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Tolerance to heavy metal stress in seedlings of three pine species from contrasting environmental conditions in Chile

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Tolerance to metal stress was studied in seedlings of three pine species (*Pinus radiata*, *P. pinaster* and *P. canariensis*) under controlled *ex vitro* conditions. Mature female cones were randomly collected at two sites in Chile (Llico and Huilquilemu) characterized by contrasting environmental conditions. One-year-old pine seedlings were immersed in trays with solutions of $CuSO_4$ (300 mM) or $AlCl_3$ (100 mM), and their survival, growth rate and decay symptoms were recorded for 60 days. Results showed large differences among provenances in seedling tolerance to $CuSO_4$ and $AlCl_3$ in terms of survival and growth. Multivariate analysis revealed a significant association ($p < 0.0001$) between the first canonical function and the following variables: provenance, species, metal stress and growing rate, as well as between the second canonical function and provenance, species, metal stress and symptomatology, indicating a high degree of genotype-environment interaction. Moreover, the activity of POX, SOD and CAT enzymes was determined 60 days after the beginning of the experiment in the Llico provenance seedlings. *P. pinaster* showed the highest activity level for all the enzymes considered, while *P. canariensis* and *P. radiata* had intermediate and lowest values, respectively. Differential gene expression among pine seedlings under metal stress with $CuSO_4$ for two genes (Cu-Zn-superoxide dismutase and RuBisCo) confirmed *P. pinaster* as the most tolerant species to $CuSO_4$ treatment. Our results are consistent with the hypothesis that abiotic stress in the maternal environment can induce a "transgenerational plasticity" which could affect progeny performances. The influence of different genetic backgrounds on the tolerance to heavy metals in pine seedlings is also discussed.

Keywords: ***Pinus*** **spp., Heavy Metals, Abiotic Stresses, Genotype-Environment Interaction**

Introduction

Soil pollution by heavy metals is a global environmental issue and a consequence of the escalation of mining and mineral processing, whose spread of metal residues contaminated large areas in Chile (Baize 2009, Parra et al. 2014). Metals accumulate in plant organs and tissues causing both physiological and biochemical changes, growth reduction and yield loss, particularly after persistent exposure (Wannaz et al. 2006, Mazen et al. 2010). An increase in reactive oxygen species (ROS) is a consequence of metal accumulation. Moreover, ROS are destructive if protective antioxidant mechanisms are not activated efficiently (Bray et al. 2000).

Copper (Cu) is an essential micronutrient with relevant physiological roles in plant growth and development. However, excessive Cu can be lethal to plants, causing a range of deleterious effects, such as the inhibition of photosynthesis and pigment synthesis, plasma membrane damage and other metabolic disturbances. The Cu concentration in plant leaves ranges from 5-30 $\mu g\ g^{-1}$ dry weight (DW). Furthermore, some plants have developed mechanisms for metal detoxification, including exclusion, compartmentalization and binding to organic ligands such as organic acids, amino acids, phytochelatins (PCs), and metallothioneins (Mts – Cobbett & Goldbrough 2002, Hall 2002). On the other hand, increasing areas of agricultural lands are acidic, where soil contains high quantities of aluminum (Al – Matsumoto 2000) which is highly toxic to plant growth. Several studies identified Al as a cause of ROS over-production and lipid peroxidation, which affect antioxidant enzyme activities in root tips (Simonovičová et al. 2004, Yadav & Mohanpuria 2009, Giannakoulas et al. 2010). Furthermore, aluminum-induced cell death has been observed after exposure to high levels of Al^{3+} (Boscolo et al. 2003, Simonovičová et al. 2004). Therefore, a strong connection between excess Al and oxidative stress has been proposed in plants (Boscolo et al. 2003, Yamamoto et al. 2003).

To survive oxidative damage, plants have developed non-enzymatic and enzymatic systems to regulate the intracellular levels of ROS (Apel & Hirt 2004, Sharma & Dubey 2007). The main non-enzymatic antioxidants are ascorbate (AsA) and glutathione (GSH), while the antioxidative enzymes are Superoxide dismutase (SOD, EC 1.15.1.1),

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Peroxidase (POD, EC1.11.1.7), Catalase (CAT, EC 1.11.1.6), AsA-GSH cycle-related ascorbate peroxidase (APX, EC 1.11.1.11), Monodehydro ascorbate reductase (MDHAR, EC 1.6.5.4), Dehydro ascorbate reductase (DHAR, EC 1.8.5.1), Glutathione peroxidase (GPX, EC 1.11.1.9) and Glutathione reductase (GR, EC 1.6.4.2 – Foyer & Noctor 2005). Higher activities of antioxidant enzymes facilitate the removal of excessive ROS and suppress lipid peroxidation, which could be beneficial for plant performance under Al stress (Apel & Hirt 2004, Mittler et al. 2004).

Current research on metal tolerance in plants is mainly focused to their adaptation to very restrictive environmental conditions. Our hypothesis was that transgenerational plasticity might influence progeny performance, resulting in a higher probability of selecting tolerant individuals. Thus, the aim of our work was to evaluate the tolerance of three *Pinus* spp. species, derived from mother plants selected in extreme agroforestry systems, to increasing concentrations of Cu and Al under controlled *ex vitro* conditions. Both biochemical and differential gene expression analyses were conducted to support the tolerance screening experiments. The final goal of thi study was the selection of Cu- and Al-tolerant *Pinus* spp. plants, suitable for greenhouse or trials under metal contamination.

Materials and methods

Plant material

Mature female cones from *P. radiata, P. pinaster* and *P. canariensis* were randomly collected from two contrasting localities: (a) the Forestry Reserve "Laguna Torca" (CONAF – *Corporación Forestal Nacional*, Chilean Forest Service) is a protected area located in Llico (34° 46′ S, 72° 6′ W), near to a marine environment with a mixture of sandy soil; (b) the Experimental Station of the Catholic University of Maule located in Huilquilemu (35° 27′ S, 71° 34′ W) in the Central Valley is characterized by arable and fertile soil. Both localities belong to the Maule Region, Chile, though they are geographically separated by the coastal mountain range.

For seed release, the cones were immersed in hot water (50 °C) for 2 min and then transferred to 40 °C for 48 h (dry treatment). Racks of 64 small pots containing a soil vermiculite mixture (1:1) were used for single-seed sowing. At the beginning of the spring season (August), seed germination was carried out in *ex vitro* controlled conditions.

Experimental treatments

One-year-old seedlings from *P. radiata, P. pinaster* and *P. canariensis* populations were selected for abiotic stress experiments with $CuSO_4$ and $AlCl_3$. Seedlings were planted in single plastic pots containing the vermiculite soil mixture. The roots of the pine plants were immersed in trays with solutions of increasing $CuSO_4$ concentrations (100 mM, 200 mM, 300 mM) or $AlCl_3$ (50 mM, 100 mM). The combinations of *Pinus* spp. genotypes (populations) with metal stress treatments resulted in a total of 15 experimental variants per site (Llico, Huilquilemu). Control treatments for the three pine species in each locality were immersed in distilled water. A total of 96 plants per treatment were placed in two replica trays (48 pots/tray) following a factorial block design. A total of 72 trays (60 for treatments, 12 for controls) were used in the experiment. During the beginning of the spring season (August-September) the experiment was conducted under *ex vitro* controlled conditions, while plantlets were totally covered with protective mesh (sunlight reduced by 50%). Both fresh weight (FW) and dry weight (DW) at T_0 (before the stress treatments) and T_F (after 60 days of stress treatments) were determined for needles (N), stem (S) and roots (R) of individual *Pinus* spp. seedlings. The number of dead plants (NDP), number of symptomatic plants (NSP) and the growing rate (GR) under treatments were estimated at 0, 15, 30, 45 and 60 days.

Samples for enzyme activity determination and DNA analysis

Approximately 1.0 g of needles (20 plants, two needles per plant) for each block (48 plants) of treatments, were pooled at 0, 15, 30, 45 and 60 days. Plants were randomly selected including both symptomatic and asymptomatic individuals. Two replicas per treatment were determined for the enzymatic activities. For RT-PCR analysis, a single sample per treatment was conducted pooling one needle of 40 randomly selected plants per treatment.

Peroxidase (POX) assay

POX activity was assessed according to Bania & Mahanta (2012). Briefly, 2 ml of phosphate buffer (pH 6.0/7.0), 100 µl of plant extract, and 1 ml of *O*-dianisidine solution were mixed. The reaction was initiated by adding 100 µl of 0.2 mM H_2O_2 and the absorbance was read at 460 nm at 30 second intervals for 5 minutes. Peroxidase activity was calculated using an extinction co-efficient of *O*-dianisidine and enzyme activity was expressed as unit per mg of protein.

Superoxide dismutase (SOD) assay

SOD activity was monitored according to the method from Roth & Gilbert (1984) and modified by Kumar et al. (2009). One ml of reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 100 mM EDTA, 20 ml enzyme extract, and 10 mM pyrogallol. Enzyme activity [U (mg protein)$^{-1}$] was calculated by monitoring the reaction mixture for 120 s (at 60 s intervals) at 420 nm in a spectrophotometer.

Catalase (CAT) assay

CAT activity was assayed by measuring the initial rate of H_2O_2 disappearance using the method of Beers & Sizer (1952) reported by Kumar et al. (2009). One ml of catalase assay reaction mixture contained 0.05 mM sodium phosphate buffer (pH 7.0), 20 ml enzyme extract and 1 mM H_2O_2. The decrease in H_2O_2 was followed by a decline in A_{240}, and the activity [U (mg protein)$^{-1}$] was calculated using a molar absorption coefficient of 40 mM^{-1} cm^{-1} for H_2O_2.

RT-PCR analysis

RT-PCRs were conducted according to Yang et al. (2010). RNA was extracted from the leaves (middle part) pooled from separate replicates. Extractions were performed using the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). One g of pooled, frozen leaf tissue was ground to a fine powder in the presence of liquid nitrogen, and then mixed with 10 ml TRIzol. RNA sample concentrations were quantified by determining the 260:280 and 260:230 nm ratios by spectrophotometry and further confirmed on a 1.2% agarose/0.4 M formaldehyde gel. Twenty ng of high quality RNA was added as template to each reaction using the Enhanced Avian HS RTPCR kit® (Sigma, St. Louis, MO, USA). For specific RT-PCR amplification, genes were selected and oligonucleotide primers were designed using sequences from the GenBank public databases (http://www.ncbi.nlm.nih.gov). Information on primer source, nucleotide sequence, and PCR annealing temperatures are listed in Tab. 1.

The PCR reaction mixture consisted of 3 µl of first strand cDNA, 5 µl of 10× PCR buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM each), 1 µl of each 10 µM primers (forward and reverse), 1 U of Taq DNA polymerase (Invitrogen), and ultrapure water (Sigma) to a final volume of 50 µl. PCRs were conducted with the following parameters: 94 °C for 3 min, 30 cycles of 94 °C for

Tab. 1 - Information of the selected genes analyzed by RT-PCR in *Pinus* spp.

No	Accession number	Protein/ Function	Primers (5'-3')	Tm (°C)	Size (bp)
1	AF434186	*P. pinaster* Cu-Zn-superoxide dismutase precursor	l - ATAGTTGCGGGTTCTGATGG r - ATGGGAGTGAGTCCAACCAC	59.96 59.82	203
2	AJ309096	*P. pinaster* ribulose-bisphosphate carboxylase (RuBisCo) small chain	l - AACAAGTGGGTGCCTTGTCT r - CACCCAGTATCTCCCATCGT	59.62 59.80	210

30 s, annealing at 59 °C for 30 s (Tab. 1), 72 °C for 1 min followed by a final incubation at 72 °C for 10 min. RT-PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

Statistical analysis

Using data recorded on day 60, the relationship between the independent variables (localities, species, metal stresses) and dependent variables (GR or growing rate, NSP or symptomatology, NDP or plant survival) were compared using non-parametric statistical tests, due to the nature of the data (categorical variables and non-normal distributions). The Kruskal-Wallis H test was used to test for differences among groups, while the Mann-Whitney U test for the pairwise comparison between groups. Additionally, data were investigated using Canonical Variant Analysis (CVA) as described by Cankaya et al. (2010), to determine the correlation between sets of variables from the same samples. According to Ter Braak (1986), CVA is an ordination technique which impose the extra restriction that the axes be linear combinations of environmental variables. Using this approach, the community variation can be directly associated with the environmental variation, and environmental variables may be quantitative or nominal (Coleman et al. 2008). The redundancy index is a measure of the variance of one set of variables predicted from the linear combination of the other set of variables. Pearson's correlation is used to analyze the linear relationship between two sets of data. Enzyme activities of POX, SOD and CAT during the experimental course were analyzed by ANOVA.

Results

Metal stress symptoms

One-year-old plants of *P. radiata, P. pinaster* and *P. canariensis* from the contrasting localities of Llico and Huilquilemu were stressed under increasing concentrations of $CuSO_4$ (100 mM, 200 mM, 300 mM) and $AlCl_3$ (50 mM, 100 mM). Tests were conducted under *ex vitro*, controlled conditions, where a protective mesh (25 m x 25 m) was used as a closed chamber to maintain the homogeneity of variable incidences associated with evapotranspiration, and to reduce possible spatial effects. After one week of stress treatments, phenotypic changes were consistently visible in the non-tolerant plants. Symptoms started from the basal part of the seedlings, and migrated to the upper fraction during the experiment. At the beginning of induced stress with either $CuSO_4$ or $AlCl_3$, the leaves were a pale yellow color, which after 60 days turned into a mixture of opaque gray and shades of red. Tolerant plants preserved the green color of leaves; however, after 60 days some of the leaves showed non-lethal symptoms in their basal and mid sections.

P. pinaster showed the best results under both 200 mM $CuSO_4$ and 300 mM $CuSO_4$ (Fig. 1), while both *P. pinaster and P. canariensis* showed tolerance to 100 mM $AlCl_3$

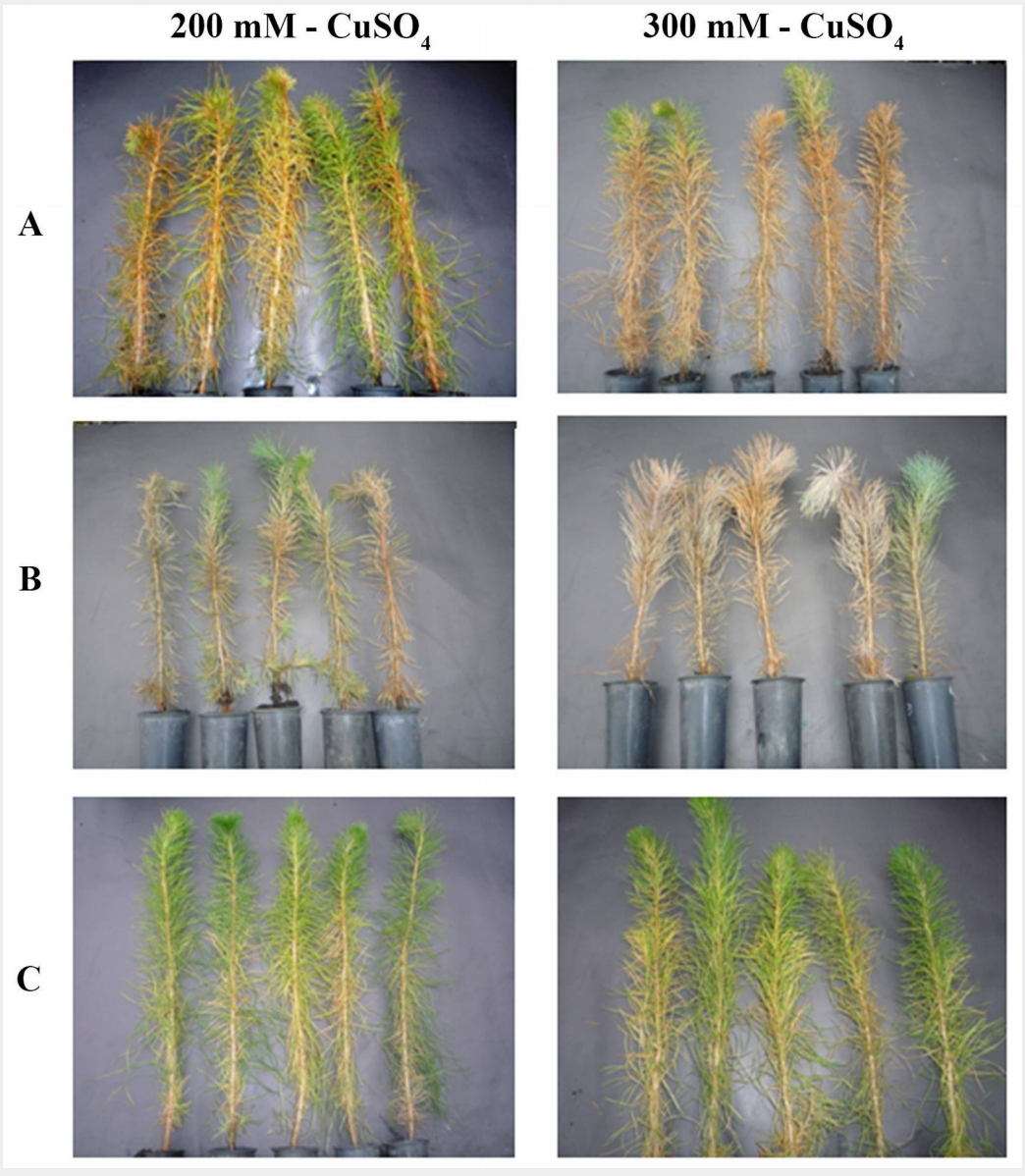

Fig. 1 - General symptoms of pine plants under abiotic stress from $CuSO_4$ treatment in controlled *ex vitro* conditions. (A): *P. radiata* ; (B): *P. canariensis*; (C): *P. pinaster*.

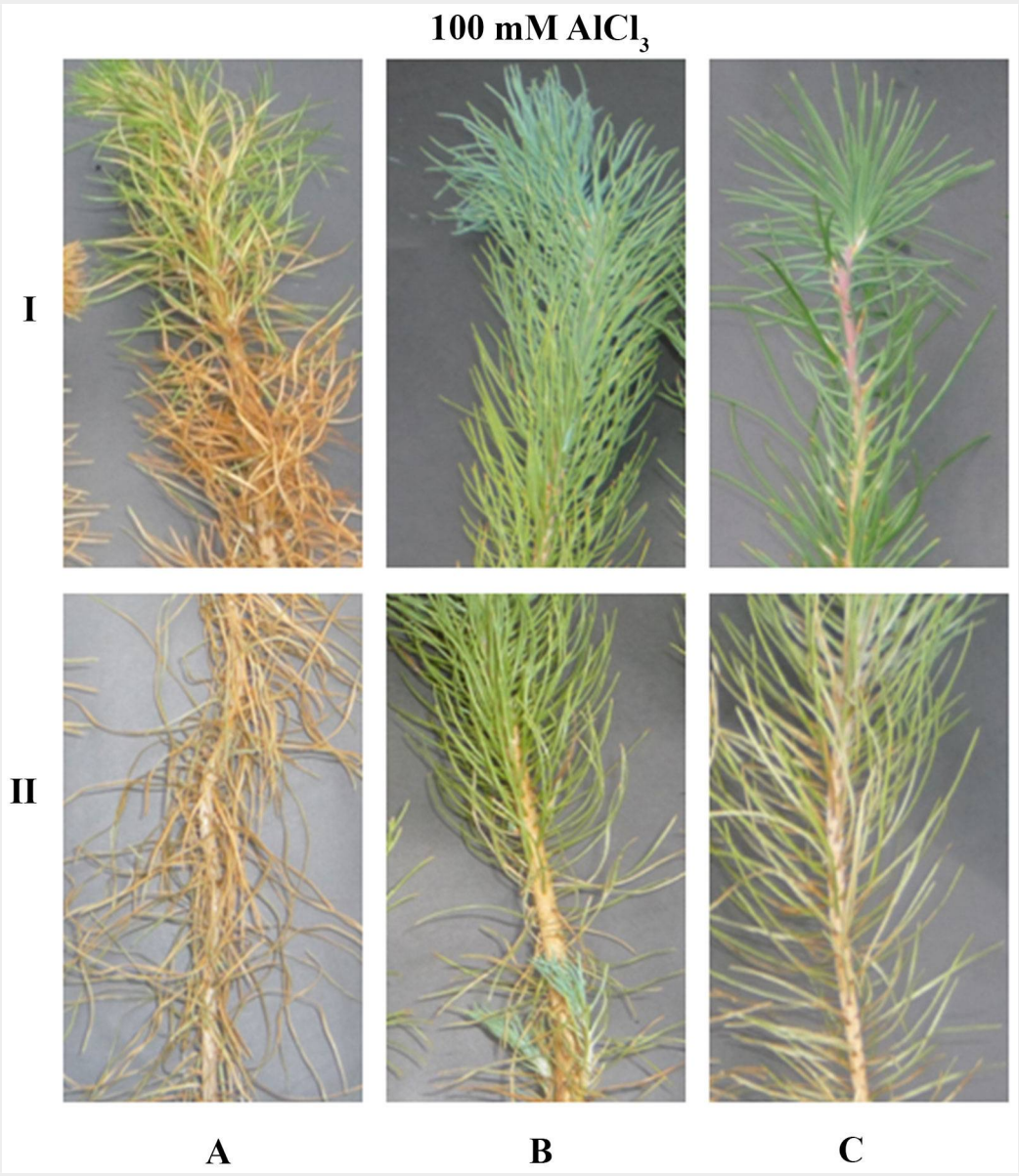

Fig. 2 - Details of symptoms in pine plants under $AlCl_3$ treatment in controlled *ex vitro* conditions. (A): *P. radiata*; (B): *P. canariensis*; (C): *P. pinaster*; (I): apical zone; (II): basal zone.

(Fig. 2). *P. radiata* had intense foliar damage, which evolved to plant death in a significant number of individuals. Contrastingly, control treatments (three pine species from each site) immersed in distilled water remained with an intense green color, indicating the optimal physiological quality of the starting materials and their expected behavior during the experimental time (data not shown).

Seedling growth and survival

The characterization of metal tolerance was estimated by the NDP and NSP after 60 days of stress. Considering the most consistent phenotypic contrast between the tolerant and non-tolerant plants, only seedlings grown under 300 mM $CuSO_4$ and 100 mM $AlCl_3$ treatments were selected for further experiments and data analysis.

Differences in the number of tolerant plants (NTP) were verified between *Pinus* spp., where *P. pinaster* displayed the highest NTP values (57.3% of individuals tolerant to $CuSO_4$ or $AlCl_3$). However, a remarkable influence of the seed provenance was detected (Tab. 2), being plantlets germinated from seeds collected in Llico more tolerant to metal stresses than those from Huilquilemu (87.4% *vs.* 12.6%, respectively).

Using exploratory univariate statistical

Tab. 2 - Tolerance to metal stresses of *Pinus* spp. seedlings from the provenances Llico and Huilquilemu after 60 days. (NSP): number of symptomatic plants; (NDP): number of dead plants; (NTP): number of tolerant plants.

Locality / metal stress / variable	Llico						Huilquilemu						Tolerant plants
	$CuSO_4$ (300 mM)			$AlCl_3$ (100 mM)			$CuSO_4$ (300 mM)			$AlCl_3$ (100 mM)			
	NSP	NDP	NTP	NSP	NDP	NTP	NSP	NDP	NTP	NSP	NDP	NTP	
P. radiata	96	76	20	96	63	33	96	91	5	96	92	4	62 (17.9%)
P. canariensis	96	80	16	58	36	60	96	89	7	96	93	3	86 (24.8%)
P. pinaster	84	6	90	57	12	84	96	82	14	96	85	11	199 (57.3%)
Tolerant plants/treatment	-	-	126	-	-	177	-	-	26	-	-	18	347 (100%)
Tolerant plants/locality	303 (87.4%)						44 (12.6%)						347 (100%)

analysis (Mann-Whitney U test), significant differences were found in symptomatology and plant survival in relation to the site and the pine species (Tab. 3). No significant differences in growth rate were detected between *P. radiata* and *P. canariensis*, while they both differed from *P. pinaster*, which had the highest values. Seedlings from Llico showed the highest growth rate and displayed significant differences in comparison with seedlings of mother plants from Huilquilemu.

A time course of the experiments with seedlings from the Llico provenance was estimated (Fig. 3). *P. pinaster* showed the best results with the lowest number of dead plants under either 300 mM $CuSO_4$ or 100 mM $AlCl_3$ treatments. Overall, the NSP was higher than the NDP, depending on the species and metal stress treatments.

Tab. 3 - Exploratory univariate analysis for different groups of variables. Different letters in the same column indicate significant differences between the means after Mann-Whitney U test ($p<0.05$).

Group	Variable	Mean		
		Growth rate (cm)	Symptomatology (scale 1 to 4)	Plant survival
Location	Llico	0.772 [a]	3.58 [a]	0.20 [a]
	Huilquilemu	0.241 [b]	3.86 [b]	0.06 [b]
Species	*P. radiata*	0.204 [b]	3.87 [c]	0.06 [c]
	P. canariensis	0.314 [b]	3.78 [b]	0.10 [b]
	P. pinaster	0.923 [a]	3.50 [a]	0.23 [a]
Metal stress	300mM $CuSO_4$	1.180	3.34	0.31
	100mM $AlCl_3$	1.241	3.26	0.35

Tab. 4 - Matrix of correlations between variables of groups 1 (locality, species and metal stress) and 2 (growth rate, symptomatology and plant survival). (***): $p<0.001$.

Response variables	Factors		
	Locality	Species	Metal stress
Growth rate	-0.181076***	0.231419***	0.275085***
Symptomatology	0.186185***	-0.196181***	-0.274277***
Plant survival	-0.211238***	0.202894***	0.295173***

Canonical variant analysis

Canonical variant analysis (CVA) was applied to test for correlations between experimental factors and response variables. To set the canonical functions, three independent variables (*i.e.*, locality, *Pinus* species and metal stress) were recognized as group 1 (genotype-environment interaction), while the three dependent variables (*i.e.*, growth rate, symptomatology and plant survival) were referred to as group 2 (tolerance to metal stress).

The Pearson's correlation analysis between the CVA dimensions of groups 1 and 2 revealed low though significant correlation coefficients (Tab. 4). The highest correlations were detected between metal stress and tolerance variables, with a maximum value of 0.295 between plant survival and metal stress.

Canonical functions reflect the correlations between latent variables in their possible combinations (Tab. 5). Since there were three variables as original criteria, only three canonical functions were extracted: (1) locality-species-metal stress-growing rate; (2) locality-species-metal stress-symptomatology; and (3) locality-species-metal stress-plant survival. Results indicated a significant ($p<0.0001$) association between these groups for functions 1 and 2, and a high degree of correlation ($r = 0.417$) for function 1.

As for function 1, the proportion of variance of the metal stress tolerance variables (set 2) explained by the genotype-environment interaction variables (set 1) was 0.173 ($R_{canonical} = 0.417$), while it was 0.020 ($R_{canonical} = 0.143$) for the canonical function 2 (Tab. 5).

The redundancy index is a measure of the ability of original variables (locality, species and metal stress) taken together, to explain the variation in the criterion variables (namely, growing rate, symptomatology and plant survival). Canonical correlation weights (*i.e.*, the impact of each variable on the canonical function) demonstrated that function 1 was mainly influenced by plant survival, metal stress, species and locality variables, while function 2 was mostly affected by the species, symptomatology and locality (Tab. 6).

Enzyme activity

In the case of seedlings from the Llico provenance, the enzyme activity of POX, SOD and CAT was determined after 60 days since the beginning of the experiment. For 300 mM $CuSO_4$ and 100 mM $AlCl_3$

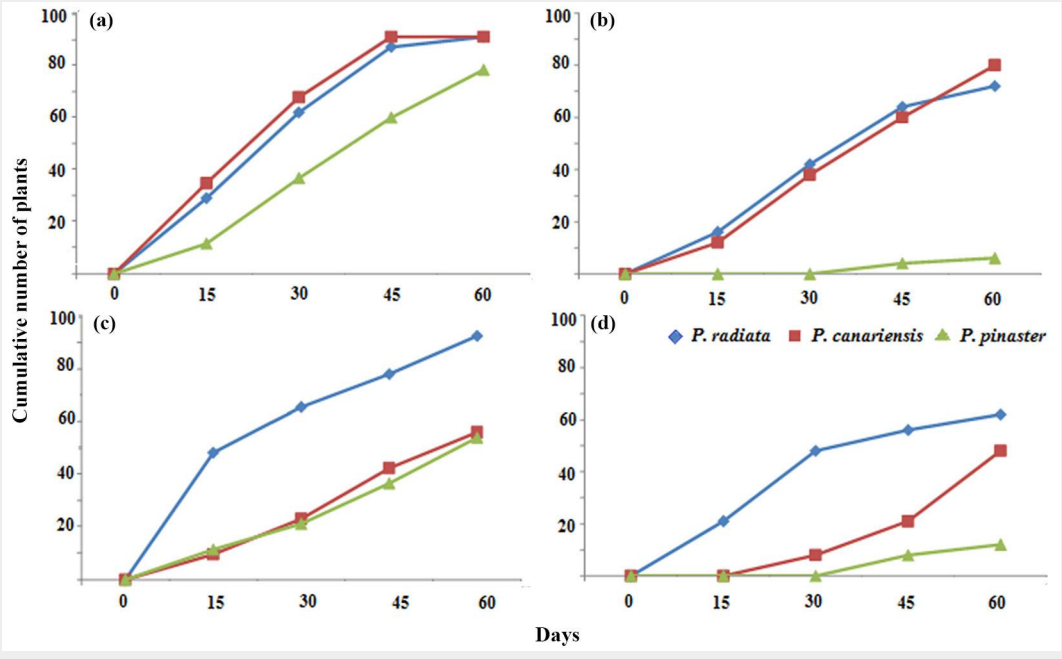

Fig. 3 - Tolerance of pine seedlings (Llico provenance) to $CuSO_4$ (a-b) and $AlCl_3$ (c-d) treatments under controlled conditions. (a) Number of symptomatic plants (NSP) at 300 mM $CuSO_4$; (b): number of dead plants (NDP) at 300 mM $CuSO_4$; (c): NSP at 100 mM $AlCl_3$; (d): NDP at 100 mM $AlCl_3$.

Tab. 5 - Significance and redundancy index of canonical functions.

Function	R canonical	Square	χ^2	dg	p-value	Prop. of variance	Redundancy
1	0.417079	0.17396	304.0054	9	<0.0001	0.923265	0.160606
2	0.142656	0.02035	29.8647	4	<0.0001	0.022106	0.000450
3	0.016064	0.00026	0.3702	1	0.5429	0.054629	0.000014

Tab. 6 - Correlations between canonical functions and the original variables.

Kind	Variables	Canonical functions		
		1	2	3
Factors	Locality	0.498843	0.50699	-0.702936
	Species	-0.505099	0.82943	0.238567
	Metal stress	-0.704108	-0.23454	-0.670241
Response variables	Growing rate	-0.245276	0.45572	0.843858
	Symptomatology	-0.224864	0.63288	0.198845
	Plant survival	-0.975096	-0.44654	0.149508

treatments, randomized samples were selected from the middle parts of five plants per treatment. For *P. pinaster* seedlings, POX activity increased in both treatments, while it decreased drastically in *P. canariensis* and *P. radiata*. In the case of SOD, enzyme activity was higher in *P. pinaster* than in *P. canariensis* and *P. radiata*. For all the three pine species, SOD activity in seedlings grown under $AlCl_3$ treatment increased during the first 30 days, whereas it severely decreased in *P. radiata* plants after 60 days (Tab. 7).

Additionally, CAT activity showed the highest values for *P. canariensis* after 15 (300 mM $CuSO_4$) and 30 days (100 mM $AlCl_3$); however, these enzymatic values decreased during the experiment. At the end of the experiments (60 days), CAT activity was highest in *P. pinaster* seedlings in both stress treatments. Overall, *P. pinaster* plants showed the highest level of enzyme activity, followed by *P. canariensis* (intermediate) and *P. radiata*, which showed the lowest enzymatic values.

RT-PCR analysis

Transcriptional activities of Cu-Zn-superoxide dismutase and Ribulose bisphosphate carboxylase (RuBisCo) were measured by semi-quantitative RT-PCRs of selected individuals. Differential gene expression analysis was conducted in pine plants treated with 300 mM $CuSO_4$. *P. radiata* showed transcript expression only at 15 days for Cu-Zn-superoxide dismutase and at 30 days in the case of RuBisCo (Fig. 4),

Tab. 7 - Time course of POX, SOD and CAT enzyme activities [in U (mg protein)$^{-1}$] in pine seedlings from the Llico provenance, treated with 300 mM $CuSO_4$ or 100 mM $AlCl_3$. Values are means ± standard deviation of three determinations per replica. Different letters in the same column indicate significant differences between the means after ANOVA ($p<0.05$).

Treatment	Enzyme	Species	T_0	15 Days	30 Days	45 Days	60 Days
300 mM $CuSO_4$	POX	*P. pinaster*	1.30 ± 0.51^a	1.48 ± 0.43^a	1.52 ± 0.62^a	1.53 ± 0.41^a	1.53 ± 0.45^a
		P. canariensis	0.81 ± 0.23^b	0.62 ± 0.13^b	0.42 ± 0.19^b	0.23 ± 0.11^b	0.08 ± 0.03^b
		P. radiata	0.48 ± 0.11^c	0.32 ± 0.09^c	0.25 ± 0.14^b	0.24 ± 0.15^b	0.07 ± 0.03^b
	SOD	*P. pinaster*	0.42 ± 0.12^a	0.44 ± 0.21^a	0.45 ± 0.11^a	0.45 ± 0.16^a	0.44 ± 0.15^a
		P. canariensis	0.43 ± 0.11^a	0.39 ± 0.16^a	0.35 ± 0.08^b	0.33 ± 0.09^b	0.25 ± 0.10^b
		P. radiata	0.35 ± 0.10^b	0.27 ± 0.13^b	0.20 ± 0.11^c	0.12 ± 0.04^c	0.09 ± 0.03^c
	CAT	*P. pinaster*	1.22 ± 0.34^a	1.21 ± 0.29^a	1.20 ± 0.21^a	1.20 ± 0.35^a	1.19 ± 0.31^a
		P. canariensis	1.25 ± 0.26^a	1.27 ± 0.31^a	1.21 ± 0.34^a	1.12 ± 0.22^a	1.05 ± 0.19^a
		P. radiata	1.24 ± 0.30^a	1.22 ± 0.36^a	1.11 ± 0.36^a	0.73 ± 0.11^b	0.62 ± 0.15^b
100 mM $AlCl_3$	POX	*P. pinaster*	0.72 ± 0.21^a	0.84 ± 0.17^a	0.85 ± 0.24^a	0.86 ± 0.29^a	0.86 ± 0.31^a
		P. canariensis	0.69 ± 0.17^a	0.65 ± 0.15^b	0.42 ± 0.19^b	0.25 ± 0.12^b	0.15 ± 0.06^b
		P. radiata	0.65 ± 0.20^a	0.49 ± 0.11^b	0.28 ± 0.14^b	0.22 ± 0.09^b	0.13 ± 0.05^b
	SOD	*P. pinaster*	0.31 ± 0.13^a	0.43 ± 0.14^a	0.42 ± 0.10^a	0.40 ± 0.18^a	0.39 ± 0.10^a
		P. canariensis	0.30 ± 0.11^a	0.35 ± 0.19^{ab}	0.34 ± 0.07^b	0.31 ± 0.08^b	0.26 ± 0.12^b
		P. radiata	0.26 ± 0.12^a	0.27 ± 0.16^b	0.21 ± 0.11^{bc}	0.18 ± 0.09^c	0.10 ± 0.03^c
	CAT	*P. pinaster*	0.85 ± 0.19^b	1.12 ± 0.22^a	1.09 ± 0.31^a	1.08 ± 0.21^a	1.07 ± 0.23^a
		P. canariensis	1.33 ± 0.19^a	1.32 ± 0.21^a	1.30 ± 0.27^a	0.75 ± 0.20^b	0.46 ± 0.16^b
		P. radiata	0.83 ± 0.22^b	0.67 ± 0.15^b	0.59 ± 0.18^b	0.38 ± 0.13^c	0.23 ± 0.09^c

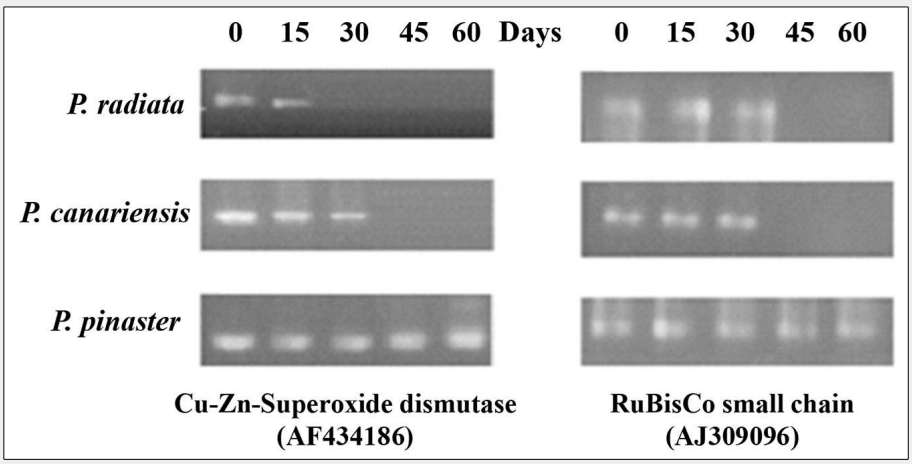

Fig. 4 - Differential gene expression in pine seedlings under stress treatment with 300 mM $CuSO_4$.

while *P. canariensis* seedlings showed gene expression in both cases at 30 days. Remarkably, consistent expression of the studied genes was demonstrated in *P. pinasters* during stress treatments (60 days). This result was in agreement with the lowest number of dead plants in this pine species.

Discussion

The present study investigated about the tolerance of *Pinus* spp. seedlings to abiotic stress ($CuSO_4$ and $AlCl_3$) in *ex vitro* controlled conditions. The interaction between different metal stresses was not considered here, because it is known that tolerances to Cu and Al are induced by separate genetic mechanisms (Cobbett & Goldbrough 2002, Simonovičová et al. 2004), and the probability of finding tolerant individuals to combined stress of $CuSO_4$/$AlCl_3$ would be very low. On the other hand, the highest concentration of salts (*i.e.*, 300 mM $CuSO_4$ and 100 mM $AlCl_3$) was applied for both the statistical and molecular analysis.

Our results showed that a higher number of tolerant seedlings originated from seeds collected from mother trees growing in a more severe environment. Indeed, the number of metal-tolerant seedlings obtained from the Llico provenance was largely higher (87.4%) than those from Huilquilemu (12.6%). Both these sites belong to the same region (Maule, Chile), but are separated by the coastal mountain range which determines highly contrasting environmental conditions between the two sites in terms of salt exposure, rain regime, soil type, etc. Moreover, Llico is a secular agroforestry system located along the coast, where pines were planted as protection barriers, while Huilquilemu is an experimental field where germplasm of different plant species is under maintenance and characterization.

The identification of the environmental factors underlying the higher metal tolerance of pine seedlings from the Llico provenance is out of the scope of this study. However, our results support the hypothesis that plant phenotype depends on genotype-environment interactions, which are specific for the area where they grow, but at the same time it could also be determined by the environment experienced by the parent trees (Boyko & Kovalchuk 2011). Indeed, the canonical variate analysis (CVA) carried out in this study seems to support the existence of a maternal effect on seedling tolerance to Cu and Al, independently from the species considered. CVA has also been used to study changes in phenolic concentrations in peach over time (Andreotti et al. 2008), as well the susceptibility to disease in monocot species and their effect on pathogen inoculum (Bithell et al. 2011).

Abiotic stress in the maternal environment has been reported to induce a "transgenerational plasticity" which could affect progeny performances (Herman & Sultan 2011). Trans-generational plastic responses to the maternal environment are transmitted to offspring phenotypes without any change in DNA (Donohue 2002). As a source of phenotypic variation, maternal environmental effects can influence the evolutionary process and the population dynamics of many plant species (Galloway 2005, Herman & Sultan 2011). Furthermore, increasing evidence suggests that transgenerational plasticity could be adaptive, enhancing offspring fitness under environments similar to that of the maternal environment (Galloway & Etterson 2007). It has also been proposed to exploit such maternal environmental effects to improve the performance of tree plantations by exposing mother trees to appropriate environmental conditions (Velasco-Conde et al. 2012). The maternal environment is known to influence many traits, such as seed traits (Violle & Jiang 2009), germination (Donohue 2002), and seedling performance (Elwell et al. 2011) in different species, including long-lived plants such as conifers (Velasco-Conde et al. 2012).

The influence of different genetic backgrounds on the tolerance to heavy metals in plants has been widely discussed (Au 2012, Prus-Glowacki et al. 2012). *Crepidorhopalon perennis* and *C. tenuis* (Linderniaceae), two metallophyte herb species from South Central Africa, have been used as models to investigate the relationship between the degree of tolerance of plants populations, bioavailable Cu content in the soil and the ecological isolation of endemic species (Faucon et al. 2012). Regarding forest species, resistant trees of both *Pinus sylvestris* L. and *Pinus nigra* Arn. have been reported to exhibit a lower degree of genetic variation than metal-sensitive trees with respect to some isozyme loci (SHDH A, PGI, PGM, MDH C and DIA). This allele depletion suggests that genetic changes induced by heavy metals may dramatically affect the adaptation process in forest tree populations (Chudzinska et al. 2014).

In this study, different activities of the enzymes POX, SOD and CAT were found among the three pine species from the Llico provenance, with *P. pinaster* showing the highest values. Moreover, the expression of genes coding for Cu-Zn-superoxide dismutase and Ribulose bisphosphate carboxylase (RuBisCo) was the highest in *P. pinaster* after 60-days metal treatments.

The protective functions of antioxidants from heavy metal stress have been reviewed by Schützendübel & Polle (2002). Based on their chemical and physical properties, different molecular mechanisms of heavy metal toxicity can be distinguished: (1) production of ROS by autoxidation and the Fenton reaction, which is typical for transition metals such as iron or copper; (2) blocking of essential functional groups in biomolecules, which has been reported for non-redox reactive heavy metals such as cadmium and mercury; (3) displacement of essential metal ions from biomolecules, a reaction occurring with different kinds of heavy metals. At the plant cell level, SODs represent the first line of defense against ROS. Ozone is produced at any location where an electron transport chain is present, and O_2 activation may occur in different compartments of the cell, including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol (Elstner 1991, Alscher et al. 2002).

Photosynthetic activity and oxidative stress are considered sensitive biological indicators of heavy metal stress (MacFarlane & Burchett 2001, Schützendübel & Polle 2002). The RT-PCR analysis carried out in this study revealed a conspicuous expression of genes coding for Cu-Zn-superoxide dismutase and RuBisCo in *P. pinaster* (Fig. 4). These results are fairly consistent with the high levels of metal tolerance observed in *P. pinaster*, while *P. canariensis* and *P. radiata* showed intermediate and low tolerance, respectively, according to their low expression of the aforementioned genes.

The results of this study are in agreement with those reported by Acquaviva et al. (2012), who demonstrated a significant differential expression of Heat shock protein 70, Hemeoxygenase and Superoxide dismutase in *P. pinaster* trees from two sites with contrasting pollution levels. These authors proposed that the increased expression of the above enzymes may exert protective effects against oxidative stress and represent an adaptative defense mechanism. Moreover, these genes could represent useful tools for monitoring the environmental contamination and better understand the mechanisms underlying plant defense to stress. Furthermore, the response of *Camellia sinensis* (L.) O. Kuntze to Cu and Al stresses was investigated by Yadav & Mohanpuria (2009). Exposure to 100 µM $CuSO_4$ or 100 µM $AlCl_3$ led to accumulation of higher levels of ROS in the *Assamica* compared to the *Chinary* genotype. Proline content was higher in *Chinary* compared to *Assamica*, while chlorophyll and protein contents decreased upon Cu and Al exposure in both varieties. Phytochelatin synthase (PCS), an enzyme involved in phytochelatin synthesis by using glutathione as a substrate, was upregulated to the highest levels in *Chinary*. These results suggest that the *Chinary* could be more Cu- and Al-tolerant than the *Assamica* genotype.

In conclusion, *P. pinaster* has shown the best tolerance to both Cu and Al under controlled conditions in terms of plant survival and detoxifying enzyme activity. Tolerant individuals of all the species studied have been selected and planted in a local mining ecosystem, which is particularly vulnerable to heavy metal pollution. Epigenetic analysis will be conducted in selected individuals in future studies.

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