
EFFECTS OF METAL IONS (Cd^{2+} , Cu^{2+} , Zn^{2+}) ON THE GROWTH AND CHELATING-COMPOUND PRODUCTION OF THREE ECTOMYCORRHIZAL FUNGI

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SUMMARY

Several studies have shown that ecotypes of ectomycorrhizal fungi from metal-contaminated sites present a high metal tolerance under *in vitro* conditions, but it is not clear whether fungi from non-contaminated sites can also develop ecotypes with high metal tolerance. Tolerance to Cd^{2+} , Cu^{2+} and Zn^{2+} ions, and production of chelating compounds as a detoxification mechanism were evaluated in ectomycorrhizal fungi collected from three uncontaminated sites (SIS, SRS and ESC). The fungi were grown in solid medium with 5 and $20\mu\text{mol}\cdot\text{l}^{-1}$ Cd, 0.1 and $1\text{mmol}\cdot\text{l}^{-1}$ Cu, and 1 and $10\text{mmol}\cdot\text{l}^{-1}$ Zn, and the tolerance index was determined. The metal-chelating compounds were determined by Chrome Azurol S (CAS) assay, and the chemical nature (hydroxamate or catecholate) of the compounds was analyzed. There was a clear

inter- and intraspecific variation in the fungal responses at low and high metal concentrations. Some ecotypes of *Rhizopogon roseolus* and *Suillus luteus* were the most tolerant at 1mM Cu and 10mM Zn. *S. luteus* SRS showed tolerance to the three metals. The addition of Cu and Cd stimulated CAS-detected metal-chelating compounds and dark pigmentation production in all isolates. In the presence of Cd the lowest pH values of the culture media were detected. Hydroxamates and catecholates were detected only in some isolates, and the catecholates were stimulated by Cd in *S. luteus* and *S. bellinii*. Among fungi collected from uncontaminated sites it was possible to find ecotypes tolerant to high metal concentrations and also producers of siderophore-type chelating compounds.

Studies have demonstrated the importance of ectomycorrhizal fungi for the plant species with which they form symbiotic associations, since under certain conditions these fungi may develop ecotypes with tolerance or resistance to high metal concentrations and thus can protect their host plants when they grow in metal contaminated soils (Hartley *et al.*, 1997; Blau-

dez *et al.*, 2000a; Jentschke and Goldbold, 2000; Urban, 2011). Metal-tolerant ecotypes may play a crucial role in the establishment of mycorrhizal tree seedlings in contaminated soils for the restoration or remediation of industrial and metalliferous sites (Goldbold *et al.*, 1998; Schützendübel and Polle, 2002; Urban 2011). Nevertheless, when the selection of fungal ecotypes is done for the aforementioned applications, it is very im-

portant that the screening of native ecotypes of ectomycorrhizal fungi be site/host specific, ecologically adapted to particular tree species and edaphoclimatic conditions of the region where the mycorrhizal seedlings will be introduced (Ray *et al.*, 2005; Carrillo-González and González-Chávez, 2012).

The mechanisms of metal tolerance described in fungi include extracellular chelation through organic com-

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pounds released by the fungal hyphae, such as organic acids and siderophores. These metal-chelating compounds play a very important role in metal acquisition by plants, fungi and bacteria from their environments (Guerinot, 1994; Winkelmann, 2002) and their participation in processes of metal detoxification has been also demonstrated (White *et al.*, 1997; Jentschke and Godbold, 2000; Machuca, 2011). Organic acids such as oxalic and citric acids are excreted by mycorrhizal fungi or plant roots into the rhizosphere, where they may both mobilize metal ions from insoluble sources or immobilize them through precipitation (Meharg, 2003; Bellion *et al.*, 2006). Siderophores are highly specific ferric iron (Fe³⁺) chelators produced by many fungi and bacteria in response to Fe deficiency, but they are also capable of forming stable complexes with many other metal ions in addition to Fe. These compounds can be classed into two major groups according to their chemical structure: hydroxamates and catecholates (Hider, 1984; Renshaw *et al.*, 2002). Although siderophores have been associated with metal detoxification and tolerance in bacteria and fungi (Rogers *et al.*, 2001; Illmer and Buttinger, 2006; Braud *et al.*, 2010; Rajkumar *et al.* 2010), production of siderophores by ectomycorrhizal fungi growing in the presence of metal ions other than Fe and their possible relation with the tolerance has not been described in the literature to date.

Several studies have shown that fungal ecotypes from contaminated sites present a higher metal tolerance than isolates from non-contaminated sites, and in some cases the tolerance displayed by the ecotypes is metal-specific to the site (Adriaensen *et al.*, 2005; Colpaert, 2008; Colpaert *et al.*, 2011; Urban, 2011). However, in many cases metal-sensitive ecotypes have also been isolated from contaminated sites and it is not clear whether fungi found in uncontaminated sites can also present tolerant ecotypes under *in vitro* conditions. The aim of this work was to evaluate the effect of cadmium (Cd²⁺), copper (Cu²⁺) and zinc (Zn²⁺) ions on the rate of growth and metal-chelating compound production in solid medium by ecotypes of ectomycorrhizal fungi collected from different uncontaminated soils. The ectomycorrhizal fungi *Rhizopogon roseolus*, *Suillus luteus* and *Suillus bellinii* used in this study were isolated from fruiting bodies collected from *Pinus radiata* plantations in different sites in the central-southern region of Chile. These fungal species were chosen as they are found with high frequency in young pine forests and primary plantations as pioneer species. The selection of metal-tolerant ecotypes and efficient producers of metal-chelating compounds will also allow these

TABLE I
LOCATION AND SOIL CHARACTERISTICS OF THE ECTOMYCORRHIZAL FUNGI COLLECTION SITES

Origin Sites	Characteristics	
	Metal content* (mg·kg ⁻¹ dry soil)	Isolates
San Isidro (SIS) Bio Bio Province 36°55'0"S; 72°0'21"O	Sandy loam soil, pH 5.9 Fe: 9.4, Zn: 0.1, Cu: 0.2	<i>Rhizopogon roseolus</i> SIS <i>Suillus luteus</i> SIS <i>Suillus bellinii</i> SIS
Santa Rosa (SRS) Bio Bio Province 37°14'19"S; 72°24'46"O	Sandy soil, pH 6.1 Fe: 4.8, Zn: 0.2, Cu: 0.1	<i>Rhizopogon roseolus</i> SRS <i>Suillus luteus</i> SRS <i>Suillus bellinii</i> SRS
Escuadron (ESC)** Concepcion Province 73°09'12.76"S; 36°58'55.21"O	Clay soil, pH 4.1 Fe: 27.2, Zn: 1.1, Cu: 4.1	<i>Rhizopogon roseolus</i> ESC <i>Suillus luteus</i> ESC

*Metal content as total concentration. **During the period when the fruiting bodies were collected, the *S. bellinii* species was not found at the ESC site.

ecotypes to be applied in future investigations on the mycorrhization of forest species, assessing the effect of the fungi when the plants are introduced in disturbed soils, poor in essential nutrients and/or contaminated with metals.

Material and Methods

Fungal isolates and collection sites

Fruiting bodies of ectomycorrhizal fungi *Rhizopogon roseolus* Fr., *Suillus bellinii* (Inz.) Kuntze and *Suillus luteus* L. Gray associated with young *Pinus radiata* D. Don plantations were collected from three forest sites located in the central-southern region of Chile, where the soils had different chemical and physical characteristics (Table I). Pure cultures of different isolates were obtained from carpophores and identified at the Fungi Biotechnology Laboratory, Universidad de Concepcion, Chile. The stock of fungal cultures was maintained and cultivated regularly on plates containing solid modified Melin-Norkrans(MMN) medium with pH adjusted to 5.8 (Marx, 1969) and incubated in the dark at 24 ±1°C for 25-30 days.

Effects of metal ions on fungal growth

Petri dishes (90mm diameter), containing 20ml of MMN medium, pH adjusted to 5.8 before autoclaving, were prepared with and without the addition of Cd²⁺, Cu²⁺ and Zn²⁺ ions, which were added as sulphate salts to the medium to give final concentrations of 5 and 20µmol·l⁻¹ Cd; 0.1 and 1mmol·l⁻¹ Cu; and 1 and 10mmol·l⁻¹ Zn. The dishes were inoculated in the centre with a mycelium-agar disk (5mm diameter) obtained from the stock plates and incubated at 24 ±1°C in the dark. The growth of the colonies was evaluated at regular intervals for 40 days and the results were expressed as average radial growth rate in mm/day (Harnett,

1987; Santiago-Martínez *et al.*, 2003). Each fungal isolate was inoculated in triplicate, with and without metal, and the means of the values reported. The tolerance of each isolate was expressed as the Tolerance Index (% TI) calculated compared to the growth of control culture (without metal). Some morphological changes in the fungal colonies were visually evaluated during the incubation period. At the end of the incubation period the changes in pH were evaluated in the solid media, with and without metal ions, using a surface pH electrode.

Detection of metal-chelating compounds in fungal extracts

At the end of the incubation period (40 days), the content of each dish, including mycelium plus MMN medium, with and without metals (Cd, Cu or Zn), was extracted with 20ml of deionised cold water for 20min under agitation. The extracts were filtered through a 0.45µm Millipore membrane and the presence of metal-chelating compounds in the filtrates was determined. To do this, 1ml of the filtered fungal extract was mixed with 1ml of Chrome Azurol S (CAS; Schwyn and Neilands 1987). This assay is based on the ability of siderophore-type metal-chelating compounds to bind and remove Fe³⁺ present in the CAS reagent, producing a change in the reagent color from blue to orange or pink. After 1h of incubation at room temperature, the absorbance of the mixture at 630nm (A₆₃₀) was measured. A reference was prepared with extracts obtained from dishes containing non-inoculated MMN solid medium (with and without metals). The percent metal-chelating compound units in the extracts were calculated by subtracting the sample (As) from the reference (Ar), so that %A₆₃₀ metal-chelating compound units=(Ar-As/Ar)×100 (Payne, 1994; Machuca and Milagres, 2003).

The presence of the hydroxamate and catecholate ligands in the filtrate extracts was determined by the colorimetric Csáky and Arnow assays, respectively. The Csáky assay (Csáky, 1948) consisted of the digestion of samples containing hydroxamic acids in sulphuric acid to detect bound hydroxylamine. A pink color indicates a positive reaction for the presence of hydroxamate structures and the absorbance of the solution was measured at 526nm. In the Arnow assay (Arnow, 1937), catecholate structures give a yellow color when they react with nitrite-molybdate in acid medium and change to an intense orange-red when the medium is made strongly basic. The solution has a maximum absorbance at 510nm. Hydroxylamine hydrochloride and 2,3-dihydroxybenzoic acid (2,3-DHBA) were used as standards for the calibration curves of the Csáky and Arnow assays, respectively, and results expressed as $\mu\text{moles}\cdot\text{l}^{-1}$.

Statistical analysis

A one-way ANOVA ($p < 0.05$) was applied to determine the differences between growth of the control (without metal) and metal treatment for each isolate. To compare the growth of the isolates from the same species at two different metal concentrations, a factorial design was used (ANOVA, $p < 0.05$) and the statistical differences between these treatments were identified using Tukey's multiple comparison test. All statistical analyses were performed with three replicates, using STATISTICA software (v. 6.0).

Results

Effects of metal ions on fungal growth

When the isolates of ectomycorrhizal fungi grew in solid MMN medium without metal ions (control cultures) different radial growth rates were observed. *S. luteus* ESC showed the highest growth rate, followed by the three isolates of *R. roseolus* (Table II). In the presence of metal ions differential responses in the growth rate and tolerance index (TI) were observed, depending on the metal ion and its concentration, and on the species/isolate (Table II). Two concentrations of Cd significantly inhibited the radial growth rate of all isolates compared to the controls, mainly at $20\mu\text{mol}\cdot\text{l}^{-1}$, and the TI were lower than those obtained with Cu and Zn. *S. luteus* ESC and *S. bellinii* SRS were the most sensitive to Cd, whereas *S. luteus* SRS displayed the highest TI at $20\mu\text{mol}\cdot\text{l}^{-1}$ (Table II). At a low Cu concentration ($0.1\text{mmol}\cdot\text{l}^{-1}$) the growth

TABLE II
RADIAL GROWTH RATE (MM/DAY) OF ECTOMYCORRHIZAL FUNGI GROWING IN SOLID MMN MEDIUM AT TWO METAL CONCENTRATIONS

	Control	Cd ²⁺ ($\mu\text{mol}\cdot\text{l}^{-1}$)		Cu ²⁺ ($\text{mmol}\cdot\text{l}^{-1}$)		Zn ²⁺ ($\text{mmol}\cdot\text{l}^{-1}$)		
		5	20	0.1	1	1	10	
<i>R. roseolus</i>								
SIS	0.80	0.46a* (58)	0.15bc*(19)	0.48b*(60)	0.00d*	0.70a (87)	0.00b*	
SRS	0.79	0.39a* (49)	0.12c* (15)	0.37c*(47)	0.00d*	0.58a (73)	0.00b*	
ESC	0.76	0.23b* (30)	0.15bc*(20)	0.64a (84)	0.39c*(51)	0.69a (91)	0.00b*	
<i>S. luteus</i>								
SIS	0.59	0.26a*(44)	0.12b*(20)	0.59b(100)	0.00e*	0.72b(122)	0.75a*(127)	
SRS	0.45	0.25a*(56)	0.23a*(51)	0.48c(107)	0.40c*(89)	0.61c*(136)	0.63c*(140)	
ESC	1.59	0.20a*(13)	0.21a*(13)	0.71a*(45)	0.31d*(19)	0.85a*(53)	0.00d*	
<i>S. bellinii</i>								
SIS	0.38	0.13a*(34)	0.07b*(18)	0.52a*(137)	0.00c*	0.49b(129)	0.00c*	
SRS	0.58	0.04b*(7)	0.04b*(7)	0.36b*(62)	0.00c*	0.63a (109)	0.00c*	

Values of growth rate followed by (*) are significantly different ($p < 0.05$) compared to the control (without metal) for each isolate.

^{a-c}At different concentrations of each metal, for isolates from each species, values followed by the same letter are not significantly different ($p < 0.05$). Values in parentheses corresponding to Tolerance Index (%) calculated compared to the control. Values are the means of three replicates \pm standard errors.

rate of some isolates were significantly reduced, while in others the growth was not affected and in *S. bellinii* SIS the growth was significantly stimulated (TI>100%). However, when the Cu was increased to $1\text{mmol}\cdot\text{l}^{-1}$, the growth of most isolates was completely inhibited and only *R. roseolus* ESC and *S. luteus* SRS and ESC showed tolerance. In no case was the growth of *S. luteus* SRS affected, showing at $1\text{mmol}\cdot\text{l}^{-1}$ the highest TI (89%) among all the isolates (Table II). Unlike Cd and Cu, at the lowest Zn concentration ($1\text{mmol}\cdot\text{l}^{-1}$) all the isolates displayed tolerance to the metal (TI>70%), except *S. luteus* ESC (TI 53%). However, when the concentration increased to $10\text{mmol}\cdot\text{l}^{-1}$ the radial growth rate of most of the isolates was completely inhibited and only *S. luteus* SIS and SRS showed tolerance (TI>00%). The radial growth rate of these isolates was significantly higher in the presence of 1 and $10\text{mmol}\cdot\text{l}^{-1}$ Zn than in its absence (Table II).

The metal ions caused modifications in some morphological characteristics of the mycelia of all the isolates (data not shown). The isolates that grew at the highest Cu and Zn concentrations displayed abundant aerial mycelium and thick mycelial cord formation. In addition, a clear influence of the metal ions on the dark pigmentation production was also observed at the highest Cd and Cu concentrations in all the isolates. Nevertheless, a very marked difference was observed between the species *R. roseolus*, which produced pigmented mycelium, and *S. luteus* and *S. bellinii*, which secreted the pigments to the medium, producing an intense dark coloration on the agar, more so in the presence of Cd. All fungal isolates decreased the pH of the medium to a range from 4 to 4.7 compared with the initial pH 5.8 of the MMN medi-

um, when grown in the absence of metal ions. In the presence of Cd the pH values were lower than those obtained with Zn and Cu, attaining values in the range 2.9-3.8, independently of the species/isolate and Cd concentration. No pH changes were detected compared to the controls in the presence of Cu or Zn, except for *S. luteus* ESC, which decreased the pH to 3 in the presence of both metal ions (Table III).

Effect of metal ions on metal-chelating compound production

All extracts from fungal cultures displayed a positive CAS reaction, but the percentages of metal-chelating compound units were different depending on species/isolates and type and concentration of metal ion. The color changes produced during the CAS assay were also different among species, and whereas the extracts of *R. roseolus* produced a change from blue to purple, with the *S. luteus* and *S. bellinii* extracts these changes were from blue to orange-reddish. In the absence of metal, the presence of the metal-chelating compounds was detected in all the extracts, with the highest percentage of metal-chelating compounds (highest CAS reaction) produced by the SIS isolates of *R. roseolus* and *S. bellinii* (Figures 1a and c). In the presence of metal ions *R. roseolus* and *S. luteus* displayed different CAS reactions, while *S. bellinii* showed a reduction in the metal-chelating compounds (Figure 1c). The presence of Cu, but not Cd or Zn, stimulated the production of the metal-chelating compounds in *R. roseolus* SIS and SRS, and in the ESC isolate the three metal ions produced a reduction in these compounds (Figure 1a). Similarly, the production of compounds by *S. luteus* ESC also was reduced by metal

TABLE III
CHANGES IN THE PH OF SOLID MMN MEDIUM DURING THE GROWTH OF ECTOMYCORRHIZAL FUNGI AT TWO METAL CONCENTRATIONS

	Control	Cd ²⁺ (μmol·L ⁻¹)		Cu ²⁺ (mmol·L ⁻¹)		Zn ²⁺ (mmol·L ⁻¹)	
	0	5	20	0.1	1	1	10
<i>R. roseolus</i> SIS	4.0 ±0.6	3.4 ±0.1	3.6 ±0.2	4.2 ±0.0	n.d.	4.2 ±0.1	n.d.
<i>R. roseolus</i> SRS	4.3 ±0.2	3.8 ±0.0	3.4 ±0.1	4.2 ±0.1	n.d.	4.0 ±0.2	n.d.
<i>R. roseolus</i> ESC	4.2 ±0.4	3.4 ±0.0	3.5 ±0.1	4.8 ±0.1	4.1 ±0.1	4.1 ±0.1	n.d.
<i>S. luteus</i> SIS	4.0 ±0.1	3.2 ±0.1	3.4 ±0.0	4.5 ±0.1	n.d.	4.2 ±0.1	5.4 ±0.1
<i>S. luteus</i> SRS	4.0 ±0.2	3.2 ±0.1	3.1 ±0.0	4.6 ±0.2	4.0 ±0.1	4.0 ±0.1	5.3 ±0.0
<i>S. luteus</i> ESC	4.5 ±0.2	3.2 ±0.1	3.2 ±0.0	3.2 ±0.2	3.0 ±0.1	3.2 ±0.1	n.d.
<i>S. bellinii</i> SIS	4.7 ±0.4	3.0 ±0.1	3.1 ±0.0	4.2 ±0.1	n.d.	4.8 ±0.2	n.d.
<i>S. bellinii</i> SRS	4.1 ±0.1	3.0 ±0.0	2.9 ±0.1	4.0 ±0.0	n.d.	3.9 ±0.1	n.d.

Initial pH of solid MMN medium was adjusted to 5.8 before autoclaving. Values are the means of three replicates ± standard errors.

n.d.: not determined because these isolates were not grown under these conditions.

ions, mainly by Cu, which was responsible for a complete absence of CAS reaction. In the SIS and SRS isolates the addition of Cd and Cu respectively, but not Zn, stimulated the production of metal-chelating compounds (Figure 1b). Those isolates that grew at 1mmol·l⁻¹ Cu and 10mmol·l⁻¹ Zn presented no CAS reaction.

The hydroxamate and catechol structures were also determined in the fungal extracts that showed a positive CAS reaction. Independently of species/isolate and of the presence or absence of metal

ions, the catechol concentrations detected by the Arnow assay were higher than hydroxamates detected by the Csáky assay. In the absence of metal, only the *S. luteus* SRS and *S. bellinii* extracts displayed the presence of both type of structures (Table IV). The addition of metal ions stimulated both catechol and hydroxamate production, but it was dependent on species/isolate and the type and concentration of metal. High catechol concentrations were detected in *S. bellinii* in the presence of the three metal ions, but only Cd stimulated the catechol

production compared to the control. In addition, the catechol production by *R. roseolus* and *S. luteus* was also stimulated by Cd and Cu, respectively, but not by Zn. The hydroxamates were detected in *R. roseolus* in the presence of Cu and Zn, and in the presence of the three metal ions in the case of *S. bellinii*. However, in *S. luteus* grown in the presence of the three metal ions hydroxamate structures were not detected (Table IV).

Discussion

Some studies have demonstrated that species of ectomycorrhizal fungi that grow on sites strongly contaminated with metal ions can exhibit high tolerance under laboratory controlled conditions in the absence of mycorrhizal association (Hartley *et al.* 1997; Meharg, 2003; Colpaert *et al.*, 2004, 2011). Nevertheless, it has not always been possible to establish a direct relationship between the presence and concentration of metal in the soils and the tolerance that fungal species collected from those sites can develop *in vitro*. This study demonstrated that the ectomycorrhizal species *R. roseolus*, *S. luteus* and *S. bellinii* associated with pine plantations from three different uncontaminated sites, presented metal tolerant isolates when cultivated *in vi-*

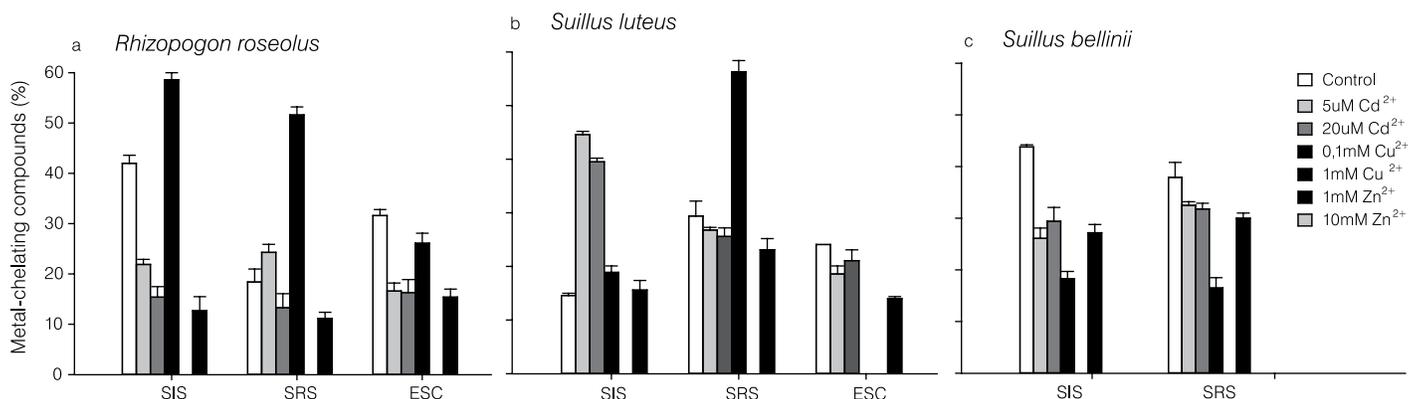


Figure 1. Metal-chelating compounds (%) siderophore-type detected by CAS assay in the extracts of cultures of ectomycorrhizal fungi isolates growing in solid MMN medium at different metal concentrations. SIS, SRS and ESC correspond to the different fungi collection sites (see Table I). Values are the means of three replicates ± standard errors.

TABLE IV
DETECTION OF CATECHOLATE AND HYDROXAMATE STRUCTURES (mmol·l⁻¹) IN EXTRACTS OF ECTOMYCORRHIZAL FUNGI GROWING IN SOLID MMN MEDIUM AT TWO METAL CONCENTRATIONS

	Cd ²⁺ (μmol·l ⁻¹)				Cu ²⁺ (mmol·l ⁻¹)		Zn ²⁺ (mmol·l ⁻¹)			
	Control		5		20		0.1		1	
	Catechol	- Hydroxam	Catechol	- Hydroxam	Catechol	- Hydroxam	Catechol	- Hydroxam	Catechol	- Hydroxam
<i>R. roseolus</i> SIS	0.0	0.0	12.3 ±0.1	0.0	3.6 ±0.1	0.0	0.0	5.3 ±0.1	0.0	5.8 ±0.2
<i>R. roseolus</i> SRS	0.0	0.0	0.0	0.0	4.5 ±0.0	0.0	0.0	8.9 ±0.2	0.0	4.3 ±0.2
<i>R. roseolus</i> ESC	3.7 ±0.1	0.0	7.5 ±0.2	0.0	19.6 ±0.3	0.0	0.0	5.6 ±0.0	0.0	6.5 ±1.0
<i>S. luteus</i> SIS	0.0	0.0	10.5 ±0.2	0.0	34.5 ±0.5	0.0	12.8 ±0.3	0.0	0.0	0.0
<i>S. luteus</i> SRS	26.7 ±0.4	4.0 ±0.1	46.7 ±2.2	0.0	71.2 ±3.3	0.0	30.0 ±1.2	0.0	0.0	0.0
<i>S. luteus</i> ESC	0.0	5.6 ±0.1	32.5 ±1.0	0.0	22.0 ±2.0	0.0	15.8 ±1.0	0.0	0.0	0.0
<i>S. bellinii</i> SIS	98.7 ±1.2	2.8 ±0.0	287.0 ±4.1	0.0	320.0 ±3.1	0.0	89.0 ±2.7	0.0	85.6 ±2.0	3.7 ±0.2
<i>S. bellinii</i> SRS	118.5 ±3.2	4.9 ±0.1	344.9 ±3.8	2.6 ±0.1	283.0 ±2.1	4.5 ±0.8	54.0 ±2.4	3.9 ±0.5	64.5 ±1.1	4.9 ±0.3

Catechol were detected by Arnow test; hydroxamate were detected by Csáky test. Values are the means of three replicates ± standard errors.

tro in solid MMN medium with Cd²⁺, Cu²⁺ and Zn²⁺ ions. The effect of each metal ion on the growth rate was evaluated at a low concentration and at another ten times higher, except Cd, a non-essential metal, which was increased only four times due to its high toxic potential on living organisms, even at very low concentrations (Trevors *et al.* 1986; Blaudez *et al.*, 2000b). This was evident when, in spite of the low concentrations of Cd used the TI values were lower than those obtained with Cu and Zn with all the isolates (Table II).

Several studies have demonstrated that fungal ecotypes from contaminated sites have a higher metal tolerance (TI) than isolates from non-contaminated sites, and in some cases the tolerance displayed by the ecotypes is metal-specific to the site. For instance, ecotypes of ectomycorrhizal fungi from Cu-contaminated sites showed tolerance *in vitro* to Cu but not to Zn (Colpaert *et al.*, 2004; Adriaensen *et al.*, 2005; Colpaert, 2008; Urban, 2011). On the other hand, the metal tolerance that a fungal species can display is dependent on the species and isolate or ecotype, as well as on the metal ion involved and its concentration (Gadd, 1993; Galli *et al.*, 1994). The soils of the forest sites from where the *R. roseolus*, *S. luteus* and *S. bellinii* isolates were collected presented low and similar levels of metals, except for the ESC site, which showed a slightly higher level of Fe, Zn and Cu (Cd contents were not determined) than the SIS and SRS sites, in addition to a texture and acidic pH different from the other sites (Table I). Although the collection sites chosen for this study were not contaminated with metal ions, some isolates were capable of growing and tolerating *in vitro* the concentrations of metals assayed, which were higher than those at their sites of origin. The *R. roseolus*, *S. luteus* and *S. bellinii* isolates displayed a different TI under the conditions assayed, depending on the type and concentration of the metal ions. The tolerance was evaluated by measuring the radial growth rate (mm/day) in solid medium, which is considered a good parameter for assessing the ability of fungal mycelia to explore or colonize soils. Among the ectomycorrhizal fungi there was a clear inter- and intra-specific variation in the responses at two concentrations of metals. According to the TI values found, *S. luteus* and *S. bellinii* were the most tolerant and sensitive species, respectively, principally at high concentrations of the three ions. Several studies carried out in axenic cultures have demonstrated interspecific variations among ectomycorrhizal fungi (Colpaert and Van Assche, 1987; Blaudez *et al.*, 2000a; Ray *et al.*, 2005). Blaudez *et al.* (2000a) observed that *S. luteus*, *S. variegatus* and *Pisolithus tinctorius* were more tolerant to Zn, Cu and

Cd than *Paxillus involutus* when grown in *in vitro* conditions, but the contrary was observed for Ni. On the other hand, an intra-specific variation has been also observed for Al among *P. tinctorius* strains (Egerton-Warburton and Griffin, 1995) and for Zn among *S. luteus* strains (Blaudez *et al.*, 2000a). In the present study, the three *R. roseolus* isolates displayed a similar sensitivity at high concentrations of metal ions, except the ESC isolate, which was the only one that grew at 1mmol·l⁻¹ Cu (51% TI). For *S. luteus*, the SIS and SRS isolates were the most tolerant at two Zn concentrations (>100% TI) and SRS showed the highest tolerance to Cd (51% TI) and Cu (89% TI) as well; however, in this species the isolate ESC was also found to be most sensitive to Cd, Cu and Zn, even though at the site of origin of this isolate the highest Cu and Zn concentrations were detected (Table I). By contrast, for *R. roseolus* ESC there seemed to be a relationship among the tolerance and the level of metal at its site of origin, at least for Cu.

Although the literature mentions that tolerance to one metal does not necessarily imply tolerance to other metals (Adriaensen *et al.*, 2005; Ray *et al.*, 2005; Colpaert, 2008), the present results show that *S. luteus* SRS from an uncontaminated site is remarkable among the various isolates studied because it showed a multiple metal tolerance for Cd, Cu and Zn in solid medium. This isolate displayed only a slight or no growth inhibition at the higher metal concentrations assayed, mainly in the presence of 10mmol·l⁻¹ Zn. This is an important characteristic if the fungal isolate is required to be applied in association with their host plant for reforestation of metal-contaminated sites, since in most cases a contaminated soil contains toxic levels of metal ion mixtures. Among the fungal species studied herein, *S. luteus* has been the most frequently reported in the literature with regard to metal tolerance. Populations of this specie from metal contaminated ecosystems have been studied for adaptive tolerance to Cu, Zn and Cd, as well as in regard to some mechanisms and genes implicated in their tolerance and the protective effect on seedlings exposed to metal (Colpaert *et al.*, 2004, 2011; Muller *et al.*, 2007; Krznanic *et al.*, 2009; Ruytinx *et al.*, 2011).

The changes observed in the pH of the media and in the pigmentation both of mycelia and media, mainly at high metal concentrations, may be related to different metal tolerance strategies developed by fungi (Gadd 1993; Jentschke and Goldbold, 2000; Martino *et al.*, 2000; Bellion *et al.*, 2006). The decrease in pH observed in all fungal cultures in the absence or presence of metal ions, mainly with Cd, is likely related to organic acid production.

It has been suggested that organic acids such as oxalic and citric acid can alleviate metal toxicity through immobilization, forming insoluble metal-complexes outside the cells of several organisms (Bellion *et al.*, 2006; Machuca, 2011). In a previous study, isolates of *S. luteus*, *R. luteolus* and *Sclerotium verrucosum* produced several organic acids (oxalic, citric, succinic and malonic) and metal siderophore type chelating-compounds when grown in liquid MMN in the absence and presence of low concentrations of Fe (35µmol·l⁻¹; Machuca *et al.*, 2007). On the other hand, depending on their concentration, oxalic and citric acids can react positively with CAS in the same form as siderophore-type iron-chelating compounds do (Oberegger *et al.*, 2001). Thus, the production of organic acids by ectomycorrhizal fungi could be contributing to the acidification of the media as well as to the positive CAS reaction.

Recently, the role of siderophores in the increase of bacterial metal tolerance has been highlighted, particularly in *Pseudomonas* ssp. (Rajkumar *et al.*, 2010; Schalk *et al.*, 2011; Cao *et al.*, 2012). Braudet *et al.* (2010) demonstrated that in *P. aeruginosa* the presence of siderophores reduced the toxicity of metals (Cu, Zn, Al and Pb among others) by extracellular chelation in media supplemented or not with Fe. Moreover, Cu at 100µmol·l⁻¹ markedly increased the siderophore production, but it was dependent on the type of siderophore. To date the stimulation or repression of the production of siderophores by many metals other than Fe has been extensively described for bacteria, but this issue has been scarcely studied in fungi such as ectomycorrhizal fungi (Illmer and Buttinger, 2006; Sinha and Mukherjee, 2008; Schalk *et al.*, 2011). In the present study, all the isolates of ectomycorrhizal fungi showed different levels of iron-chelating compounds as detected by the CAS assay, when grown in MMN solid medium supplemented with 18µmol·l⁻¹ Fe, in the absence or presence of Cd, Cu and Zn (Figure 1). Differences in the colors of positive CAS reaction were observed among *R. roseolus* and two *Suillus* species, which could be related to chelating compounds with different chemical structures. In the presence of metals the production of these compounds was reduced, stimulated or not affected, depending on the species/isolate. Zn reduced the production of chelating compounds for the three species and a reduction was also observed in *R. roseolus* in the presence of Cd, but not Cu, which increased these compounds in the SIS and SRS isolates. For *S. luteus*, Cd and Cu stimulated the production of these compounds in the SIS and SRS isolates, respectively. In contrast, in *S. bellinii* the three metal ions reduced the chelating compounds produc-

tion. Although it was not possible to establish any relationship between the metal tolerance and chelating compound production for the different species/isolates, it is important to note that the chelating compounds were determined at the end of the incubation period (40 days) and a peak of production may have occurred before that time. Of the three ectomycorrhizal fungi studied herein, only in *S. luteus* has the purification and characterization of hydroxamate siderophores been described when the fungus was grown in Fe-free liquid medium (Haselwandter *et al.*, 2011). Studies into Fe regulation and characterization are being conducted on our isolates to determine whether or not the chelating compounds with the ability to remove Fe³⁺ from the CAS reagent correspond to true siderophores.

The chemical determination of the chelating compounds present in the fungal extracts with positive CAS reaction showed the presence of hydroxamate and catecholate structures, depending on the species/isolate. Nevertheless, the concentrations of catecholates were always higher than hydroxamates. Although the literature indicates that fungi produce siderophores only of the class hydroxamates (Hider, 1984; Renshaw *et al.*, 2002), the high concentrations of catecholates detected in the extracts of ectomycorrhizal fungi by the Arnow assay may be related to the production of phenolic pigments mainly by *S. luteus* and *S. bellinii* in the presence of Cd. Phenolic pigments with the ability to chelate several metal ions, including iron, have been detected in the fruiting bodies and mycelia cultures of a great number of higher fungi from genera such as *Suillus*, *Rhizopogon* and *Pisolithus*, among others (Zhou and Liu, 2010; Velíšek and Cejpek, 2011). Thus, the production of pigments by ectomycorrhizal isolates could contribute to the positive reactions with the CAS and Arnow assays. During the growth of ectomycorrhizal fungi in the media containing toxic concentrations of metal ions, there is possibly a combination of different strategies used to alleviate the toxicity, depending on fungal isolate and the type of metal and its concentration. These strategies could include the production of organic acids, siderophores and pigments.

Copper mining and forestry activities are among Chile's main industries, generating extensive areas of disturbed soils, poor in mineral nutrients and/or contaminated with toxic concentrations of metal ions other than Cu. This is where native ecotypes of ectomycorrhizal fungi such as the *S. luteus* SRS and *R. roseolus* ESC isolates, both metal-tolerant and chelating-compound producers, display potential for future research about reforestation with *P. radiata*.

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EFEECTO DE IONES METÁLICOS (Cd²⁺, Cu²⁺, Zn²⁺) EN EL CRECIMIENTO Y PRODUCCIÓN DE COMPUESTOS QUELANTES DE METAL EN TRES HONGOS ECTOMICORRÍZICOS

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RESUMEN

Diversos estudios han demostrado que hongos aislados de sitios contaminados con metales presentan alta tolerancia *in vitro*, pero no está claro si hongos provenientes de sitios no contaminados también pueden presentar ecotipos tolerantes. La tolerancia a Cd²⁺, Cu²⁺ y Zn²⁺ y la producción de compuestos quelantes como posible mecanismo de detoxificación, fue evaluada en tres hongos ectomicorrízicos recolectados en tres sitios no contaminados (SIS, SRS y ESC). Los hongos fueron cultivados en medio sólido con 5 y 20 μmol·l⁻¹ Cd; 0,1 y 1 mmol·l⁻¹ Cu; y 1 y 10 mmol·l⁻¹ Zn, y se determinó el índice de tolerancia. Los compuestos quelantes fueron determinados con Chrome Azurol S (CAS), y su naturaleza química (hidroxamatos o catecolatos) fue analizada. Entre los hongos existió una clara variación inter e intraespecífica

en las respuestas a bajas y altas concentraciones de metales. Algunos ecotipos de *Rhizopogon roseolus* y *Suillus luteus* fueron los más tolerantes a 1 mM Cu y 10 mM Zn. *S. luteus* SRS presentó tolerancia a los tres metales. La adición de Cu y Cd estimuló la producción de compuestos quelantes detectados con CAS y la producción de pigmentos oscuros en todos los ecotipos. Los menores valores de pH de los medios de cultivo fueron detectados en presencia de Cd. Hidroxamatos y catecolatos fueron detectados sólo en algunos ecotipos, y los catecolatos fueron estimulados por Cd en *S. luteus* y *S. bellinii*. Entre los hongos recolectados en sitios no contaminados fue posible encontrar ecotipos tolerantes a altas concentraciones de metal y además productores de compuestos quelantes del tipo sideróforos.

EFEITO DE ÍONS METÁLICOS (Cd²⁺, Cu²⁺, Zn²⁺) NO CRESCIMENTO E PRODUÇÃO DE COMPOSTOS QUELANTES DE METAL EM TRÊS FUNGOS ECTOMICORRÍZICOS.

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RESUMO

Diversos estudos têm demonstrado que fungos isolados de locais contaminados com metais apresentam alta tolerância *in vitro*, mas não está claro se fungos provenientes de locais não contaminados também podem apresentar ecótipos tolerantes. A tolerância a Cd²⁺, Cu²⁺ e Zn²⁺ e a produção de compostos quelantes como possível mecanismo de detoxificação, foi avaliada em três fungos ectomicorrízicos recolhidos em três locais não contaminados (SIS, SRS e ESC). Os fungos foram cultivados em meio sólido com 5 e 20 μmol·l⁻¹ Cd; 0,1 e 1 mmol·l⁻¹ Cu; e 1 e 10 mmol·l⁻¹ Zn, e se determinou o índice de tolerância. Os compostos quelantes foram determinados com Cromo Azurol S (CAS), e sua natureza química (hidroxamatos ou catecolatos) foi analisada. Entre os fungos existiu uma clara variação inter e in-

traespecífica nas respostas em baixas e altas concentrações de metais. Alguns ecótipos de *Rhizopogon roseolus* e *Suillus luteus* foram os mais tolerantes a 1 mM Cu e 10 mM Zn. *S. luteus* SRS apresentou tolerância aos três metais. A adição de Cu e Cd estimulou a produção de compostos quelantes detectados com CAS e a produção de pigmentos escuros em todos os ecótipos. Os menores valores de pH dos meios de cultivo foram detectados em presença de Cd. Hidroxamatos e catecolatos foram detectados somente em alguns ecótipos, e os catecolatos foram estimulados por Cd em *S. luteus* e *S. bellinii*. Entre os fungos recolhidos em locais não contaminados foi possível encontrar ecótipos tolerantes a altas concentrações de metal e além disso produtores de compostos quelantes do tipo sideróforos.