos, F., Kagawa, A., Yukawa, T. 2010. Mycoheterotrophy evolved from mixotrophic ancestors: evidence in *Cymbidium* (Orchidaceae). *Annals of botany*, **106**, 573-581.

Mucha, A., Almeida, C., Bordalo, A., Vasconcelos, M. 2010. LMWOA (low molecular weight organic acid) exudation by salt marsh plants: natural variation and response to Cu contamination. *Estuarine, Coastal and Shelf Science*, **88**, 63-70.

Mujica, M., Saez, N., Cisternas, M., Manzano, M., Armesto, J., Pérez, F. 2016. Relationship between soil nutrients and mycorrhizal associations of two *Bipinnula* species (Orchidaceae) from central Chile. *Annals of Botany*, mcw082.

Münzenberger, B., Heilemann, J., Strack, D., Kottke, I., Oberwinkler, F. 1990. Phenolics of mycorrhizas and non-mycorrhizal roots of Norway spruce. *Planta*, **182**, 142-148.

Nath, M., Bhatt, D., Prasad, R., Gill, S., Anjum, N., Tuteja, N. 2016. Reactive oxygen species generation-scavenging and signaling during plant-arbuscular mycorrhizal and *Piriformospora indica* interaction under stress condition. *Frontiers in Plant Science*, **7**, 1574.

Nian, H., Yang, Z., Ahn, S., Cheng, Z., Matsumoto, H. 2002. A comparative study on the aluminium-and copper-induced organic acid exudation from wheat roots. *Physiologia Plantarum*, **116**, 328-335.

Nigam, R., Srivastava, S., Prakash, S., Srivastava, M. 2001. Cadmium mobilisation and plant availability–the impact of organic acids commonly exuded from roots. *Plant and Soil*, **230**, 107-113.

Nontachaiyapoom, S., Sasirat, S., Manoch, L. 2010. Isolation and identification of *Rhizoctonia*-like fungi from roots of three orchid genera, *Paphiopedilum*, *Dendrobium*, and *Cymbidium*, collected in Chiang Rai and Chiang Mai provinces of Thailand. *Mycorrhiza*, **20**, 459-471.

Novoa, P., Espejo, J., Cisternas, M., Rubio, M., Dominguez, E. 2015. Guía de campo de las orquídeas chilenas. Segunda edicion ampliada., Corporacion Chilena de la Madera (CORMA). Santiago.

Ogura-Tsujita, Y., Yokoyama, J., Miyoshi, K., Yukawa, T. 2012. Shifts in mycorrhizal fungi during the evolution of autotrophy to mycoheterotrophy in *Cymbidium* (Orchidaceae). *American Journal of Botany*, **99**, 1158-1176.

Ogura-Tsujita, Y., Yukawa, T. 2008. *Epipactis helleborine* shows strong mycorrhizal preference towards ectomycorrhizal fungi with contrasting geographic distributions in Japan. *Mycorrhiza*, **18**, 331-338.

Okayama, M., Yamato, M., Yagame, T., Iwase, K. 2012. Mycorrhizal diversity and specificity in *Lecanorchis* (Orchidaceae). *Mycorrhiza*, **22**, 545-553.

Oldroyd, G., Harrison, M., Paszkowski, U. 2009. Reprogramming plant cells for endosymbiosis. *Science*, **324**, 753-754.

Otero, J., Ackerman, J., Bayman, P. 2004. Differences in mycorrhizal preferences between two tropical orchids. *Molecular Ecology*, **13**, 2393-2404.

Otero, J., Flanagan, N., Herre, E., Ackerman, J., Bayman, P. 2007. Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). *American Journal of Botany*, **94**, 1944-1950.

Otero, J., Thrall, P., Clements, M., Burdon, J., Miller, J. 2011. Codiversification of orchids (Pterostylidinae) and their associated mycorrhizal fungi. *Australian Journal of Botany*, **59**, 480-497.

Otero, T., Ackerman, J., Bayman, P. 2002. Diversity and host specificity of endophytic *Rhizoctonia*-like fungi from tropical orchids. *American Journal of Botany*, **89**, 1852-1858.

Paduano, C., Rodda, M., Ercole, E., Girlanda, M., Perotto, S. 2011. Pectin localization in the Mediterranean orchid *Limodorum abortivum* reveals modulation of the plant interface in response to different mycorrhizal fungi. *Mycorrhiza*, **21**, 97-104.

Pandey, M., Sharma, J., Taylor, D., Yadon, V. 2013. A narrowly endemic photosynthetic orchid is non-specific in its mycorrhizal associations. *Molecular Ecology*, **22**, 2341-2354.

Park, J., Choppala, G., Bolan, N., Chung, J., Chuasavathi, T. 2011. Biochar reduces the bioavailability and phytotoxicity of heavy metals. *Plant and Soil*, **348**, 439.

Pecoraro, L., Girlanda, M., Kull, T., Perini, C., Perotto, S. 2012. Analysis of fungal diversity in *Orchis tridentata Scopoli*. *Central European Journal of Biology*, **7**, 850-857.

Pereira, G., Albornoz, V., Muñoz-Tapia, L., Romero, C., Atala, C. 2015. Asymbiotic germination of *Bipinnula fimbriata* (Orchidaceae) seeds in different culture media. *Seed Science and Technology*, **43**, 367-377.

Pereira, G., Romero, C., Suz, L., Atala, C. 2014. Essential mycorrhizal partners of the endemic Chilean orchids *Chloraea collicensis* and *C. gavilu*. *Flora*, **209**, 95-99.

Pereira, O., Rollemberg, C., Borges, A., Matsuoka, K., Kasuya, M. 2003. *Epulorhiza epiphytica* sp. nov. isolated from mycorrhizal roots of epiphytic orchids in Brazil. *Mycoscience*, **44**, 0153-0155.

Perotto, S., Rodda, M., Benetti, A., Sillo, F., Ercole, E., Rodda, M., Girlanda, M., Murat, C., Balestrini, R. 2014. Gene expression in mycorrhizal orchid protocorms suggests a friendly plant–fungus relationship. *Planta*, **239**, 1337-1349.

Peterson, R., Uetake, Y., Bonfante, P., Faccio, A. 1996. The interface between fungal hyphae and orchid protocorm cells. *Canadian Journal of Botany*, **74**, 1861-1870.

Peumans, W., Van Damme, E. 1995. Lectins as plant defense proteins. *Plant Physiology*, **109**, 347.

Pham, V., Kim, J. 2012. Cultivation of unculturable soil bacteria. *Trends in Biotechnology*, **30**, 475-484.

Ponstein, A., Bres-Vloemans, S., Sela-Buurlage, M.. van den Elzen, P.. Melchers, L., Cornelissen, B. 1994. A novel pathogen-and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiology*, **104**, 109-118.

Porras-Alfaro, A., Bayman, P. 2007. Mycorrhizal fungi of *Vanilla*: diversity, specificity and effects on seed germination and plant growth. *Mycologia*, **99**, 510-525.

Puente, M., Bashan, Y. 1994. The desert epiphyte *Tillandsia recurvata* harbours the nitrogen-fixing bacterium *Pseudomonas stutzeri*. *Canadian Journal of Botany*, **72**, 406-408.

Rajkumar, M., Sandhya, S., Prasad, M., Freitas, H. 2012. Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnology Advances*, **30**, 1562-1574.

Rasmussen, H. 2002. Recent developments in the study of orchid mycorrhiza. *Plant and Soil*, **244**, 149-163.

Rasmussen, H. 1995. *Terrestrial orchids: from seed to mycotrophic plant*. Cambridge University Press. 460p.

Rasmussen, H., Rasmussen, F. 2009. Orchid mycorrhiza: implications of a mycophagous life style. *Oikos*, **118**, 334-345.

Rasmussen, H., Rasmussen, F. 2007. Trophic relationships in orchid mycorrhiza-diversity and implications for conservation. *Lankesteriana International Journal on Orchidology*, **7,** 334-341.

Rasmussen, H., Whigham, D. 2002. Phenology of roots and mycorrhiza in orchid species differing in phototrophic strategy. *New Phytologist*, **154**, 797-807.

Regvar, M., Likar, M., Piltaver, A., Kugonič, N., Smith, J. 2010. Fungal community structure under goat willows (*Salix caprea* L.) growing at metal polluted site: the potential of screening in a model phytostabilisation study. *Plant and Soil*, **330**, 345-356.

Reinhardt, D. 2007. Programming good relations-development of the arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology*, **10**, 98-105.

Roberts, D., Dixon, K. 2008. Orchids. *Current Biology*, **18**, R325-R329.

Roesch, L., Fulthorpe, R., Riva, A., Casella, G., Hadwin, A., Kent, A., Daroub, S., Camargo, F., Farmerie, W., Triplett, E., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal*, **1**, 283-290.

Roy, M., Watthana, S., Stier, A., Richard, F., Vessabutr, S., Selosse, M. 2009a. Two mycoheterotrophic orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of ectomycorrhizal fungi. *BMC Biology*, **7**, 51.

Roy, M., Yagame, T., Yamato, M., Iwase, K., Heinz, C., Faccio, A., Bonfante, P., Selosse, M. 2009b. Ectomycorrhizal *Inocybe* species associate with the mycoheterotrophic orchid *Epipogium aphyllum* but not its asexual propagules. *Annals of Botany*, **104**, 595-610.

Salmani-Ghabeshi, S., Palomo-Marín, M., Bernalte, E., Rueda-Holgado, F., Miró-Rodríguez, C., Cereceda-Balic, F., Fadic, X., Vidal, V., Funes, M., Pinilla-Gil, E. 2016. Spatial gradient of human health risk from exposure to trace elements and radioactive pollutants in soils at the Puchuncaví-Ventanas industrial complex, Chile. *Environmental Pollution*, **218**, 322-330.

Salomons, W. 1995. Environmental impact of metals derived from mining activities: processes, predictions, prevention. *Journal of Geochemical Exploration*, **52**, 5-23.

Sánchez, S., Bills, G., Zabalgogeazcoa, I. 2008. Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. *Fungal Diversity*, **33**, 87-100.

Sathiyadash, K., Muthukumar, T., Uma, E., Pandey, R. 2012. Mycorrhizal association and morphology in orchids. *Journal of Plant Interactions*, **7**, 238-247.

Sattayasai, N., Sudmoon, R., Nuchadomrong, S., Chaveerach, A., Kuehnle, A., Mudalige-Jayawickrama, R., Bunyatratchata, W. 2009. *Dendrobium findleyanum* agglutinin: production, localization, anti-fungal activity and gene characterization. *Plant Cell Reports*, **28**, 1243-1252.

Schatz, B., Geoffroy, A., Dainat, B., Bessière, J., Buatois, B., Hossaert-McKey, M., Selosse, M. 2010. A case study of modified interactions with symbionts in a hybrid Mediterranean orchid. *American Journal of Botany*, **97**, 1278-1288.

Schelkle, M., Ursic, M., Farquhar, M., Peterson, R.L. 1996. The use of laser scanning confocal microscopy to characterize mycorrhizas of *Pinus strobus* L. and to localize associated bacteria. *Mycorrhiza*, **6**,, 431-440.

Schliemann, W., Ammer, C., Strack, D. 2008. Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. *Phytochemistry*, **69**, 112-146.

Schmalenberger, A., Duran, A., Bray, A., Bridge, J., Bonneville, S., Benning, L., Romero-Gonzalez, M., Leake, J., Banwart, S. 2015. Oxalate secretion by ectomycorrhizal *Paxillus involutus* is mineral-specific and controls calcium weathering from minerals. *Scientific Reports*, **5**, 12187.

Schödelbauerová, I., Roberts, D., Kindlmann, P. 2009. Size of protected areas is the main determinant of species diversity in orchids. *Biological Conservation*, **142**, 2329-2334.

Schultes, R. 1990. Medicinal orchids of the Indians of the Colombian Amazon. *American Orchid Society Bull*, **59**, 159-161.

Schützendübel, A., Polle, A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany*, **53**, 1351-1365.

Schweiger, R., Baier, M., Persicke, M., Müller, C. 2014. High specificity in plant leaf metabolic responses to arbuscular mycorrhiza. *Nature Communications*, **5**, 3886.

Seaton, P., Hu, H., Perner, H., Pritchard, H. 2010. Ex situ conservation of orchids in a warming world. *Botanical Reviews*, **76**, 193–203.

Selosse, M., Faccio, A., Scappaticci, G., Bonfante, P. 2004. Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. *Microbial Ecology*, **47**, 416-426.

Selosse, M., Roy, M. 2009. Green plants that feed on fungi: facts and questions about mixotrophy. *Trends in Plant Science*, **14**, 64-70.

Selosse, M., Martos, F., Perry, B., Maj, P., Roy, M., Pailler, T. 2010. Saprotrophic fungal symbionts in tropical achlorophyllous orchids: Finding treasures among the 'molecular scraps'? *Plant Signaling & Behavior*, **5**, 349-353.

Shefferson, R., Kull, T., Tali, K. 2008. Mycorrhizal interactions of orchids colonizing Estonian mine tailings hills. *American Journal of Botany*, **95**, 156-164.

Shefferson, R., Weib, M., Kull, T., Taylor, D. 2005. High specificity generally characterizes mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Molecular Ecology*, **14**, 613-626.

Shimura, H., Koda, Y. 2005. Enhanced symbiotic seed germination of *Cypripedium macranthos* var. rebunense following inoculation after cold treatment. *Physiologia Plantarum*, **123**, 281-287.

Shimura, H., Matsuura, M., Takada, N., Koda, Y. 2007. An antifungal compound involved in symbiotic germination of *Cypripedium macranthos* var. rebunense (Orchidaceae). *Phytochemistry*, **68**,, 1442-1447.

Simard, S., Perry, D., Jones, M., Myrold, D., Durall, D., Molina, R. 1997. Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature*, **388**, 579-582.

Simard, S., Durall, D. 2004. Mycorrhizal networks: a review of their extent, function, and importance. *Canadian Journal of Botany*, **82**, 1140-1165.

Smith, S., Read, D. 2010. *Mycorrhizal symbiosis*. Academic press.

Smith, S. 1966. Physiology and ecology of orchid mycorrhizal fungi with reference to seedling nutrition. *New Phytologist*, **65**, 488-499.

Sneh, B. 1996. Non pathogenic isolates of *Rhizoctonia* spp.(np-R) and their role in biological control. in: *Rhizoctonia species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*, Springer Netherlands, pp. 473-483.

Sommer, J., Pausch, J., Brundrett, M., Dixon, K., Bidartondo, M., Gebauer, G. 2012. Limited carbon and mineral nutrient gain from mycorrhizal fungi by adult Australian orchids. *American Journal of Botany*, **99**, 1133-1145.

Song, Y., Ye, M., Li, C., He, X., Zhu-Salzman, K., Wang, R., Su, Y., Luo, S., Zeng, R. 2014. Hijacking common mycorrhizal networks for herbivore-induced defence signal transfer between tomato plants. *Scientific Reports*, **4**, 3915.

Stark, C., Babik, W., Durka, W. 2009. Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*. *Mycological Research*, **113**, 952-959.

Steinfort, U., Verdugo, G., Besoain, X., Cisternas, M. 2010. Mycorrhizal association and symbiotic germination of the terrestrial orchid *Bipinnula fimbriata* (Poepp.) Johnst (Orchidaceae). *Flora*, **205**, 811-817.

Stewart, S., Kane, M. 2006. Symbiotic seed germination of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell, Tissue and Organ Culture*, **86**, 159-167.

Stöckel, M., Těšitelová, T., Jersáková, J., Bidartondo, M., Gebauer, G. 2014. Carbon and nitrogen gain during the growth of orchid seedlings in nature. *New Phytologist*, **202**, 606-615.

Suárez, J., Weiß, M., Abele, A., Garnica, S., Oberwinkler, F., Kottke, I. 2006. Diverse tulasnelloid fungi form mycorrhizas with epiphytic orchids in an Andean cloud forest. *Mycological Research*, **110**, 1257-1270.

Suryanarayanan, T., Kumaresan, V. 2000. Endophytic fungi of some halophytes from an estuarine mangrove forest. *Mycological Research*, **104**, 1465-1467.

Swarts, N., Sinclair, E., Francis, A., Dixon, K. 2010. Ecological specialization in mycorrhizal symbiosis leads to rarity in an endangered orchid. *Molecular Ecology*, **19**, 3226-3242.

Swarts, N., Dixon, K. 2009. Terrestrial orchid conservation in the age of extinction. *Annals of Botany*, **104**, 543-556.

Tabatabai, M., Bremner, J. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biology and Biochemistry*, **1**, 301-307.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, **30**, 2725-2729.

Taylor, D., Bruns, T. 1997. Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences*, **94**, 4510-4515.

Taylor, D., Bruns, T., Leake, J., Read, D. 2002. Mycorrhizal specificity and function in myco-heterotrophic plants. in: *Mycorrhizal Ecology*, Springer, Berlin Heidelberg, pp. 375-413.

Taylor, D., McCormick, M. 2008. Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. *New Phytologist*, **177**, 1020-1033.

Tešitelová, T., Tešitel, J., Jersáková, J., Říhová, G., Selosse, M. 2012. Symbiotic germination capability of four *Epipactis* species (Orchidaceae) is broader than expected from adult ecology. *American Journal of Botany*, **99**, 1020-1032.

Timke, M., Wang-Lieu, N., Altendorf, K., Lipski, A. 2005. Community structure and diversity of biofilms from a beer bottling plant as revealed using 16S rRNA gene clone libraries. *Applied and Environmental Microbiology*, **71**, 6446-6452.

Trudell, S., Rygiewicz, P., Edmonds, R. 2003. Nitrogen and carbon stable isotope abundances support the myco-heterotrophic nature and host-specificity of certain achlorophyllous plants. *New Phytologist*, **160**, 391-401.

Tsai, W., Hsiao, Y., Pan, Z., Kuoh, C., Chen, W., Chen, H., 2008. The role of ethylene in orchid ovule development. *Plant Science*, **175**, 98-105.

Turnau, K., Gawroński, S., Ryszka, P., Zook, D. 2012. Mycorrhizal-based phytostabilization of Zn–Pb tailings: lessons from the Trzebionka mining works (Southern Poland). in: *Bio-Geo Interactions in Metal-Contaminated Soils*, Springer, Berlin Heidelberg, pp. 327-348.

Valadares, R., Pereira, M., Otero, J., Cardoso, E. 2011. Narrow fungal mycorrhizal diversity in a population of the orchid *Coppensia doniana*. *Biotropica*, **44**, 114-122.

Valadares, R., Perotto, S., Santos, E., Lambais, M. 2014. Proteome changes in *Oncidium sphacelatum* (Orchidaceae) at different trophic stages of symbiotic germination. *Mycorrhiza*, **24**, 349-360.

Valadares, R. 2014. Identification of genes and proteins involved in the regulation of orchid mycorrhiza, Doctoral thesis. Universidade de São Paulo.

van Loon, L.C. 1985. Pathogenesis-related proteins. *Plant Molecular Biology*, **4**, 111-116.

Vendramin, E., Gastaldo, A., Tondello, A., Baldan, B., Villani, M., Squartini, A. 2010. Identification of two fungal endophytes associated with the endangered orchid *Orchis militaris* L. *Journal of Microbiology and Biotechnology*, **20**, 630-636.

Verdejo, J., Ginocchio, R., Sauvé, S., Salgado, E., Neaman, A. 2015. Thresholds of copper phytotoxicity in field-collected agricultural soils exposed to copper

mining activities in Chile. *Ecotoxicology and Environmental Safety*, **122**, 171-177.
Verdugo, G., Marchant, J., Cisternas, M., Calderón, X., Peñaloza, P. 2007. Caracterizacion morfometrica de la germinacion de *Chloraea crispa* lindl. (Orchidaceae) usando un analisis de imagen. *Gayana Botánica*, **64**, 232-238.
Veresoglou, S., Chen, B., Rillig, M. 2012. Arbuscular mycorrhiza and soil nitrogen cycling. *Soil Biology and Biochemistry*, **46**, 53-62.
Voyron, S., Roussel, S., Munaut, F., Varese, G., Ginepro, M., Declerck, S., Marchisio, V. 2009. Vitality and genetic fidelity of white-rot fungi mycelia following different methods of preservation. *Mycological Research*, **113**, 1027-1038.
Walkley, A., Black, I. 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Science*, **37**, 29-38.
Wang, E., Yu, N., Bano, S., Liu, C., Miller, A., Cousins, D., Zhang, X., Ratet, P., Tadege, M., Mysore, K. 2014. A H+-ATPase that energizes nutrient uptake during mycorrhizal symbioses in rice and *Medicago truncatula*. *The Plant Cell*, **26**, 1818-1830.
Wang, W., Vignani, R., Scali, M., Cresti, M. 2006. A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis*, **27**, 2782-2786.
Wang, X., Bauw, G., Van Damme, E., Peumans, W., Chen, Z., Van Montagu, M., Angenon, G., Dillen, W. 2001. Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. *The Plant Journal*, **25**, 651-661.
Warcup, J., Talbot, P. 1967. Perfect states of *Rhizoctonias* associated with orchids. *New Phytologist*, **66**, 631-641.
Warcup, J. 1981. The mycorrhizal relationships of Australian orchids. *New Phytologist*, **87**, 371-381.
Waterman, R., Bidartondo, M. 2008. Deception above, deception below: linking pollination and mycorrhizal biology of orchids. *Journal of Experimental Botany*, **59**, 1085-1096.
Watkinson, J., Welbaum, G. 2002. Characterization of gene expression in roots of *Cypripedium parviflorum* var. pubescens incubated with a mycorrhizal fungus. *XXVI International Horticultural Congress: Elegant Science in Floriculture 624*. pp. 463-470.
Weber, R., Webster, J. 2001. Teaching techniques for mycology: 14. Mycorrhizal infection of orchid seedlings in the laboratory. *Mycologist*, **15**, 55-59.
White, T., Bruns, T., Lee, S., Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, **18**, 315-322.
Wiemken, V., Boller, T. 2002. Ectomycorrhiza: gene expression, metabolism and the wood-wide web. *Current Opinion in Plant Biology*, **5**, 355-361.
Xing, X., Ma, X., Deng, Z., Chen, J., Wu, F., Guo, S. 2013. Specificity and preference of mycorrhizal associations in two species of the genus *Dendrobium* (Orchidaceae). *Mycorrhiza*, **23**, 317-324.
Yam, T., Arditti, J. 2009. History of orchid propagation: a mirror of the history of biotechnology. *Plant Biotechnology Reports*, **3**, 1.
Yamaji, N., Xia, J., Mitani-Ueno, N., Yokosho, K., Ma, J. 2013. Preferential delivery of zinc to developing tissues in rice is mediated by P-type heavy metal ATPase OsHMA2. *Plant Physiology*, **162**, 927-939.

Yamato, M., Iwase, K., Yagame, T., Suzuki, A. 2005. Isolation and identification of mycorrhizal fungi associating with an achlorophyllous plant, *Epipogium roseum* (Orchidaceae). *Mycoscience*, **46**, 73-77.

Yoneyama, K., Xie, X., Sekimoto, H., Takeuchi, Y., Ogasawara, S., Akiyama, K., Hayashi, H., Yoneyama, K. 2008. Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. *New Phytologist*, **179**, 484-494.

Yuan, Z., Chen, Y., Yang, Y. 2009. Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid (*Dendrobium nobile*): estimation and characterization. *World Journal of Microbiology and Biotechnology*, **25**, 295-303.

Yukawa, T., Ogura-Tsujita, Y., Shefferson, R., Yokoyama, J. 2009. Mycorrhizal diversity in *Apostasia* (Orchidaceae) indicates the origin and evolution of orchid mycorrhiza. *American Journal of Botany*, **96**, 1997-2009.

Zhao, X., Zhang, J., Chen, C., Yang, J., Zhu, H., Liu, M., Lv, F. 2014. Deep sequencing–based comparative transcriptional profiles of *Cymbidium hybridum* roots in response to mycorrhizal and non-mycorrhizal beneficial fungi. *BMC Genomics*, **15**, 747.

Zimmer, K., Meyer, C., Gebauer, G. 2008. The ectomycorrhizal specialist orchid *Corallorhiza trifida* is a partial myco-heterotroph. *New Phytologist*, **178**, 395-400.