Mycorrhization protects Betula pubescens Ehr. from metal-induced oxidative stress increasing its tolerance to grow in an industrial polluted soil

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Highlights

Mycorrhization increased *B. pubescens* biomass but reduced its metal accumulation.

Mycorrhization led to a mitigation of the metal-induced oxidative stress.

Ascorbate peroxidase along with catalase modulate ROS levels for signaling.

Superoxide dismutase has a key role in *B. pubescens* antioxidant defense.

Mycorrhization protects *Betula pubescens* Ehr. from metal-induced oxidative stress increasing its tolerance to grow in an industrial polluted soil

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Abstract

In recent years, the use of woody plants in phytoremediation have gained popularity due to their high biomass production and their association with mycorrhizal fungi, which can improve their survival and development rates under stress conditions. In this study, mycorrhized and non-mycorrhized white birch plants (*Betula pubescens* Ehr.) were grown in control and a metal-polluted industrial soil. After 60 days of culture, plant growth and metal accumulation, the content of photosynthetic pigments and oxidative-stress markers, as well as the enzymatic activities and gene expressions of antioxidant enzymes were measured.

According to our results, mycorrhized birch plants grown in control soil showed an increased activity and gene expression of catalase and ascorbate peroxidase, along with hydrogen peroxide overproduction, which could support the importance of the reactive oxygen species as signaling molecules in the regulation of plant-fungus

interactions. Additionally, in polluted soil mycorrhized plants had higher biomass but lower metal accumulation, probably because the symbiotic fungus acted as a barrier to the entrance of metals into the host plants. This behavior led to mitigation in the oxidative challenge, reduced hydrogen peroxide content and diminished activities of the antioxidant enzymes in comparison to non-mycorrhized plants.

Keywords

Heavy metals, mycorrhization, oxidative stress, gene expression, phytoremediation

1. Introduction

Metal contamination due to human or industrial activities has become a major environmental problem nowadays [1,2]. Some heavy metals (HM), such as Zn or Cu, are essential for normal plant growth and development, however all are toxic at elevated concentrations. Therefore, high concentrations of HM in soil may produce not only toxicity symptoms in plants, growth inhibition and even plant death, but also pose a threat to human health [3].

Heavy metal stress causes an enhanced generation of reactive oxygen species (ROS) and high concentrations of cytotoxic molecules that cause oxidative stress. This can block functional groups of biomolecules, disturb metabolic pathways and produce severe damage to proteins, membrane lipids, and DNA [4–6]. However, other studies [7–9] have shown that ROS also play an important role as signaling transduction molecules regulating the responses to a broad range of stresses and certain stages of development. In plants, the synergic role of the antioxidant enzymes is one of the main mechanisms involved in alleviating oxidative stress, although other non-enzymatic mechanisms, such as free proline accumulation, can also be involved [5,10,11]. Among the most studied antioxidant enzymes are glutathione reductase (GR, EC 16.42.2), catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (POD, EC.1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11). Therefore, the equilibrium between the oxidative and anti-oxidative capacities is of major importance and must be carefully regulated to avoid cellular damage, which occurs when ROS production exceeds the scavenging capacity of the antioxidant processes and mechanisms.

In recent years, the use of trees in phytoremediation has gained popularity. Because of their deeper root system, considerable tolerance to HM and greater biomass,

they can improve the effectiveness of phytoremediation [1,12,13]. In natural conditions, roots of trees associate with soil fungi forming ectomycorrhizas [14,15]. The presence of these structures greatly increases their root absorption area and the bioavailability of water and other important nutrients. They are of great importance when plants grow in nutrient-poor or metal-polluted soils, where the mycorrhizas may improve the survival and development rates of trees under stress conditions. It has also been demonstrated that plants mycorrhized with metal-tolerant fungi perform better at elevated HM concentrations [16–18]. However, the effect of mycorrhization on plants stress tolerance or HM accumulation is not clear since it strongly depends on the plant, fungus or metal involved and even on several environmental factors [13,15].

In previous *in vitro* studies [19], it was observed that mycorrhized Iberian white birch plants (*Betula celtiberica* Rothm & Vasc.*)* accumulated more Cd than the non-mycorrhized plants. Nevertheless, most of the studies are focused on artificially contaminate soils or substrates with just one HM studying its effect on metal accumulation or the physiological responses of model plants. However, in nature we rarely find just one contaminant but several of them and in different concentrations, so that the responses of plants in real conditions widely differ from those observed under lab conditions. Therefore, the aim of this work is to study the growth and physiological responses of birch plants, mycorrhized (M) and non-mycorrhized (NM), when growing in an industrial metal-polluted soil. For this purpose, we selected plants of European white birch (*Betula pubescens* Ehrh.), a closely related species of *B. celtiberica* (previously known as *B. pubescens subsp. celtiberica*), with a wider distribution. In these plants, we measured the growth and metal accumulation, the content of photosynthetic pigments and oxidative stress markers, as well as the enzymatic activities and gene expressions of enzymes related to the antioxidant systems.

2. Materials and methods

2.1. Plant material, fungus inoculum and growth conditions

Plants of a clone of *B. pubescens* were micropropagated *in vitro* in half-strength Murashige and Skoog medium (pH 5.7) [20], with 0.2 g L^{-1} of sequestrene 138-Fe (Ciba-Geigy AG), 30 g L^{-1} of sucrose and 7 g L^{-1} agar. Cultures were kept in growth chamber at 25 °C and a 16 h photoperiod. After five weeks, plants were inoculated with an isolation of the ectomycorrhizal fungus *Paxillus ammoniavirescens* Contu & Dessì, collected in a metal-polluted soil and selected for its *in vitro* growth and high Cd accumulation capacity. The inoculation was performed by placing two 1 cm diameter discs of actively growing fungal mycelium directly on plant culture medium surface. After 4 weeks, mycorrhizae formation was checked. Then, birch plants, M and NM, were acclimated to greenhouse conditions and transferred to Rootrainer book-like containers (Ronaash, Ltd.) of 20 cm cell depth filled with a peat:perlite mixture (3:2, v/v). After 15 days, plants were finally transferred into 1 L pots (diameter and height = 14 cm) filled with peat:perlite (3:1, v/v) mixture (control soil) or polluted soil. Polluted soil was previously autoclaved twice at an interval of 48 h at 100 °C for 1h, to eliminate existing arbuscular mycorrhizal propagules. Polluted soil was collected from a deserted nitrate factory in Langreo (Asturias, Spain) and it was contaminated with several toxic elements including Cu (1181 ± 39 mg kg^{-1} dw soil), Zn (1841 ± 136 mg kg^{-1} dw soil), As (1630 ± 99 mg kg^{-1} dw soil), Cd (7.1 ± 1.2 mg kg^{-1} dw soil), Hg (1.4 ± 0.3 mg kg^{-1} dw soil) and Pb (10183 ± 2265 mg kg^{-1} dw soil). The experiment was carried out under greenhouse conditions for 60 days in a 2 x 2 completely randomized factorial design.

2.2. Growth parameters and metal content

At the end of the culture period, plants were carefully extracted from pots and rinsed exhaustively with tap water and washed twice with double de-ionized water. To

determine the influence of the treatments on plant growth, fresh and dry weights and lengths of shoots and roots were measured. Afterwards plants were separated into leaves, stem and roots and collected as pools formed by at least four different plants, homogenized with liquid nitrogen and stored at -80 °C.

For metal content determination, 100 mg of oven dried plant material (at 40 °C for 48 h) was digested with HNO_3 in a microwave oven and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) as described in [19].

2.3. Analysis of photosynthetic pigments and chlorophylls fluorescence

Chlorophylls and carotenoids were extracted from 500 mg of frozen leaves using 10 mL of 80% acetone. Homogenates were centrifuged at 3,000 g for 10 min before measuring the absorbance. The pigment content was calculated according to the equations of Porra [21] and Lichtenthaler and Wellburn [22].

Chlorophyll fluorescence was measured with an OS1-FL portable fluorometer (Opti-Sciences) on second fully expanded leaves previously dark-adapted for 20 min. The Fv/Fm ratio was determined according to the method described in [23].

2.4. Hydrogen peroxide content, lipid peroxidation and free proline content

For the hydrogen peroxide (H_2O_2) content determination, 500 mg of frozen leaves were mixed with 2.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 g for 15 min, following the method described in [10]. To a 0.5 mL of the supernatant, 1 mL of 1M KI and 0.5 mL of 100 mM phosphate buffer (pH 7.6) were added. The absorbance of samples was measured at 390 nm and the H_2O_2 content was determined using a calibration curve and expressed as μg H_2O_2 g^{-1} fresh weight.

Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) [24,25]. MDA was extracted from 500 mg of frozen leaves with 2.5 mL of 0.1% TCA and the homogenates centrifuged at 10,000 g for 15 min. To

a 0.5 mL of the supernatant, 2 mL of 20% TCA with 0.5% TBA was added and the mixture heated at 95 °C for 30 min. Then, samples were quickly cooled in ice and centrifuged at 10,000 g for 15 min. The absorbance of the supernatant was measured at 535 nm (ε = 155 mM^{-1} cm^{-1}). The absorbance at 600 nm was used to correct the non-specific turbidity. MDA content was expressed as nmol g^{-1} fresh weight.

Free proline content was determined according to the methods described in [24,26]. A 500 mg aliquot of fresh leaf material was homogenized in 3% (w/v) sulfosalicylic acid and centrifuged at 10,000 g for 15 min. A 1 mL aliquot of supernatant was mixed with 1 mL of glacial acetic acid and acid ninhydrin (1:1, v/v). The mixture was heated for 1 h at 100◦C in a water bath and cooled quickly on ice to stop the reaction. Then proline was extracted in toluene (5 mL) and the absorbance read at 520 nm. Free proline concentration was determined using a calibration curve and expressed as μmol g^{-1} fresh weight.

2.5. Antioxidant enzyme activities

The determination of antioxidant enzyme activities was carried out by spectrophotometric assays as described in [10,27]. Crude extracts were obtained from 500 mg of frozen leaves macerated in 100 mM Tris-HCl buffer (pH 7.8) containing 3 mM of dithiothreitol, 2% (w/w) insoluble polyvinylpolypyrrolidone, and 1 mM of ethylenediaminetetraacetic acid (EDTA). After centrifugation at 10,000 g for 15 min at 4 °C, extracts were aliquoted and stored at -20 °C. Protein concentration was determined as described in [28]. Enzymatic activity was expressed as units of enzymatic activity mg^{-1} protein.

Guaiacol peroxidase activity (POD, EC.1.11.1.7) was determined by the oxidation of guaiacol in a reaction with a mixture containing 30 mM 2-methoxyphenol (guaiacol) and 4 mM H_2O_2 in 0.2 M sodium acetate buffer (pH 6.0) as described in

[10,27]. One unit of enzymatic activity was defined as the consumption of 1 μmol of guaiacol min^{-1} cm^{-3} at room temperature with an extinction coefficient for tetraguaiacol of 26.6 mM^{-1} cm^{-1}.

Catalase (CAT, EC 1.11.1.6) activity was measured by the consumption of H_2O_2 in an assay with a solution containing 10 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) as described in [10,27]. One unit of enzymatic activity was defined as the consumption of 1 μmol H_2O_2 min^{-1} cm^{-3} using an extinction coefficient of 39.4 mM^{-1} cm^{-1}.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined in a reaction with a solution containing 0.5 mM xanthine, 0.1 mM EDTA, 0.05 mM ferricytocrhome-c, and xanthine oxidase in 100 mM potassium buffer (pH 7.6) as described in [10,27]. One unit of enzymatic activity was defined as the enzyme quantity needed to inhibit the reduction of ferricytochrome-c by 50 % min^{-1}.

The activity of glutathione reductase (GR, EC 16.42.2) was determined measuring the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) via glutathione (GSH) previously reduced by GR as described in [10,27]. To do this, a reaction mixture containing 3 mM DTNB, 2 mM NADPH, and 20 mM GSSG in 100 mM phosphate buffer (pH 7.6) was used. One unit of enzymatic activity was defined as the consumption of 1 μmol DTNB min^{-1} cm^{-3} using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined in a reaction mixture containing 0.25 mM ascorbic acid and 0.3 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and the absorbance measured at 290 nm as described in [10,27]. The enzymatic activity was defined as the consumption of 1 μmol ascorbate min^{-1} cm^{-3} using a coefficient of absorbance of 2.8 mM^{-1} cm^{-1}.

2.6. Gene expression analysis

Isolation of the RNA was carried out using the protocol described in [29] with slight modifications. A 100 mg aliquot of frozen leaves was homogenized in 550 µl of buffer containing 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidinone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermidine and 2% β-mercaptoethanol. Then, it was extracted twice by adding 550 µl of chlorophorm:isoamyl alcohol (24:1) mixture and centrifuged at 14,000 g for 20 minutes at 4 °C. After addition of 10 µl LiCl (10 M), RNA was precipitated overnight at 4°C and harvested by centrifugation at 14,000 g for 20 minutes at 4 °C. The pellet obtained was washed with 75% ethanol and resuspended in RNase free water. The concentration of the RNA was determined spectrophotometrically at 260 nm using Nanodrop equipment (Isogen Life Science) and RNA quality was tested by using Experion™ automated electrophoresis system (Bio-Rad). Genomic DNA was removed using TURBO DNA-free Kit (Ambion) and the cDNA synthesis was performed using PrimerScript RT reagent Kit (Takara) with equal amounts of RNA input (1µg). Then cDNA was ten-fold diluted using a 1/10 dilution of TE buffer (1 mM Tris-HCl, EDTA 0.1 mM, pH 8.0) and stored at -20 °C.

Reverse Transcription quantitative PCR (RT-qPCR) was performed with ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. Gene primers (Table 1) were designed using Primer 3 [30,31], according to sequences of genes obtained in the NCBI nucleotide database of the closely related species *Betula pendula.* The qPCR efficiency and amplification specificity of the primers was validated by melting curves. PCR amplifications were done in a total volume of 10 µl containing 2 µl cDNA sample, 5 µl SYBR Green, 0.6 µl primers (10 µM) and 2.4 µl RNase free water. The reaction cycle was as follows: 20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C. Gene expression was calculated

relatively as $2^{-\Delta Cq}$. As suggested by Graynorm algorithm [32], gene expression was normalized with a normalization factor based on the expression of reference genes of *Betula sp.* and the closely related species *Populus Trichocarpa* (Table 1).

2.7. Statistical analysis

Two-way Analysis of variance (ANOVA) was used to evaluate the effect of mycorrhization, soil on the measured variables. Log transformation was used to approximate normality when necessary. When F ratio was significant ($p \leq 0.05$), Tukey's least significant difference test ($p \leq 0.05$) was applied. Results were mean ± standard error of at least three independent replicates. Each sample was obtained from 4 different plants per treatment. Data were analyzed using SPSS19 software (IBM).

3. Results

3.1. Plant growth and metal accumulation

After 60 days of culture, no external symptoms of phytotoxicity, nor biomass decrease (Fig. 1), were observed between NM *B. pubescens* plants grown on control or metal-polluted soil. Mycorrhization influenced both fresh and dry weight (Fig. 1) and these parameters were always higher in M plants.

Plants grown in polluted soil accumulated high concentrations of Zn, Pb, Cu, and As both in roots and leaves, and, metal accumulation was generally higher in roots (Table 2). When growing in polluted soil, NM plants showed the highest metal accumulation (Table 2). In leaves, Zn accumulated at the highest concentration (Table 2), while in roots Pb reached concentrations over 2,200 mg kg^{-1} DW (Table 2) without the plants showing any toxicity symptoms.

3.2. Analysis of photosynthetic pigments and photochemical parameters

Mycorrhization decreased the total chlorophyll content of plants grown in control soil but increased it in plants grown in polluted soil (Table 3), mainly due to changes in chlorophyll a content. With regard to carotenoids, mycorrhization increased this parameter in plants grown in polluted soil while no differences were observed in plants grown in control soil. Photosynthetic efficiency, measured as the Fv/Fm index, was not affected by the treatments assayed (Table 3).

3.3. Parameters related to oxidative stress

The results of parameters related to oxidative stress are represented in Table 4. When compared to the NM plants, mycorrhization increased H_2O_2 concentration in control soil while it was decreased in polluted soil. Furthermore, the H_2O_2 levels of NM plants grown in polluted soil showed an increase of 20 % with respect to the same plants grown in control soil.

The highest MDA levels were measured in M plants grown in control soil while no differences were observed between M and NM plants grown in polluted soil.

Both free proline and soluble protein content decreased between a 44-67% in plants grown in contaminated soil when compared with control soil while mycorrhization did not affect these parameters.

3.4. Antioxidant enzymes activities

In general, mycorrhization had no effect (GR, SOD, POD) or a slightly increasing effect (APX, CAT) on enzyme activity in plants grown under control conditions (Fig. 2). When the enzyme activity of plants was compared between control and metal-polluted conditions, it was clear that the activities of all enzymes analyzed were strongly increased in plants grown on metal-polluted soil. Mycorrhization diminished this increase in enzyme activity completely (GR) or partially (CAT, POD, APX) when

plants were grown on metal-polluted soil. Only for SOD (Fig. 2C), no difference was observed between M and NM plants grown on metal-polluted soil.

3.5. Gene expression

Mycorrhization induced *APX* gene expression but had no effect on the transcription levels of *GR*, *CSD* or *GST* (Fig. 3). Gene expression of *CAT* was also up-regulated but only in M plants grown under control conditions. When comparing the gene expression of plants grown in control and metal-polluted soil, it was observed that *CSD* and *GST* transcription levels were increased and reduced, respectively under metal-polluted conditions (Fig. 3). The expression levels of *GR*, *CAT* or *APX* were not affected by metal pollution.

4. Discussion

B. pubescens shows a high tolerance to HM present in the soil since no differences in fresh or dry weight were observed between NM plants grown in control or polluted soil. The high metal tolerance of birch was also reported by other researchers, who observed that birch clones or species were capable of growing at high metal concentrations without serious toxicity symptoms [19,33,34]. In addition, some authors support that the ectomycorrhizal symbiosis enhances plant fitness and improves plant growth under HM stress [13,17,18,35]. In our case, the mycorrhization of *B. pubescens* had a positive effect on plant growth both in control and polluted soils. This is probably due to the increased root absorption area of trees and therefore the uptake of water and other important nutrients [14]. This improved absorption is of major importance in nutrient-deficient and degraded soils such as metal-polluted soils, increasing the resistance of plants to cope with metal toxicity.

Whereas we detected all the elements in leaves, the maximum metal accumulation was generally found in roots. In this way, Kopponen et al. [33] reported

that metal-tolerant birch trees can apparently live in polluted soils by reducing their metal translocation rate from roots to shoots. Similar results were also reported in other plant species [36–38], reflecting the difficulty of plants to translocate metals to the aboveground tissues. This response is logic taking into account that for most of the metals analyzed there is a lack of specific membrane transporters. In addition, the apoplastic pathway is restricted by the Casparian strips in the root endodermis, hence the elements mainly accumulate in the roots [14]. There, the retention in the cell wall seems to be one of the main detoxification strategies, as is has been reported for Cd [39]. In contrast, Zn showed the highest root-to-shoot translocation, especially in plants grown in control soil. Zinc is an essential micronutrient necessary for the correct plant growth and development and it is actively taken up by specific membrane transporters [14,40] while other toxic elements such as Cd, As, or Pb do not have any known function in plant cells and their uptake is possible due to their chemical similarity to other essential elements like Ca, P, Fe or Zn [14].

With regard to the effect of mycorrhization on metal accumulation, this was lower in M plants cultured in polluted soil than in NM plants. These results are in accordance to Krznaric et al. [16,41] and Colpaert et al. [18] who reported that metal-tolerant fungal strains restricted metal translocation leading to an increased tolerance of the host plant. In contrast, Han et al. [42] reported that an increased Cd accumulation led to a reduced Cd tolerance on M Poplar. How mycorhization reduce metal accumulation in the host plants can be explained by many mechanisms. Fungal mycelia can act as a physical barrier in the absorbing roots, metal sorption to hyphal sheath or mycelium can occur, and metal translocation to the host can be limited because of metal accumulation in fungal vacuoles or extracellular chelation by exudation of organic acids, metabolites or slime [14,15,43–45]. In spite of all, the effect of mycorrhization on

plant metal accumulation cannot be generalized. It is dependent on the interaction between plant and fungus and/or related to the concentration of metals in the soil [14,46]. It is also possible that part of the controversy around the reported results is due to the type of medium, or substrate used in these experiments. Using substrates or soils contaminated with just one metal is not comparable to a natural soil polluted with a wide range of HM. Polluted industrial soils are complex systems in which different metals or fungi can interact with each other and with soil particles, so the results observed may vary in a complex way [15,47]. Therefore, we think that results described here highlight the importance of carrying out studies under natural conditions in order to validate the results obtained *in vitro* or hydroponics as was also suggested by other authors [12].

Plants growing under HM stress usually present photosynthesis inhibition due to chlorophyll degradation or reduced biosynthesis, which can lead to disturbed carbon fixation and therefore reduced growth [48]. In this way, metal-induced decreases in chlorophyll concentrations have been reported in many plant species [27,49–53]. However, in our study we observed that soil pollution did not affect neither chlorophyll and carotenoid concentrations nor the photosynthetic performance (Table 3). This lack of toxicity shows that birch plants have a good metal tolerance given the high metal accumulation of Zn in leaves or Pb in roots. The concentrations of these elements were several times higher than the critical leaf concentration for toxicity in non-tolerant plants [54]. Mycorrhization even further protected the plants from toxic effects of HM (Table 3), which might be related the lower metal concentrations in M plants (Table 2). Adriaensen et al. [17] also reported that mycorrhization with a Zn-tolerant isolate of *Suillus bovinus* protected Scots pine plants against the chlorophyll reduction caused by the exposure to elevated Zn concentrations.

Although in some studies HM, such as Cd, are involved in protein degradation and protein synthesis inhibition [53,55], in others [56], an increase in total protein content has been reported since they also induce the synthesis of several proteins related to the plant defense mechanisms. It has also been hypothesized that proline plays an important role in plants stress tolerance, since, apart from being an osmolyte, it can play a role in ROS and HM scavenging. Thus, accumulation of free proline is expected under HM exposure as it has been observed in several studies [1,57]. However, we observed proline decrease in plants grown in polluted soil, which was concomitant with a decrease in the soluble protein concentration observed in the same conditions. By contrast, Yılmaz and Parlak [58] reported proline accumulation in *Groelandia densa* exposed to Cd even though a reduction in the soluble protein content was observed. Therefore, we can deduce that proline may not be important in birch metal-induced stress tolerance.

Excessive accumulation of metals in plant cells causes ROS overproduction leading to an oxidative challenge that, depending on its severity, can produce oxidative damage [5,6,59]. Membrane lipids are very sensitive to ROS oxidation and so they are considered as primary targets for metal toxicity [6,60]. According to our results the absence of lipid peroxidation observed in NM plants grown in polluted soil (Table 4), suggests that the birch antioxidant system was capable to cope with the metal-induced H_2O_2 overproduction. In plants, one of the main mechanisms involved in alleviating this oxidative challenge is the synergic role of the antioxidant enzymes [4,7]. The activities of these enzymes usually experience stimulation after metal exposure in dose-dependent responses, although they can also suffer depletion and even inhibition at toxic concentrations [10,44]. Given the strong increase in the activities of all the enzymes analyzed in plants grown in polluted soil (Fig. 2), we can deduce that in *B. pubescens*

the role of this enzymes is crucial in the protection against the metal-induced oxidative stress. Huang et al. [61] also reported that the rapid induction of the antioxidant enzymes was responsible of the high Pb tolerance of an ecotype of *Sedum alfredii*. As part of the oxidative stress defense, GST is involved in the detoxification of the toxic by-products of lipid peroxidation [62,63]. In this way, elevated *GST* transcription levels in response to metal stress have been previously reported in *B. papyrifera* [63]. However, we observed *GST* down-regulation in plants grown under polluted conditions. This inconsistency might be the consequence of either negative feedback regulation of *GST* expression or the possibility of multiple isoforms being expressed.

Mycorrhization had a beneficial effect alleviating the metal-induced oxidative stress on plants grown in polluted soil, reducing both the excess of H_2O_2 and the activities of GR, CAT, POD and APX. This effect may be a result of the lower metal accumulation found in these plants. Only for SOD, we did not observe a reduction of enzymatic activity (Fig. 2C). This observation, along with the up-regulation of *CSD* observed in metal-polluted conditions, suggests that SOD plays a central role in birch ROS scavenging system. This result seems logic given the broad importance of SOD, rapidly converting superoxide radicals, highly toxic and reactive molecules, into less toxic agents, which are substrate of other ROS-scavenging components [4,62].

Interestingly, mycorrhization induced ROS overproduction that led to membrane damage in plants under control conditions (Table 4). In this situation slight increases in CAT and APX activities were observed, along with transcriptional up-regulations of their coding genes. This could be explained by the fact that in early stages of the mycorrhizal infection plant undergo a redox imbalance and hence oxidative stress as it has been observed in nodule formation in leguminous [25]. Furthermore, Baptista et al. [64] suggested that ROS generation may be involved in the regulation of plant-fungi

interactions during the development of the ectomycorrhizal symbiosis. In this scenario, a strict control of ROS levels is of major importance [7], and according to our results, in *B. pubescens* CAT and APX may play an important role in this control. Given the higher affinity of APX for H_2O_2, this enzyme has been proposed as a key player in the fine modulation of ROS for signaling [4,60]. The fact that *APX* up-regulation was observed only in mycorrhized conditions, suggests a role of this enzyme in the regulation of plant-fungi interactions during the mycorrhizal symbiosis.

5. Conclusions

Mycorrhization of white birch with *P. ammoniavirescens* resulted in an improved growth and tolerance to HM that is based in a restricted translocation of metals to the host plant tissues. This mechanism leads to a reduced metal-induced oxidative stress. In this scenario APX, along with CAT, may have a key role in the fine modulation of ROS production.

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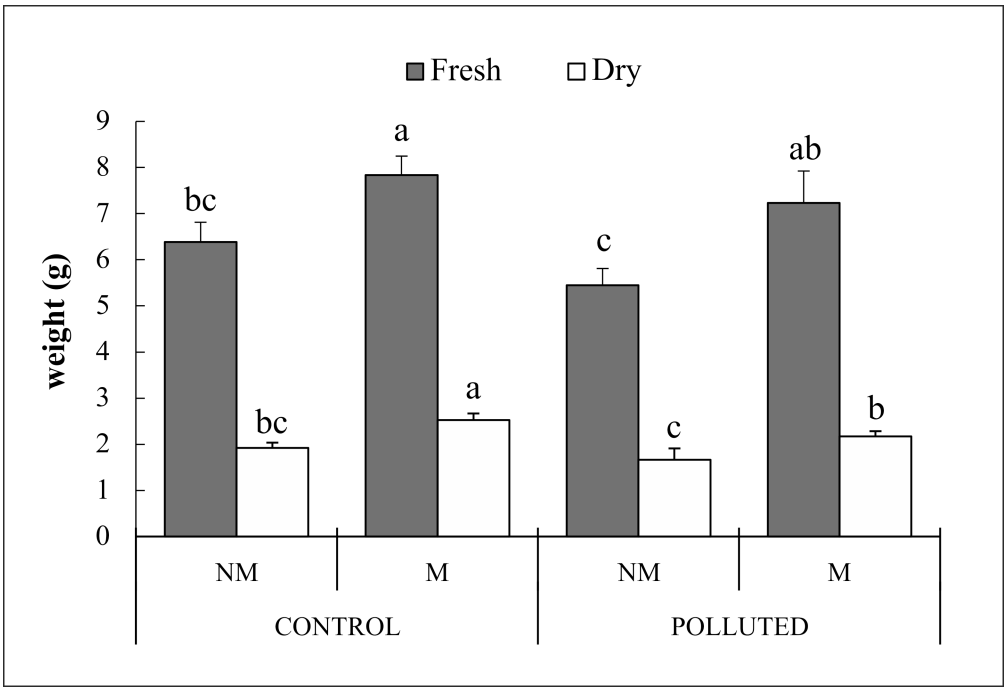
Figures captions

Figure 1. Fresh and dry weight of mycorrhized (M) and non-mycorrhized (NM) *B. pubescens* grown for 60 days in control or polluted soil. Different letters indicate significant differences at $p<0.05$.

Figure 2. Enzymatic activities of glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (POD) and ascorbate peroxidase (APX) in leaves of mycorrhized (M) and non-mycorrhized (NM) *B. pubescens* grown for 60 days in control or polluted soil. Different letters indicate significant differences at $p<0.05$.

Figure 3. Gene expression of glutathione reductase (*GR*), catalase (*CAT*), Cu-Zn-superoxide dismutase (*CSD*), ascorbate peroxidase (*APX*) and glutathione-S-transferase (*GST*) in leaves of mycorrhized (M) and non-mycorrhized (NM) *B. pubescens* grown for 60 days in control or polluted soil. Results expressed as relative to the non-mycorrhized control. * Significant up or down-regulation at $p<0.05$.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NM
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CONTROL
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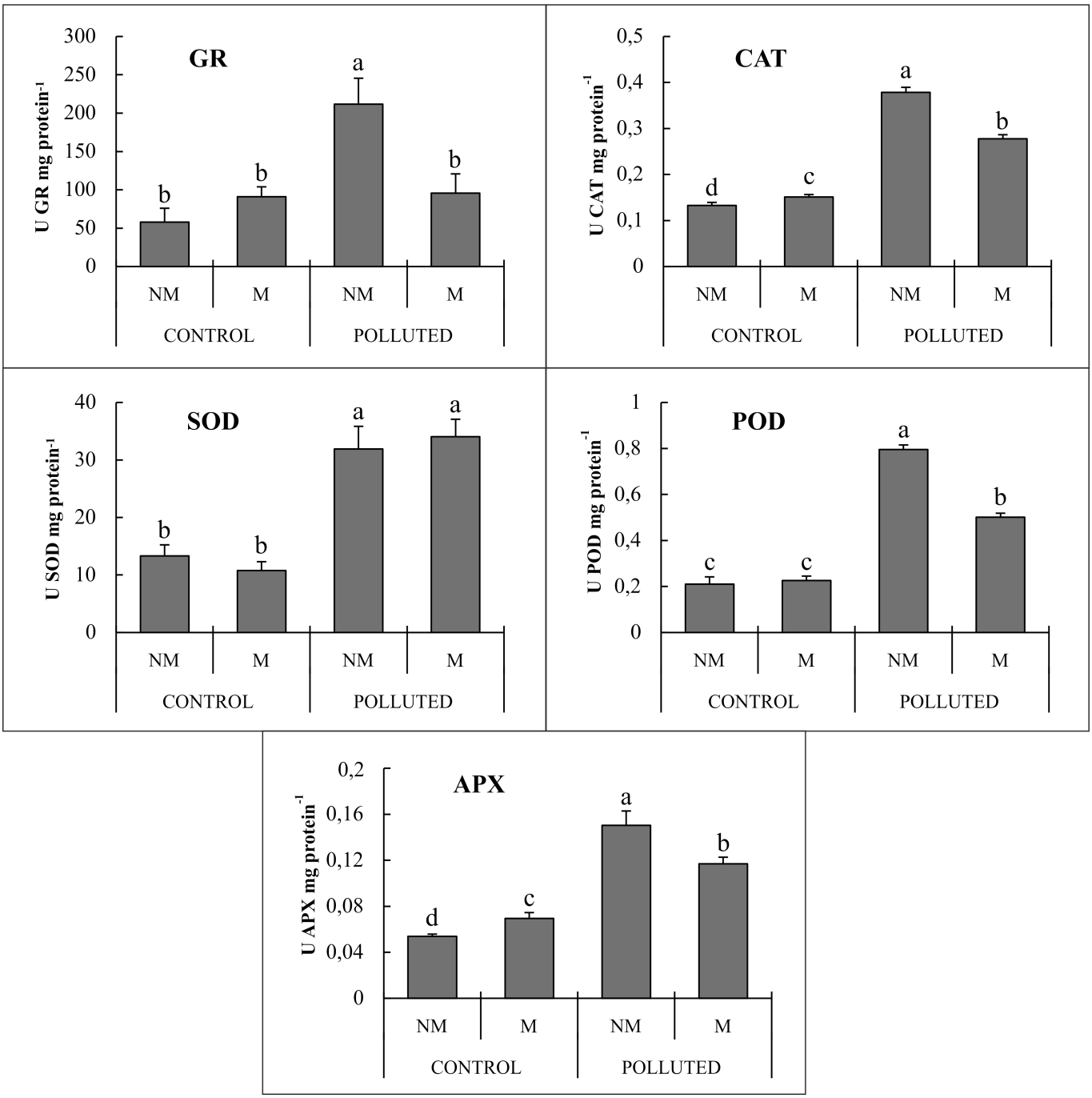
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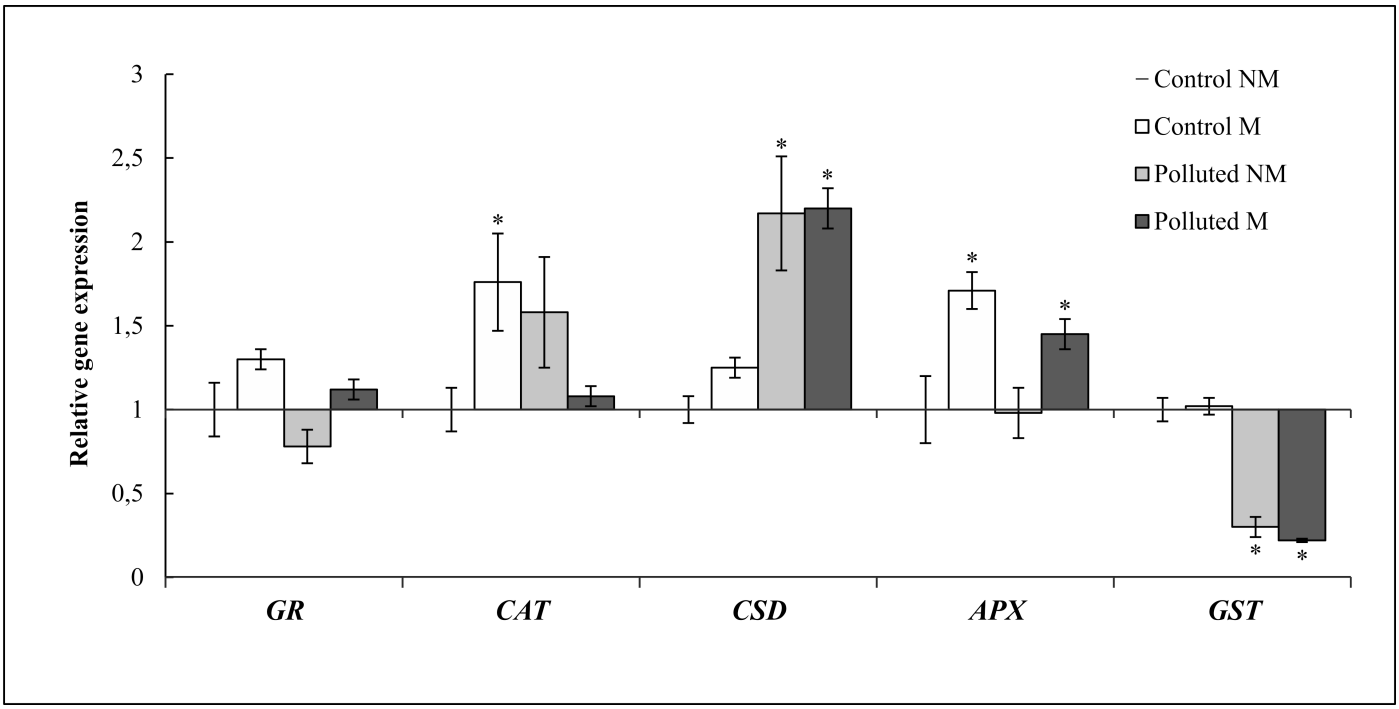
Relative gene expression
3
2,5
2
1,5
1
0,5
0
Control NM
Control M
Polluted NM
Polluted M
GR
CAT
CSD
APX
GST

Table 1

Primer sequences used for the real time RT-PCR analyses.

Gene	Protein	Species	Primer sequences forward/reverse	Product size (bp)	Accession number
GR	Glutathione reductase	*Betula pendula*	GGCTGTGGGTGATGTTACGA/ TGGTTTGCTAGGTTCCCCAC	103	AJ279690
CSD	Cu-Zn-superoxide dismutase	*Betula pendula*	GCAACAGTCAGGGTGTCAGT/ TGTTGTGTCCCCAAGTGCAT	134	AJ279694
APX	Ascorbate peroxidase	*Betula pendula*	GTTCTGGCTTTGAGGGACCC/ GTCAGACAGAGGTGCCTTGT	91	AJ279686
CAT	Catalase	*Betula pendula*	CCATTGTGGTTCCTGGTGTCT/ GCTGGGAGTTGCAGGTAGTT	115	AJ295295
GST	Glutathione-S-transferase	*Betula pendula*	CCTGAAAACAAGGTGCCAGC/ CCAACTCTTCAGCAAACGCC	145	KF246508
PSAH	Photosystem I psaH protein	*Populus trichocarpa*	CCAAGTATGGTGACAAGAGTG/ TGGAGCTGCAAATGTCTC	145	XM_002304170
ATUB	Alpha-tubulin	*Betula pendula*	AACTCATCAGCGGCAAGGAA/ TTGTCTGCAAGCTTCCGGAT	112	AJ279695
E2	Ubiquitin-conjugating enzyme	*Betula luminifera*	TTGATGTGGCATGGCAGGAT/ TCACCAGCGTCATCACCATC	145	KM586056

Table 2

Metal accumulation in leaves and roots of mycorrhized (M) and non-mycorrhized (NM) *B. pubescens* grown for 60 days in control or polluted soil. Different letters within the same column and organ denote significant differences at $P<0.05$.

Organ	Growth condition	Metal concentration (mg kg $^{-1}$ DW)					
		Cu	Zn	As	Cd	Hg	Pb
Leaves	Control NM	2.25 ± 0.14 c	112.91 ± 6.81 c	0.53 ± 0.08 c	0.24 ± 0.01 c	0.01 ± 0.00 c	0.50 ± 0.03 c
	Control M	2.38 ± 0.22 c	112.76 ± 0.74 c	0.33 ± 0.03 c	0.20 ± 0.01 c	0.02 ± 0.00 b	0.29 ± 0.01 d
	Polluted NM	16.71 ± 0.91 a	1228 ± 149 a	21.62 ± 4.89 a	9.45 ± 0.83 a	0.09 ± 0.00 a	140.08 ± 4.23 a
	Polluted M	7.63 ± 0.57 b	929.9 ± 27.5 b	12.05 ± 1.24 b	6.34 ± 0.64 b	0.01 ± 0.00 bc	48.85 ± 2.86 b
Roots	Control NM	4.43 ± 0.01 d	43.44 ± 0.17 c	0.08 ± 0.01 c	0.11 ± 0.00 c	0.001 ± 0.000 c	0.002 ± 0.000 c
	Control M	9.28 ± 1.05 c	54.71 ± 6.54 c	0.004 ± 0.000 d	0.14 ± 0.00 c	0.006 ± 0.001 c	0.002 ± 0.000 c
	Polluted NM	567.7 ± 29.4 a	1208 ± 89 a	281.1 ± 43.5 a	22.99 ± 3.28 a	1.43 ± 0.14 a	2239 ± 230 a
	Polluted M	364.6 ± 15.3 b	803.8 ± 40.6 b	196.5 ± 22.4 b	12.07 ± 0.94 b	0.87 ± 0.07 b	1450 ± 147 b

Table 3

Photosynthetic efficiency and pigments concentration in leaves of mycorrhized (M) and non-mycorrhized (NM) *B. pubescens* cultured for 60 days in control or polluted soil. (Chl: chlorophyll). Different letters indicate significant differences at $P<0.05$.

Growth condition	Total Chl (mg g^{-1} FW)	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Fv/Fm
Control NM	30.98 ± 1.11 ab	23.29 ± 0.78 a	7.69 ± 0.35 ab	5.18 ± 0.14 ab	0.74 ± 0.01 a
Control M	28.58 ± 0.49 c	21.37 ± 0.41 b	7.21 ± 0.16 b	4.78 ± 0.13 b	0.73 ± 0.01 a
Polluted NM	29.04 ± 0.77 bc	21.43 ± 0.71b	7.61 ± 0.16 ab	4.75 ± 0.25 b	0.75 ± 0.01 a
Polluted M	32.36 ± 0.75 a	24.22 ± 0.64 a	8.13 ± 0.18 a	5.44 ± 0.18 a	0.71 ± 0.02 a

Table 4

Hydrogen peroxide (H_2O_2), malondialdehyde (MDA), free proline and soluble protein concentrations in leaves of mycorrhized (M) and non-mycorrhized (NM) *B. pubescens* cultured for 60 days in control or polluted soil. Different letters indicate significant differences at $P<0.05$.

Growth condition	H_2O_2 (µmol g^{-1} FW)	MDA (nmol g^{-1} FW)	Free Proline (µmol g^{-1} FW)	Soluble Protein (mg g^{-1} FW)
Control NM	2.44 ± 0.08 c	8.65 ± 0.21 b	1.00 ± 0.09 a	1.11 ± 0.34 a
Control M	2.83 ± 0.08 ab	9.48 ± 0.22 a	0.96 ± 0.04 a	0.96 ± 0.16 a
Polluted NM	3.07 ± 0.16 a	8.55 ± 0.23 b	0.43 ± 0.05 b	0.37 ± 0.07 b
Polluted M	2.52 ± 0.15 bc	8.41 ± 0.32 b	0.31 ± 0.04 b	0.53 ± 0.03 b