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ORIGINAL ARTICLE

Adaptation and tolerance mechanisms developed by mycorrhizal *Bipinnula fimbriata* plantlets (Orchidaceae) in a heavy metal-polluted ecosystem

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Abstract

The adaptation and performance of orchid mycorrhizae in heavy metal-polluted soils have been poorly explored. In the present study, proteomic and metabolic approaches were used to detect physiological changes in orchid roots established in a heavy metal-polluted soil and to ascertain whether mycorrhizal fungi affect the metabolic responses of roots. Young *Bipinnula fimbriata* plantlets were established in control and heavy metal-polluted soils in a greenhouse. After 14 months, exudation of root organic acids, phenolics, percentage of mycorrhization, mineral content, and differential protein accumulation were measured. More root biomass, higher root colonization, and higher exudation rates of citrate, succinate, and malate were detected in roots growing in heavy metal-polluted soils. Higher accumulation of phosphorus and heavy metals was found inside mycorrhizal roots under metal stress. Under non-contaminated conditions, non-mycorrhizal root segments showed enhanced accumulation of proteins related to carbon metabolism and stress, whereas mycorrhizal root segments stimulated protein synthesis related to pathogen control, cytoskeleton modification, and sucrose metabolism. Under heavy metal stress, the proteome profile of non-mycorrhizal root segments indicates a lower induction of defense mechanisms, which, together with the stimulation of enzymes related to carotenoid biosynthesis and cell wall organization, may positively influence mycorrhizal fungi colonization. The results point to different metabolic strategies in mycorrhizal and non-mycorrhizal root segments that are exposed to heavy metal stress. The results indicate that root colonization by mycorrhizal fungi is stimulated to alleviate the negative effects of heavy metals in the orchids.

Keywords Orchid mycorrhiza · Organic acid exudation · Proteome · Contaminated soil

Introduction

Ecosystem degradation due to mining activities has well known detrimental effects on the environment and human health (Salomons 1995; Fang et al. 2003). 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

This study is dedicated for the memory of the German/Spanish mycorrhizae researcher, Horst Vierheilig (1960–2011) of CSIC, Spain

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ecosystems (Ginocchio et al. 2004; Arenson et al. 2015; 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). These contaminating processes have induced shifts in species composition, altering the normal functioning of ecosystems. Specifically, the species richness decreased considerably towards the smelter, and the established plants are partly dependent on mycorrhizal interaction to survive in metal-polluted soils (Cornejo et al. 2008; Ginocchio 2000; Meier et al. 2012b).

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 and Dixon 2008). The orchid's life cycle is associated with specific symbiotic associations with fungi of the polyphyletic *Rhizoctonia* group, as well as some endophytic and ectomycorrhizal-forming fungi (preferably in fully mycoheterotrophic orchids), which are key to supply carbon, nutrients, and water mainly during transition from seed to protocorm (Bayman and Otero 2006; Cameron et al. 2006, 2007; Valadares et al. 2014). Production of seeds with an endosperm lacking essential nutrients for an embryo's development renders orchid 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 and Roy 2009). Additionally, the pathogenicity level of fungal species should be considered, as some interactions can easily and often shift from mutualism to parasitism (Rasmussen and Rasmussen 2009; Bender et al. 2014). This requires a balance between the amount of nutrients received and the potential pathogenicity of some orchid mycorrhizal fungi (OMF) (Valadares et al. 2014).

Although little is known about the molecular regulations in orchid mycorrhizae (ORM), 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 upregulation of genes related to mutualism, and that plant genes typically involved in defense responses against fungi were not induced in orchid mycorrhiza. Zhao et al. (2014) reported genes expressed in symbiotically germinated seeds of the orchid *Dendrobium officinale* related to carbon metabolism, plant defense, signaling, and plant development. The proteome of symbiotic *Oncidium sphacelatum* protocorms during transition from mycoheterotrophy to autotrophy has shown differential expression of proteins related to the stress response, energy metabolism, carotenoid and phytoalexin biosynthesis, and signaling (Valadares et al. 2014). López-Chávez et al. (2016) identified upregulation of orchid proteins related to cell cycle, ribosome biogenesis, energy metabolism, and secretion, as well as upregulation of symbiotic proteins related to stress response, protein-protein interaction, saccharides, and protein biosynthesis. Valadares (2014) analyzed the proteome and RNA profile of mycorrhizal and non-mycorrhizal root segments of *Oeceoclades maculata* which suggested alleviation of defense response and enhanced nitrogen metabolism in mycorrhizal roots.

Under stress, several plants depend on symbiotic associations to first establish and then to improve nutrition (Rajkumar et al. 2012; Belimov et al. 2015). In this context, ORM are specific mycorrhizal associations 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 ORM (in partially mycoheterotrophic plants) 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 phenolics; and (3) associations with OMF adapted to the ecological conditions where orchids become established (Dearnaley 2007; Herrera et al. 2017; Shefferson et al. 2008). The association of orchids with specific OMF is fundamental to colonize new ecological niches in heavy metal-polluted areas (Jurkiewicz et al. 2001; Shefferson et al. 2008). There are no reports of molecular mechanisms developed by orchids to grow in heavy metal-polluted soils. We hypothesized that the orchid *B. fimbriata* may develop specific metabolic mechanisms to establish in a heavy metal-polluted soil and these mechanisms are different for mycorrhizal and non-mycorrhizal tissues. Therefore, in this study, we address how mycorrhizal colonization and exposure to heavy metal-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.

Materials and methods

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 (Novoa et al. 2015). This orchid produces highly viable seeds and can be

cultivated in symbiotic and asymbiotic media (Steinfort et al. 2010; Herrera et al. 2017). Their distribution includes the Ventanas industrial complex, with some individuals growing in this polluted ecosystem, commonly associated with pseudometalophyte species (Ginocchio et al. 2004). Sampling of plants 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, with similar root biomass, and without signals of abundant mycorrhizal colonization (brownish zones) were collected near the Ventanas industrial complex (32° 46′ 08.3″ S, 71° 28′ 17.1″ W) and in the Concón dunes (32° 56′ 35.9″ S, 71° 33′ 00.6″ W), placed in paper bags, and transported to the greenhouse (< 12 h) for individual experiments.

Soil sampling

Soil samples of five random microsites were collected from two semiarid Mediterranean ecosystems: (1) ultisol top soil (20 cm) affected by heavy metal pollution from Puchuncaví Valley in central Chile, 1.5 km southeast of the Ventanas copper smelter, which was exposed to atmospheric deposition of heavy metal-enriched particles (Fuentes et al. 2016), and (2) dunesand ultisol soil from Concón, which was defined as control, because its natural conditions promote development of naturally germinated plants.

Plant growth conditions

Four-month-old *B. fimbriata* plantlets ($n = 20$) obtained by rhizome replication of young plantlets sampled at both ecosystems (control and heavy metal-polluted), similarly to the reported by Jiang et al. (2015), were transferred to plastic pots (30 cm × 15 cm; per soil) with 800 g of sieved soil (2 mm; with the same original soils) and cultivated (Fig. 1a). 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) (Fig. 1b, c) in roots developed in the control (soil from Concón) and the heavy metal-polluted soil (soil from Puchuncaví).

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 systems, comprising mycorrhizal and non-mycorrhizal root segments ($n = 5$), under both soil conditions and quantified by RP-HPLC, as described in Meier et al. (2012a) with minor modifications. The entire root system of the plants 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 for 1 h. The solution was filtered (0.45 μm), freeze-dried, and roots were kept separately for mycorrhizae and metal analysis. The residue was resuspended in 500 μL deionized sterile water and filtered again (0.22 μm). Calibration curves were prepared using an organic acids kit (47,264, Supelco, Bellefonte, PA) and the standard of the phenolic compounds 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 analyzed according to Meier et al. (2012a).

Plant and soil chemical analyses

For soil chemical analyses ($n = 5$), total phosphorus was determined by the alkaline oxidation method (Dick and Tabatabai 1977). 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 and Duquette, 1986) and exchangeable Al was extracted with 1 M KCl. All were analyzed by atomic absorption spectroscopy (PinAAcle 900 T, 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 1. The mineral content in roots ($n = 5$, pooled from different plants) 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).

Molecular analysis and characterization of mycorrhizal fungi

After extraction of organic acids, root tissues of five plants (from control and metal-polluted soils) were analyzed 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). Root cross-sections of 5 random plants (~ 10 mm diameter; $n = 200$) were classified, based on the area of infection: 0–20%, 20–40%, 40–60%, 60–80%, 80–100% infection. To isolate and identify compatible

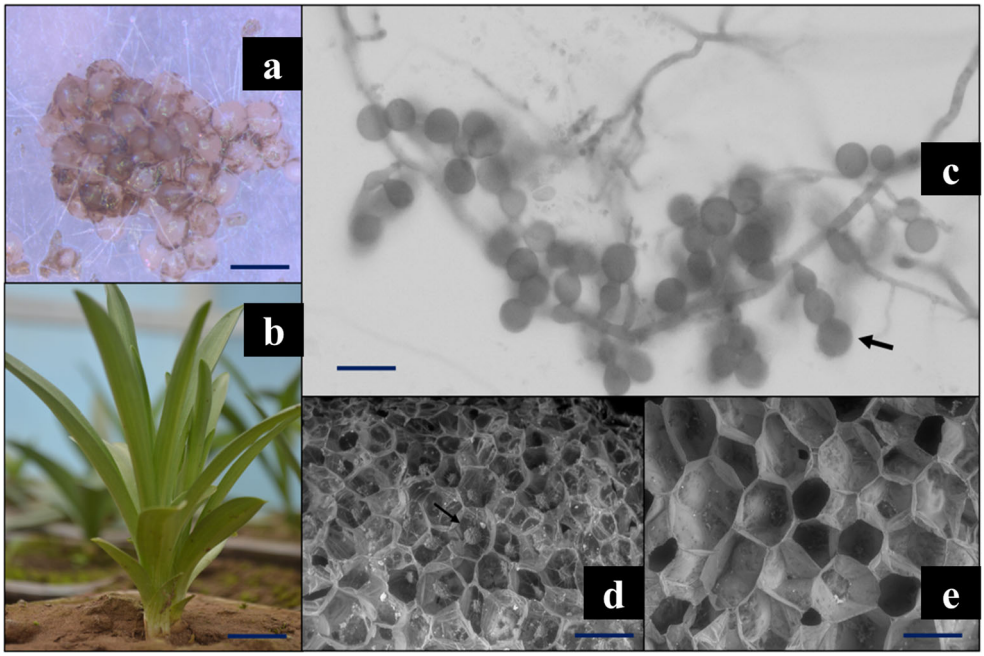

Fig. 1 **a** *Bipinnula fimbriata* plantlets established in the heavy metal-polluted soil (scale bar = 1 cm); Mycorrhizal (**b**) and non-mycorrhizal root tissues (**c**) showing pelotons (black arrow) (scale bars = 200 μm); **d** Mycorrhizal fungi isolated from orchids developed in heavy metal-polluted soil, showing moniliod cell (black arrow) (scale bar = 20 μm); and **e** *Bipinnula fimbriata* protocorms obtained by symbiotic germination (scale bar = 500 μm)

mycorrhizal fungi candidate for bioaugmentation strategies, a culture-dependent approach was used. One day after sampling, roots were washed in distilled water and cut into 5 cm segments. These fragments were superficially disinfected by washing in 70% ethanol for 1 and 4 min in 20% sodium hypochlorite solution (0.5% active chlorine), followed by five washings with sterilized water. Root pieces containing pelotons (mycorrhizal roots) had their velamen removed and discarded. Eight to ten fragments were placed on Petri dishes and incubated at 25 °C. Growing colonies were observed daily. Septate fungi with 90° hyphal branching, constrictions close to the branching point and no spore formation were classified as *Rhizoctonia*-like fungi and transferred to dishes containing fresh potato dextrose agar (PDA) plus streptomycin (100 mgL^{-1}) and modified oatmeal agar (OMA) media (4 g of oats L^{-1}, 10 g of agar, pH 5.6 supplemented with streptomycin 100 mg L^{-1}). Fungi were cultured in solid PDA medium for 7 days at 27 °C and DNA was extracted to amplify the internal transcribed spacers of the ITS1 region (5′-TCCGTAGGTGAACCTGCGG) and ITS4 region (5′-TCCTCCGCTTATTGATATGC) of genomic rDNA according to Herrera et al. 2017.

Table 1 Chemical characteristics from control and Cu-polluted soil associated with *Bipinnula fimbriata* ($n = 5$)

	Control soil	Heavy metal-polluted soil
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]	1.49	196

[a] mg kg^{-1}

[b] In H_2O

[c] %

[d] (meq/100 g)

Protein extraction from roots

Proteins were extracted following the procedure described by Wang et al. (2006) with minor modifications. Mycorrhizal root fragments were selected based on the presence of pelotons (brownish zones characteristic of ORM colonization), whereas non-mycorrhizal roots (white roots without OMF-like structures) were also selected to detect specific protein expression. Mycorrhizal and non-mycorrhizal root segments from different plants ($n = 5$), developed in control and heavy metal-polluted soils (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. 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 with 70% ethanol. The final pellet was dried and proteins were suspended in 8 M urea-7 M thiourea buffer. Protein pooled from root segments of several plants growing in polluted and control soils was performed independently and each sample ($n = 5$) was injected five times. Protein quantification was carried out with the Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA).

Proteome analysis

For protein samples of mycorrhizal and non-mycorrhizal root segments from plants developed in control and heavy metal-polluted soils ($n = 5$), 50 μg of proteins was dissolved in denaturing buffer containing 5 mM dithiothreitol (DTT; Sigma-Aldrich) at 56 °C for 25 min. Then, 14 mM iodoacetamide was added and incubated at room temperature for 30 min. 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 micrograms of peptides was injected into a chromatograph (NanoACQUITY 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 × 20 mm) 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 to 2000 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 filtered according detection in four analytical replicates and $p < 0.05$. Proteins were considered upregulated or downregulated 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 and Götz 2008) was used to categorize proteins according to their biological process, molecular functions, and cellular location.

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 (R Core Team 2018; https://www.R-project.org).

Results

Identification of mycorrhizal fungi

Sixteen fungal groups out of 67 isolates were isolated. BLAST analyses showed that three were closely related to *Tulasnella calospora* (GenBank Accession EU218888) (Fig. 1d), 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 2). The fungal sequences from *B. fimbriata* roots (from both soil treatments) showed high similarity to other Tulasnellaceae fungi isolated from the same orchid species (Table 2, References therein). All *Tulasnella* spp. isolated in this study can promote *B. fimbriata* seed germination to different degrees, showing embryo swelling, rhizoid presence, and foliar primordia formation (Fig. 1e). Colonization percentages in roots were variable and were higher in plants developed in the polluted soils (Table 3), showing most of root fragments with 0–20% (control soil) and 20–40% (contaminated soil) colonization percentage; however, the three colonization levels were found in both soils (Fig. S1).

Chemical analyses

In the polluted ecosystem, heavy metal content exceeds, by many times, the content in control soil, specifically copper, arsenic, and zinc (Table 3). Higher accumulation of phosphorus and heavy metals was found inside mycorrhizal root segments growing under heavy metal stress, specifically copper, aluminum, iron, and zinc (Table 3).

Table 2 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-CC[a]001	39	26	*KP306589* (Uncultured Tulasnellaceae)	98	Photosynthetic orchid	GenBank
BF-PV[b]002	24	16	*EU218888* (*Tulasnella calospora*)	99	Not specified	Taylor and McCormick 2008
BF-PV[b]003	9	6	*KP278160* (*Leptodontidium* sp.)	97	*Chloraea longipetala*	Herrera et al. 2017
BF-CC[a]004	17	11	*KP278157* (*Phomopsis columnaris*)	99	*Chloraea gavilu*	Herrera et al. 2017
BF-CC[a]005	11	7	Unidentified	< 95	–	–

[a] *Bipinnula fimbriata* plants sampled in Concon soil

[b] *Bipinnula fimbriata* plants sampled near Ventanas industrial complex

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 3). Concomitant with root development that was significantly stimulated 1.4-fold more in contaminated soil than in controls (Table 3; Table S1), biomass of shoots was not significantly different between plants from control and contaminated soil at the end of the cultivation period. However, the root: shoot biomass ratio (7.5 vs. 14.6) between plants from control and metal-polluted soil, respectively, indicates an important difference in C allocation (Table 3). Roots developed in heavy metal-polluted soil showed more biomass (shorter roots and more individual roots than control soils) (Table 3). Furthermore, root colonization by OMF was higher in heavy metal-polluted soils, in spite of the metals, and in the presence of higher N, P, and K (Table 3).

Root exudates

We detected exudation of four organic acids from *B. fimbriata* plants grown in both soils: citrate, succinate, malate, and oxalate. *B. fimbriata* plantlets grown in the polluted soil had the higher overall exudation rate of citrate, succinate, and malate, but lower concentrations of oxalic acid (Table 3). For phenolic compounds, our analysis only detected cinnamic acid, which was exuded in similar quantities from roots growing in control or contaminated soils (Table 3). Linear regression analysis showed that cinnamic acid, succinate, and oxalate were positively related to OMF colonization, whereas 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).

Proteome analyses

The proteome of roots was different between control and contaminated soil and was strongly affected by mycorrhization (Fig. 2; Fig. S2). Higher protein diversity was detected in root segments developed in control soils, including 119 protein clusters in mycorrhizal and 91 in non-mycorrhizal segments. Proteome of root segments developed in the polluted soil was less complex, with 86 protein clusters in mycorrhizal and 55 in non-mycorrhizal root segments. Description of gene ontology categories (biological processes, molecular function, and cellular locations) of proteins are listed in (Table S2).

The expressed proteins found in mycorrhizal and non-mycorrhizal root segments were different in plants developed in the heavy metal-polluted soil (Fig. 2). Specifically, comparing non-mycorrhizal root segments from polluted and control soils, we observed 320 proteins, grouped in 108 protein clusters. Seven protein clusters were exclusively detected in root segments from heavy metal-polluted soil, 16 in root segments from control soils, and 85 were differentially expressed. After filtering, 22 proteins were selected: three exclusively present in segments from heavy metal-polluted soil, 4 in segments from the controls, and 15 with differential accumulation (Fig. 2). Upregulated (polluted/control soil) proteins 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 downregulated 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 defense (orcinol O-methyltransferase, lipoxygenase, allene oxidase, ubiquitin-like protein) (Table 5).

In mycorrhizal root segments, we also found specific protein expression/accumulation (polluted/control soil), identifying 391 proteins, grouped in 138 protein clusters.

Table 3 Different variables measured in the orchid *Bipinnula fimbriata* developed in control (Concon) and heavy metal-polluted (Puchuncavi) soils. Results are mean ± SD ($n = 5$)

Variable		Control soil	Heavy metal-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*	64.5 ± 6.70*
Shoot biomass[b]		6.5 ± 3.1 [ns]	4.4 ± 2.8 [ns]
Total protein content[c]	*M*	337 ± 5*	303 ± 4*
	NM	316 ± 4**	224 ± 7**
Heavy metals in soil[d]			
	Cu	3.45 ± 0.95**	197.5 ± 32.2**
	As	2.8 ± 1.13*	18.25 ± 6.7*
	Zn	80.29 ± 7.5**	168.9 ± 12.3**
Heavy metals in root[d]			
Cu	*M*	0.25 ± 0.03**	2.02 ± 0.54**
	NM	0.34 ± 0.08**	1.14 ± 0.02**
Al	*M*	736 ± 158*	1324 ± 345*
	NM	455 ± 34[ns]	536 ± 77[ns]
Fe	*M*	1268 ± 224[ns]	1718 ± 352[ns]
	NM	1366 ± 136*	1518 ± 105*
Zn	*M*	91.3 ± 12.4*	53.8 ± 5.3*
	NM	48.2 ± 2.9*	65.3 ± 8.2*
P in root [d]	*M*	1328.0 ± 143*	1904.7 ± 305*
	NM	532.4 ± 27**	281.8 ± 45**
Colonization percentage[e]		17.8 ± 3.1*	24 ± 2.5*
Seed viability (%)		70 ± 15*	53 ± 8*

[ns] Non-significant

**$p < 0.01$

*$p < 0.05$

M, mycorrhizal root; *NM*, non-mycorrhizal root

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

[b] mg

[c] Total proteins identified on each sample

[d] μg g^{-1}

[e] Mean of percentage of pelotons from random mycorrhizal root segments

We detected 24 proteins exclusively in mycorrhizal root segments of heavy metal-polluted soil, 53 in controls, and 61 with differential accumulation. After filtering, 20 proteins were selected; one protein was exclusively related to segments from heavy metal-polluted soils and three to control root segments, whereas 16 proteins showed differential accumulation between treatments (Fig. 2; Table 5). Upregulated proteins in mycorrhizal root segments from heavy metal-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 defense (HSP70), whereas downregulated proteins were related to energy (ribulose-1,5-bisphosphate carboxylase/oxygenase), flavonoid metabolism (chalcone synthase), defense (3-ketoacyl-CoA thiolase, orcinol O-methyltransferase), translation (ribosomal protein S3a), protein modification (peptidyl-prolyl cis-trans isomerase), and redox homeostasis (ascorbate peroxidase) (Table 5). Spectrum, peptide, and protein identification and quantitation reports are provided (Online Resource 1).

Table 4 Correlation matrix of some selected parameters in *Bipinnula fimbriata* plantlets growing in heavy metal-polluted soils ($n = 5$)

Variables	Malic acid[a]	Succinic acid[a]	Citric acid[a]	Cinnamic acid[a]	Root biomass[b]	Total protein[c]	Cu in soil[d]	Cu in root[d]	Available Cu[e]	% 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
Root 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[d]								–	0.89	0.86
Available Cu[e]									–	0.58

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

[b] mg

[c] Total proteins identified in each sample

[d] μg g^{-1}

[e] μg L^{-1}

Discussion

The Puchuncaví Valley received intense heavy metal pollution since the installation of the Ventanas copper smelter in 1964, which has limited establishment of vegetation. In these soils, heavy metals 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). Furthermore, we detected more mineral nutrients in the heavy metal-polluted than in control soils, as well as a higher presence of some mineral nutrients that influence positively the mycorrhizal colonization. The increase in OMF colonization likely explains the efficient nutrient uptake in roots and the high metal content, because of immobilization of heavy metals by the fungal mycelium (Jurkiewicz et al. 2001).

Orchids developed in terrestrial substrates depend on mycorrhizal interactions to complement carbon and obtain other nutrients, specially under stress conditions and during early developmental stages, 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 root segments under both soil conditions were affected by the presence of OMF,

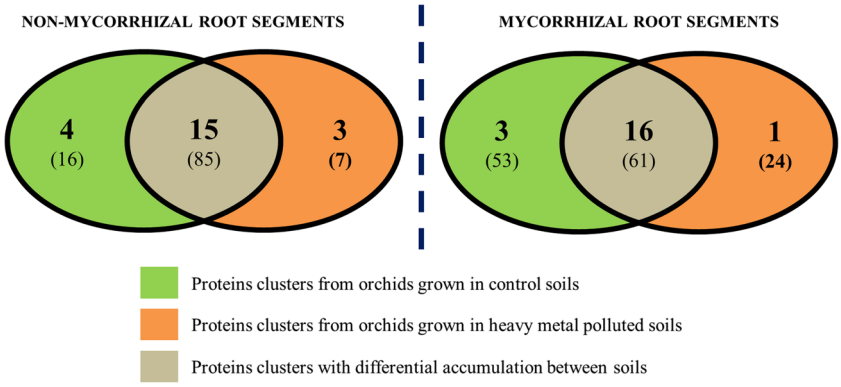

Fig. 2 Venn diagrams showing distribution of the main protein clusters of *Bipinnula fimbriata* obtained by comparisons of mycorrhizal and non-mycorrhizal root segments developed in control and heavy metal-polluted soil. Number between parentheses refers to the protein clusters before protein filtering

Table 5 Most representative proteins identified in *Bipinnula fimbriata* mycorrhizal and non-mycorrhizal roots developed in control and heavy metal-polluted soils

Protein description	Organism	Accession (protein)	Molecular weight	(*p* value)	Fold change
Non-mycorrhizal roots (heavy metal-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 (heavy metal-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	*Paphiopedilum armeniacum*	AFU07709	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

increasing synthesis of membrane transporters and proteins having a role in controlling mycorrhizal fungi spread, such as orcinol O-methyltransferase, chalcone synthase, and phenylalanine ammonia lyase. Further analyses are necessary to clarify the adaptation mechanisms developed by the mycorrhizal fungi to grow under these soil conditions, and to identify whether the observed OMF are heavy metal accumulators, similar to what is known from AM fungi, and from ORM of *Epipactis atropurpureum*, *Epipactis helleborine*, and *Dactylorhiza majalis* (Jurkiewicz et al. 2001; Aguilera et al. 2011; Turnau et al. 2012).

Exudates in *B. fimbriata* roots were different, related to the soil where they grow, showing a different exudation pattern of organic acids and a phenolic compound that are important mechanisms to promote metal exclusion in heavy metal-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 as stimulators of native soil microorganisms (Nigam et al. 2001; Schmalenberger et al. 2015). Therefore, *B. fimbriata* roots may respond in a similar way as other plant species that modify their exudates as a response to heavy-metal stress (Nian et al. 2002; Mucha et al. 2010). In the case of cinnamic acid, we did not detect any significant differences in exudation.

In orchids, non-mycorrhizal root segments are different from mycorrhizal ones, mainly by the presence of mycorrhizal fungi in pelotons. Non-mycorrhizal root segments are expected to act as a starch sink, whereas mycorrhizal roots host the OMF (Cameron et al. 2006, 2008; Valadares 2014). Our results suggest that, under either control or metal-contaminated soil, the proteome was affected by the presence of the OMF. The higher concentrations of proteins detected in mycorrhizal roots are related to a higher activity of essential cellular processes, such as growth, membrane transport, pathogen control, and oxidative stress control (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 carbon of fungal-origin, promoting growth and control of OMF spread (Valadares et al. 2014; Zhao et al. 2014).

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 heavy metal-polluted soils, non-mycorrhizal root segments had lower protein related to defense responses against the fungus. This may explain the higher peloton abundance in roots of heavy metal-polluted soils. In addition, the production of enzymes able to metabolize carotenoid-derived molecules may also play a role in signaling and functionality of the orchid mycorrhizal interaction (Valadares et al. 2014). Furthermore, the enzyme epoxycarotenoid dioxygenase detected in non-mycorrhizal root segments, together with abscisic acid, may play a role in mycorrhizal fungi attraction and anatomic modifications, similar to which is reported in arbuscular mycorrhizal fungi (Herrera-Medina et al. 2007), as well as regulator of abiotic stress responses against heavy metals, using abscisic acid as mediator for multiple pathways that contribute to decrease the plant toxicity (Bücker-Neto et al. 2017).

Still under heavy-metal stress, in non-mycorrhizal root segments, we also detected a decrease in protein synthesis of defense-related mechanisms (flavonoid, phytoalexins, and ethylene) and production of enzymes related to the metabolism of strigolactones, which have been defined as hormones involved in root and shoot branching, as well as promoters of fungal root colonization in AM symbioses (Smith and Waters 2012; 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 and Parniske 2013). Furthermore, in mycorrhizal roots, the presence of OMF 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 signaling and transport, as in the case of the orchid *Cymbidium hybridum* (Zhao et al. 2014). Under heavy metal stress, we found enhanced accumulation of transmembrane ATPases involved in nutrient transport across the plasma membrane, similar to other mycorrhizal interactions (Yamaji et al. 2013; Wang et al. 2014).

We detected proteins related to copper transport that were over-expressed in non-mycorrhizal root segments developed in heavy metal-polluted soils, which reflected an active cross-membrane transit of copper in root cell membranes. However, in mycorrhizal root segments, protein synthesis related to copper transport was less abundant. This may be explained by the presence of OMF which may reduce the available concentrations of some metals (Jurkiewicz et al. 2001). Besides, the presence of OMF may explain the copper content in mycorrhizal root segments, that almost double to the content measured in non-mycorrhizal root segments. We also expect that, under heavy metal stress, mycorrhizal fungi (and maybe also other beneficial endophytes) 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 colonization of OMF in orchids requires specific and efficient responses against infection of vital tissues, including biosynthesis of defense molecules, biosynthesis,

and perception of ethylene and jasmonic acid. In our experiments, mycorrhizal root segments showed over-expression of proteins related to jasmonic acid biosynthesis, such as lipoxygenase and allene oxidase, and ethylene production, which trigger specific responses against pathogen attack (Song et al. 2014).

This study showed that establishment of *B. fimbriata* in heavy metal-polluted soil requires compatible orchid mycorrhizal fungi adapted to specific soil microsites, which influence exudation of specific organic acids. The pollution induces proteomic changes in mycorrhizal and non-mycorrhizal root segments that allow the establishment of orchids at this heavy metal-polluted soil.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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