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Research paper

Symbiotic association between *Salix purpurea* L. and *Rhizophagus irregularis*: modulation of plant responses under copper stress

Adriana M. Almeida-Rodríguez[1,4], Marcelo P. Gómes[2], Audrey Loubert-Hudon[1], Simon Joly[1,3] and Michel Labrecque[1,3]

[1]Département de Sciences Biologiques, Institut de Recherche en Biologie Végétale (IRBV), Université de Montréal, 4101 Sherbrooke East, Montréal, QC, Canada H1X 2B2; [2]Institut des Sciences de l'environnement, Université du Québec à Montréal, Succ. Centre-Ville, C.P. 8888, Montréal, QC, Canada H3C 3P8; [3]Montreal Botanical Garden, 4101 Sherbrooke East, Montréal, QC, Canada H1X 2B2; [4]Corresponding author (adriana@ualberta.ca)

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There are increasing concerns about trace metal levels such as copper (Cu) in industrial sites and the broader environment. Different studies have highlighted the role of mycorrhizal associations in plant tolerance to trace metals, modulating some of the plant metabolic and physiological responses. In this study, we investigated the role of the symbiotic association between *Rhizophagus irregularis* and *Salix purpurea* L. in modulating plant responses under Cu stress. We measured Cu accumulation, oxidative stress-related, photosynthetic-related and hydraulic traits, for non-inoculated (non-arbuscular mycorrhizal fungi) and inoculated saplings exposed to different Cu concentrations. We found that *S. purpurea* is a suitable option for phytoremediation of Cu, acting as a phytostabilizer of this trace metal in its root system. We observed that the symbiotic association modulates a broad spectrum of metabolic and physiological responses in *S. purpurea* under Cu conditions, including (i) a reduction in gas exchange associated with chlorophyll content changes and (ii) the sequestration of Cu into the cell walls, modifying vessels anatomy and impacting leaf specific conductivity (K_L) and root hydraulic conductance (L_P). Upholding K_L and L_P under Cu stress might be related to a dynamic Aquaporin gene regulation of *PIP1;2* along with an up-regulation of *TIP2;2* in the roots of inoculated *S. purpurea*.

Keywords: Aquaporin (AQP), arbuscular mycorrhizal fungi, bioremediation, leaf specific conductivity (K_L), root hydraulic conductance (L_P).

Introduction

A vast concern is emerging worldwide related to the management and restoration of natural and industrial sites affected by the accumulation of complex mixtures of trace metals (such as copper (Cu)) and organic compounds as a result of industrial activities. Soil remediation using fast growing plants and their associated microbes is a relatively low cost, versatile and non-invasive alternative for decontamination of those sites (Chen et al. 2012). Copper is universally absorbed by plants, since Cu has an essential role in a wide range of metabolic activities in plants due to its ability to cycle between oxidized Cu(II) and reduced Cu(I) states (Theil and Raymond 1994). Its essential role in metabolism seems to have evolved during the development of dioxygen-generating photosynthesis in cyanobacteria, since the Cu homeostasis machinery is highly conserved among photosynthetic organisms (Burkhead et al. 2009). Copper plays key roles in photosynthesis, respiration, CO_2 fixation, gas exchange, perception of ethylene, reactive oxygen metabolism and cell wall remodelling (Mocquot et al. 1996, Burkhead et al. 2009). Copper levels in plants vary among species, with normal concentrations in plant shoots ranging from 2 to 50 μg $(g\ DW)^{-1}$ (Cohu and Pilon 2007). However, an excess of Cu could be detrimental for plants. One of the first symptoms of Cu toxicity is a reduction in root-to-shoot ratio, since this metal mainly accumulates in the roots (Mocquot et al. 1996, Navari-Izzo et al. 2006), being up-taken by COPT

transporters (Sancenón et al. 2003). In shoots, the most common symptoms of Cu toxicity are chlorosis (Burkhead et al. 2009), reduction of photosynthetic activity and growth cessation. These are due to the fact that Cu triggers the formation of reactive oxygen species (ROS) that disrupt the thylakoid membrane, especially the photosystem II (PSII), and chlorophylls (Kuper et al. 2004, Bernal et al. 2006). A detrimental effect of trace metals has also been observed in plants' water status and gas exchange (Llamas et al. 2008, Kholodova et al. 2011). Ice plants (*Mesembryanthemum crystallinum* L.) exposed to high concentrations of Cu have shown that osmotic adjustments occurred in line with inhibition of Aquaporin (AQP) gene expression and with a sharp drop in transpiration. However, gas exchange slowly stabilized below normal levels over time, suggesting potential plant phenotypic plasticity under Cu stress (Kholodova et al. 2011).

At the cellular level, plants have developed different mechanisms for coping with Cu toxicity. Chelation, by cysteine-rich metallothionein proteins, is one of them. Exclusion of Cu from the cytosol into the cell wall, the chloroplasts and the tonoplast by cation transporters like P-type ATPase HMA5 or PAA1 may also buffer cytosolic Cu concentrations (Cobbett and Goldsbrough 2002, Hall 2002, Bernal et al. 2006, Brunner et al. 2008). The movement and accumulation of Cu in various cellular organelles and the cell wall might occur with a dynamic osmoregulation facilitated by AQPs. The Tonoplast Intrinsic Proteins (TIPs) and the Plasma Membrane Intrinsic Proteins (PIPs) might be key facilitators of water movement during this process (Zhang et al. 2008). Aquaporins are active transmembrane proteins that facilitate the movement of water and small solutes in living membranes (Maurel et al. 2009). Some of these proteins are actively regulated under abiotic stress (Almeida-Rodriguez et al. 2010). In addition, a member of the PIP1s has been associated with resistance to trace metals in tobacco (Zhang et al. 2008).

Different studies have highlighted the role of mycorrhizal associations with *Glomus* spp. in plant tolerance to trace metals (Chen et al. 2007, Novoa et al. 2009, Cicatelli et al. 2010, 2012, Lin et al. 2014). Arbuscular mycorrhizal fungi (AMF) associations occur in >90% of vascular plants (Wang and Qui 2006) and they were crucial during plant colonization of terrestrial habitats (Helgason and Fitter 2005). Arbuscular mycorrhizal associations are symbiotic, where AMF are able to extend throughout much more soil surface areas than plant roots alone, providing water and nutrients, particularly phosphorus and micronutrients, to the plant (Willis et al. 2013). Arbuscular mycorrhizal fungi are, thus, particularly beneficial for plants growing not only under depleted soil conditions but also under biotic and abiotic stresses (Cicatelli et al. 2010). In exchange, the plant transfers photosynthetic carbon products to the fungi and provides them with an ecological niche (Novoa et al. 2009). Cicatelli et al. (2010) have shown the beneficial effects of AMF *Funneliformis mosseae* (formerly *Glomus mosseae*) and *Rhizophagus irregularis* (formerly *Glomus intraradices*) in restoring growth in *Populus alba* L. under Cu and zinc stress. *Populus alba* showed a strong transcriptional response to high concentrations of these metals that is modulated by the presence of AMF, resulting in a protective effect at the metabolic level (Cicatelli et al. 2012).

Populus and *Salix* belong to the Salicaceae family. This family is characterized by their fast growth rates and high biomass production, their easy vegetative propagation and their tolerance to high metal concentrations (Todeschini et al. 2007, Kuzovkina and Volk 2009, Cicatelli et al. 2012). They can also be colonized to different degrees by arbuscular and ecto-mycorrhizal fungi (Karliński et al. 2010). These features make poplars and willows suitable for phytoremediation of Cu-polluted sites (Labrecque and Teodorescu 2001, Cloutier-Hurteau et al. 2014). In this regard, the identification of phytostabilizer plants for trace metals and their use in remediation practices is of great importance, since phytostabilizer plants might reduce the leaking of trace metals into water bodies (Todeschini et al. 2007). In addition, the low translocation of trace metals to the aerial parts of the plant is an important trait for preventing herbivores from ingesting high amounts of these elements during feeding, limiting the movement of metals into the food chain.

In this study, we investigated the role of the symbiotic association between *R. irregularis* and *Salix purpurea* in the decontamination of soils containing three different levels of Cu. We evaluated the way in which *S. purpurea* accumulates and stores this trace metal in different organs. In addition, we studied the different physiological responses of *S. purpurea* under Cu stress, with special attention to the modulation of the hydraulic relations of this plant species by the AMF association, including root hydraulic conductance (L_P), leaf specific hydraulic conductivity (K_L) and root-AQP gene expression profiles.

Materials and methods

Plant growth conditions and treatments

Stem cuttings of *S. purpurea* L. cv. 'Fish Creek' were rooted in 130 ml rootrainer books (Stuewe & Sons, Inc., Tangent, OR, USA) containing an autoclaved soil mixture of two parts potting soil, one part expanded vermiculite and half a part sand. Stem cuttings were randomly assigned to two groups. Cuttings of the first group were planted on the same day and used for above-ground physiological measurements, molecular assays and root colonization rates. Cuttings of the second group were planted in groups (blocks) of six plants every day for eight consecutive days. This group was used for hydraulic, anatomical and biomass measurements, and trace elemental analysis. Half of the rooted cuttings for both groups were randomly selected and inoculated with spores of the AMF *R. irregularis* (~80 spores per millilitre of soil) 3 days after being planted (Mycorhise® ASP, Premier Tech Biotechnologies, Rivière-du-Loup, QB, Canada). Inoculated (AMF) and non-inoculated (non-AMF) rooted cuttings were kept separated in small chambers

during 3 weeks in the greenhouse (see growing conditions below). They were watered every 2 days.

Approximately 135 l of autoclaved soil (same mixture as described above) was spiked with different concentrations of Cu 1 month previous to repotting for equilibration. One-third was watered with deionized water (control); one-third was spiked to a final concentration of 66.7 p.p.m. free Cu (117.6 p.p.m. $CuSO_4$ and 2.4 p.p.m. $CuCl_2$ dissolved in deionized water), referred to as low copper (low Cu); and the last third was spiked to a final concentration of 156.78 p.p.m. free Cu (274.37 p.p.m. $CuSO_4$ and 5.599 p.p.m. $CuCl_2$ dissolved in deionized water), referred to as mild Cu treatment (mild Cu). Three-week-old saplings from non-AMF and AMF treatments were randomly repotted in the three different Cu treatments starting on 21 August 2012. Plants were at most 20 cm tall at the outset of the Cu experiment. Individual pots were placed in Sunbags (Sigma-Aldrich(R), St Louis, MO, USA) for avoiding spore contamination. Saplings were grown for 5 months before the analyses under a semi-controlled greenhouse environment (18 h/6 h light/dark, 22/20 °C day/night, 60% relative humidity), watered once a week and fertilized once a week using Hocking's modified complete fertilizer.

Above-ground measurements

Basal stem diameter (BSD) and plant height (*H*) were measured right before conducting the above-ground physiological measurements. Basal stem diameter was measured on the lower portion of the stem above the initial cutting using calipers. Leaves for stomatal conductance (g_s), chlorophyll content and chlorophyll fluorescence measurements were selected according to the leaf plastochron index (LPI) (Larson and Isebrands 1971). The aforementioned measurements were performed for leaves between LPI 7 and LPI 10, using a SC-1 leaf porometer (Decagon Devices Inc., Pullman, WA, USA) and a LEAF-chlorophyll metre (FT Green LLC, Wilmington, DE, USA), following the respective manufacturer's instructions. All measurements were performed between 9 am and 11 am. Chlorophyll fluorescence was assessed in fully expanded leaves equilibrated in the dark for 20 min using a pulse-amplitude modulation (PAM-2500, Heinz Walz GmbH, Effeltrich, Germany) chlorophyll fluorometer (Heinz Walz GmbH). An 11-step rapid light curve was performed. Saturating pulses were triggered at 48-s intervals using an increasing actinic light intensity at each step (0, 11, 60, 100, 170, 270, 420, 600, 970, 1410 and 2110 μmol photons m^{-2} s^{-1}). The electron transport through PSII (ETR) (Krall and Edwards 1992), the photochemical quenching (qP), the non-photochemical quenching (NPQ) (Redondo-Gómez et al. 2007) and the maximal PSII photochemical yield ($F_vF_m^{-1}$) were calculated using fluorescence parameters. The ETR and NPQ were calculated at 420 μmol photons m^{-2} s^{-1}, since it is the closest irradiation in relation to light growth conditions. All measurements were taken between 9 am and 11 am.

Organ collection

Immediately following the non-disrupting physiological measurements, fully expanded leaves were harvested and stored at –80 °C. These leaves were used for biochemical assays, oxidative responses and antioxidant systems. Total roots for each plant were also harvested, quickly washed and split into two groups. Half of the roots were immediately frozen in liquid nitrogen for molecular assays and stored at –80 °C until processing. The other half were directly fixed in a solution containing 4% formaldehyde, 2% glutaraldehyde and 1× phosphate-buffered saline (PBS) and stored at 4 °C for root colonization rate quantification until processing. A sample of the rhizosphere was also collected, frozen in liquid nitrogen and stored at –80 °C until processing for molecular assays.

Conductive stem areas, leaf area and biomass

The group of plants selected for these measurements were harvested in the same order as originally planted, six plants per day, each one of them representing one of the six treatments, during 8 days. Complete stems with leaves were harvested and kept in a humid dark chamber to avoid dehydration. In the laboratory, a stem segment of 17 cm was cut under water above 20 cm height from the root collar. The distal 2 cm of it was cut under water and kept in 70% ethanol at 4 °C for wood anatomical measurements. The remaining 15 cm of the stem segment was used for hydraulic measurements. Each segment was fitted to a tubing apparatus (Sperry et al. 1988) where the initial net flow rate through the segment was measured, using degasified and filtered solution containing 20 mM KCl + 1 mM $CaCl_2$. Hydraulic conductance (K_h) was calculated as the relationship between the flow rate through the segment and the pressure gradient along the segment, according to Hacke et al. (2010). Right after the stem flow rate was measured; the stem was perfused under vacuum pressure (10 kPa) with 0.1% safranin dying solution for 30 min according to Sperry and Tyree (1988). Transverse sections of the middle part of the safranin dye segment were observed under a microscope for the determination of conductive stem areas (μm^2). Images were taken with ZEISS Axio Imager 1 microscope equipped with a ZEISS Axiocam HRc camera at ×20 (Carl-Zeiss, Oberkochen, Germany). The net hydraulic conductivity (K_S) was determined as the K_h per conductive stem area (Sperry et al. 1988). After the transverse sectioning, the remaining pieces of the stem segment were pooled along with the rest of the stem(s) for dry biomass determination. All the downstream leaves from the harvested segment were collected and their leaf area (LA) was determined (m^2) using a ΔT area meter MK2 (Delta-T Devices, Cambridge, UK). The net leaf specific conductivity (K_L) was determined as the K_h per LA supplied by the measured xylem segment (Sperry et al. 1988).

The downstream leaves, stems, most of the roots and a rhizosphere sample of each plant were separately stored in paper bags or a metallic tray and oven-dried for 4 days at 60 °C for dry biomass and trace elements analyses.

Vessel and fibre diameters and double cell wall thickness measurements

Stem sections were fixed in 4% formaldehyde, 50% ethanol, 5% glacial acetic acid, 0.1% Tween 20, processed, embedded in paraplast and sectioned as described previously (Sutton et al. 2007). Cross sections (8–10 µm) were prepared using a microtome (Leica RM2155, Leica Biosystems, Wetzlar, Germany). Sections were mounted, dewaxed and rehydrated as described previously (Almeida-Rodriguez et al. 2011). Slides were incubated in 0.01% toluidine blue solution for 3–5 min. Images were taken using a ZEISS Axio Imager 2 microscope equipped with a ZEISS AxioCam MR3 camera (Carl-Zeiss). Three to five biological replicates were included for each treatment. Area and diameters were measured in at least 300 vessels, distributed in at least three distinct areas of the section. Fibre areas and diameters, as well as vessel and fibre double cell wall thickness, were measured in 50 cells in at least three distinct areas of the section. Magnification for vessels and fibre area and diameters was ×200. Double cell wall thickness was measured under magnification ×400. Pictures were analysed using ImageJ software (rsbweb.nih.gov).

Root hydraulic conductance

Individual white root tips were harvested for hydraulic L_P calculations according to Almeida-Rodriguez et al. (2011). Briefly, three root segments with intact root tips were collected for plant. Roots were trimmed to a final length of 5 cm under water leaving the root cap intact. The cut end of each root was inserted into a high-density polyethylene (HDPE) tubing system. The free space between the root and the HDPE was filled with dental impression material and sealed with cyanoacrylate glue. The root segment and HDPE tubing were then fitted to a tubing system connected to a vacuum pump. The root segments were immersed in a reservoir of filtered (0.2 µm) ultrapure water placed on an analytical balance (0.1 mg to 220 g; Sartorius, Gottingen, Germany). The root-tubing system was stabilized for 10 min, and then weight changes were recorded every 5 s. Water flow through the root segment was promoted by applying a partial vacuum to the open end of the tubing system. Pressure was consecutively decreased to −10, −20, −30 and −40 kPa. The flow rate (Q_v, $m^3\ s^{-1}$) was recorded at each pressure after it stabilized. The roots were then exposed to 0.1 mM $HgCl_2$ (an AQP inhibitor) following the above-mentioned steps and the flow rate was recorded. Finally, the roots were transferred back to water and the same procedure was repeated. After the whole procedure, the root integrity was confirmed using a ZEISS SteREO Discovery.V12 stereomicroscope equipped with a ZEISS Axiocam HRc digital camera (Carl-Zeiss). The surface area of the root segment was calculated using ImageJ (surface area = $2\pi r^2 + 2\pi r \times$ length). Root hydraulic conductance was calculated as the slope of the linear regression of the volumetric flux density ($J_v = Q_v$ per unit surface area of the root segment, $m\ s^{-1}$) on the vacuum pressure (North et al. 2004). The order in which the roots from each treatment were measured was randomized.

Copper and other trace elemental analysis

Oven-dried above-ground organs (leaves and stems) were pooled, while roots and rhizosphere were processed separately. All samples were ground to a fine powder using a coffee grinder. Two hundred milligrams of each sample, along with controls and blanks, were digested in 2 ml nitric acid overnight. The tubes were then incubated in a dry bath at 120 °C for at least 5 h until each sample was clear and no fumes were visible. Above-ground pooled samples were diluted in ultrapure water to 4%. Roots and soil samples were diluted to 5%. Trace elemental analysis was performed using a plasma mass spectrometer (ICP-MS) at the Trace Metal Analysis Laboratory (Department of Agricultural and Environmental Sciences, McGill University, Montreal, QC, Canada).

Oxidative responses and antioxidant systems

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents, as well as the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), were determined in three plants per treatment, according to Gomes et al. (2014). Briefly, H_2O_2 and MDA contents were evaluated as described in Velikova et al. (2000) and Hodges et al. (1999). For antioxidant enzyme characterization, 0.2 g of macerated leaves was extracted with a buffer containing 100 mmol l^{-1} potassium phosphate (pH 7.8), 100 mmol l^{-1} ethylenediaminetetraacetic acid, 1 mmol l^{-1} l-ascorbic acid and 5% PVP10 (m/v). Protein content was determined using Bradford method, and the activities of SOD (EC 1.15.1.1; Beyer and Fridovich 1987), CAT (EC 1.11.1.6; Azevedo et al. 1998) and APX (EC 1.11.1.11; Nakano and Asada 1981) were determined.

Root colonization rates

One gram of small fixed roots with 4% formaldehyde, 2% glutaraldehyde and 1× PBS was selected from total roots and added to a 15 ml tube. Roots were clarified twice in hot 10% KOH and incubated on a hot plate for 2 h. Roots were rinsed three times in dH_2O and stored in 1% trypan blue dying solution in the dark until microscopy slides were mounted (Phillips and Hayman 1970). The stained roots were gently washed in 50% glycerol. Sections of 1 cm were mounted. At least 125 cm of roots for three to five biological replicates were observed for colonization rate determination for each treatment. Images were taken with ZEISS Axio Imager 2 microscopes equipped with a ZEISS Axiocam HRc camera (Carl-Zeiss). Magnifications were ×200, ×400 and ×640. Root length was measured using ImageJ.

Glomeraceae mitochondrial DNA quantification

Mitochondrial DNA quantification in roots and soil samples using a *Glomus*-specific primer for the conserved unigene NADH

dehydrogenase subunit 1 (*NAD1*) was performed by a TaqMan quantitative real time polymerase chain reaction (qRT-PCR) assay (forward primer: 5′-GCTATGAACTTCCTTTGGCTATGGT-3′, reverse primer: 5′-GCCACACCCCTTGTTGAAATTC-3′) and a TaqMan probe (5′-FAM-TCAATAACAGGTTCGCTATCC-3′) (Micali C. et al. at Marc St-Arnaud's Lab, University of Montreal, unpublished data). Briefly, 100 mg of ground root tissue was extracted using DNeasy Plant Mini kit DNA extraction (Qiagen, Venlo, Netherlands) following manufacturer's protocol. Two hundred and fifty milligrams of soil was extracted using Power soil® microbial genomic DNA extraction kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. Total DNA was run in 1% agarose gel for quality control and quantified using Qubit dsDNA HS assay (Life Technologies, Carlsbad, CA, USA). Copy number of mitochondrial *NAD1* DNA was quantified using a standard curve with eight serial dilution points starting at 4.8×10^8 DNA copies of this gene. Real-time PCR was performed on an Applied Biosystems viiA™ Real-Time PCR system (Life Technologies). Three biological replicates, each with two technical replicates, were assayed for each sample. The PCR was carried out in a final volume of 20 µl including 38 ng DNA, 1× TaqMan® Environmental master mix 2.0 (Applied Biosystems, Foster City, CA, USA) and 1 µl of TaqMan polymerase solution containing *NAD1* primer. PCR conditions were as follows: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min. Samples were subjected to auto-C_t (cycle threshold) for analysis.

Phylogenetic analysis of AQPs in S. purpurea

Salix purpurea AQP gene models were identified from a *de novo* assembled transcriptome (A.M. Almeida-Rodriguez, E. Gonzalez, S. Joly, unpublished data) (the nucleotide sequences reported in this article have been submitted to GenBank with accession numbers: KR866280, KR866281, KR866282, KR866283, KR866284, KR866285, KR866286, KR866287, KR866288, KR866289, KR866290, KR866291, KR866292, KR866293, KR866294, KR866295, KR866296, KR866297, KR866298, KR866299, KR866300, KR866301, KR866302, KR866303, KR866304, KR866305, KR866306, KR866307, KR866308, KR866309, KR866310), using *Arabidopsis thaliana* L. and *Populus trichocarpa* L. AQP amino acid sequences as tBLASTn queries. Aquaporin-deduced amino acid sequences from *S. purpurea* and *P. trichocarpa* were aligned using MUSCLE in MEGA v5.2.2 (Tamura et al. 2011). A phylogenetic tree was constructed using maximum likelihood in MEGA with 1000 bootstrap replicates for branch support, using the WAG substitution model.

Gene expression profile of Salix AQPs

Total RNA from roots was extracted using the hexadecyltrimethylammonium bromide extraction protocol (Chang et al. 1993). Two micrograms of total RNA was treated with deoxyribonuclease I (New England Biolabs(R), Ipswich, MA, USA) and used as template for first-strand cDNA synthesis using oligo$(dT)_{23}$ VN and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Amino acid sequences of three reference genes previously reported in *P. trichocarpa* (Almeida-Rodriguez et al. 2011) were used to search for reference gene candidates in the *S. purpurea* transcriptome mentioned above, using tBLASTn (Elongation Factor-1-alpha (*EF1A*), Eukaryotic Translation Initiation Factor 5A (*TIF5A*) and Protein Serine/Threonine Phosphatase (*PP2A*); see Table S1 available as Supplementary Data at *Tree Physiology* Online. Gene-specific qRT-PCR primers and probes were designed including the 3′ end of the coding region and the 3′UTR region of reference genes and 13 AQP candidate sequences (*PIP1;1*, *PIP1;2*, *PIP2;1*, *PIP2;2*, *PIP2;3*, *TIP1;2*, *TIP1;3*, *TIP1;4*, *TIP1;5*, *TIP2;2*) using Geneious bioinformatic software platform (Biomatters, CA, USA) (see Table S1 available as Supplementary Data at *Tree Physiology* Online). Real-time PCR was performed on an Applied Biosystems viiA™ Real-Time PCR system (Life Technologies). For each gene, PCR efficiency (E) was determined from a four-point cDNA serial dilution, according to $E = 10^{[-1/\text{slope}]}$. Although different pairs of primers and probes were tested, primer validation or efficiency tests failed for *SpPIP1;3*, *SpNIP2;1* and *SpXIP1;2* (data not shown) and these AQPs were not included in the qRT-PCR study. Three biological replicates, each with three technical replicates, were assayed for each sample. Assays were carried out in 384-well plates. Three reference genes and four target genes were included on every plate, using one of the non-AMF/control Cu sample as a calibrator. PCR was carried out in 10 µl reaction with 9.375 ng of cDNA and 1× TaqMan master mix (Life Technologies) under the following conditions: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min. Samples were subjected to auto-C_t (cycle threshold) for analysis. Relative transcript abundance for each target gene was calculated using the comparative C_t method ($\Delta\Delta C_t$), in which each target gene was normalized against the arithmetic mean of transcript abundance corresponding to the three reference genes, and data were expressed in relative terms.

Statistical analysis

Data were analysed using JMP 11 (SAS Institute, Cary, NC, USA). For most of the measurements, a complete randomized design analysis of variance was followed by Tukey honest significance difference (HSD) test when whole test was significant. For the roots and soil samples in the Cu trace element analysis, analysis of variance was performed in Box–Cox transformed data. For all parametric tests, differences were considered statistically significant at $P < 0.05$. Non-parametric analysis comparing different pairs was done for vessel area (VA) and NAD1 measurements, respectively. P-values were adjusted to the number of comparisons (Bonferroni correction).

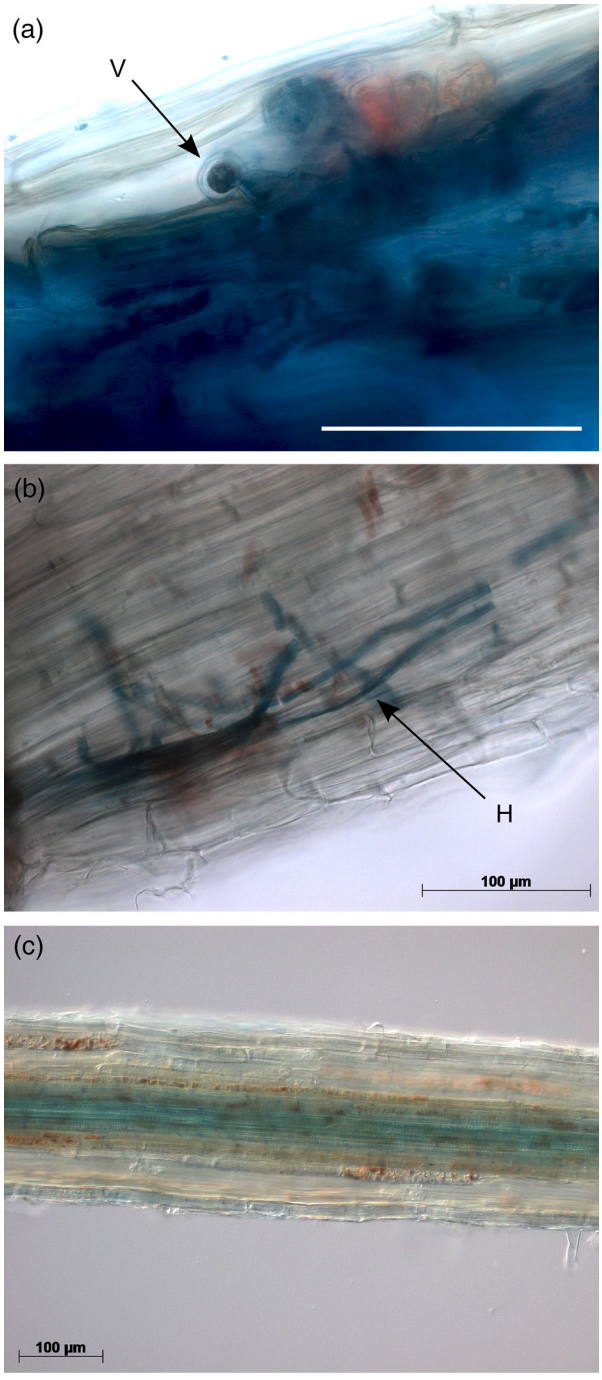

Figure 1. Light microscope images of *S. purpurea* fine roots showing *R. irregularis* colonization (a) and (b) or a non-colonized root (c). Horizontal bars = 100 µm. Arrows point to vesicle (v) and hyphae (h) structures of the mycorrhizal fungi.

Results

Mycorrhizal association and Cu accumulation in plant tissues

Fungus intracellular structures were observed in low abundance during the colonization rate exploration in the AMF treatment, mainly hyphae and vesicles (Figure 1). Colonization rates were supported by quantitative estimation of *Glomus* sp. mitochondrial DNA in the roots and rhizosphere. Significant differences were observed in the number of NAD1 molecules between the roots and rhizosphere of AMF plants when compared with non-AMF ones (Table 1). The rhizosphere of AMF saplings had the highest amount of NAD1 molecules when compared with other samples. The NAD1 copies observed in the AMF roots were 5.6% for control Cu, 16.7% for low Cu and 2.49% for mild Cu that of their respective rhizosphere values (Table 1), confirming the low colonization rate observations. Some NAD1 copies were sometimes detected in non-AMF soil and roots, but in a very low amount when compared with the AMF saplings. Guaranteeing complete control of cross-contamination in this type of study is very difficult, since plants were grown under a semi-controlled environment and they were randomized in the greenhouse space. We tried to control cross-contamination by enclosing the plant pots into sunbags, but a better isolation system might be considered in follow up experiments. Nonetheless, we assume that the physiological responses of these plants should not be influenced by the scarce presence of the AMF, since very low rates of DNA copies of the mitochondrial NAD1 were detected in the non-AMF plants.

Table 1. *Glomus* sp. NAD1 quantification of *S. purpurea* roots and rhizosphere samples by a TaqMan® qRT-PCR assay (Applied Biosystems). Saplings were inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Number of copies of mitochondrial NAD1 were determined using a standard curve with eight serial dilution points starting at 4.8×10^8 DNA copies. Three biological replicates, each with two technical replicates, were assayed for each sample. Values represent means ± SD. Different letters indicate that the means were significantly different between AMF treatments at each different type of sample (roots and rhizosphere). Non-parametric test followed by comparison of each pair using Wilcoxon test ($P < 0.05$).

AMF treatment	Cu treatment	Roots	Rhizosphere
		NAD1 molecules $\times 10^5$	NAD1 molecules $\times 10^5$
Non-AMF	Control Cu	0.000 ± 0.000^A	0.133 ± 0.128^a
Non-AMF	Low Cu	0.139 ± 0.137^A	0.066 ± 0.115^a
Non-AMF	Mild Cu	0.066 ± 0.115^A	0.086 ± 0.150^a
AMF	Control Cu	1.246 ± 0.625^B	22.026 ± 12.093^b
AMF	Low Cu	0.310 ± 0.198^B	1.853 ± 0.397^b
AMF	Mild Cu	0.406 ± 0.430^B	16.310 ± 17.640^b

Salix purpurea mainly accumulates Cu in its root system (Figure 2b), where it is directly related to the amount of the metal present in the rhizosphere (Figure 2c). When little Cu is present in the soil (control treatment), the shoot accumulates ~50% of what is accumulated in the roots. However, saplings exposed to Cu treatments accumulated in their shoots only 5 and 3% of the total Cu accumulated in their roots when exposed to low and mild Cu, respectively. We found no significant differences in the accumulation of Cu between non-AMF and AMF plants ($P = 0.06$).

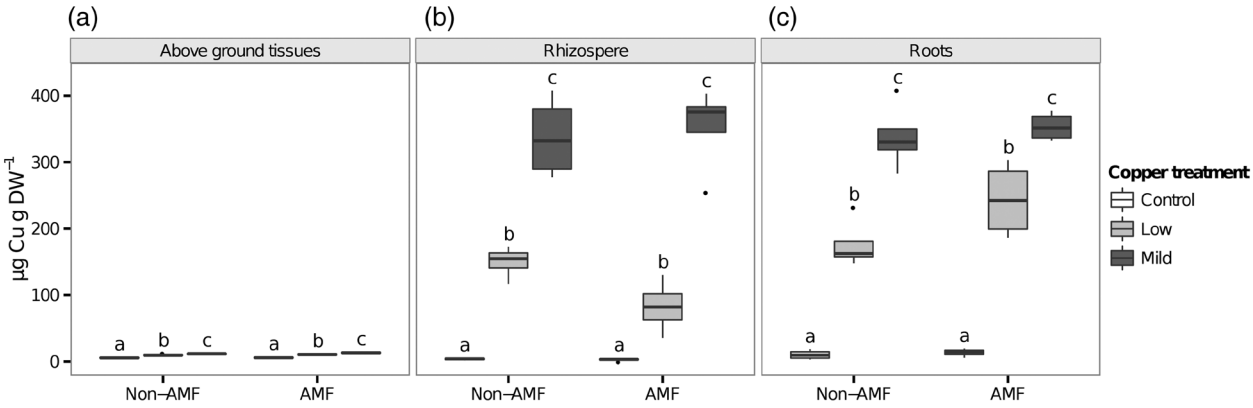

Figure 2. Total Cu concentration (µg g^{-1}) in pooled above-ground tissues (a), roots (b) and rhizosphere (c) of *S. purpurea* saplings inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Box-and-whisker plots. Different letters indicate that the means were significantly different between treatments. The best transformation of data was selected by Box–Cox transformation for both (b) and (c) for normalizing the data. Analysis of variance followed by Tukey HSD test ($P < 0.05$).

Table 2. Basal stem diameter, plant height, LA and root-to-shoot ratio. *Salix purpurea* saplings inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Five to eight biological replicates were assayed for each treatment. Values represent means ± SD. Analysis of variance test ($P < 0.05$).

Mycorrhizal	Cu	BSD (mm)	Height (cm)	LA (m^2)	Root-to-shoot ratio
Non-AMF	Control Cu	5.78 ± 0.21	136.40 ± 14.29	0.02300 ± 0.00487	0.218 ± 0.035
Non-AMF	Low Cu	5.82 ± 0.32	136.50 ± 5.71	0.02240 ± 0.00584	0.171 ± 0.056
Non-AMF	Mild Cu	5.67 ± 0.85	132.18 ± 20.07	0.02323 ± 0.00459	0.186 ± 0.027
AMF	Control Cu	5.58 ± 0.22	144.00 ± 15.44	0.02412 ± 0.00439	0.172 ± 0.043
AMF	Low Cu	5.25 ± 0.40	124.36 ± 15.90	0.02377 ± 0.00228	0.191 ± 0.049
AMF	Mild Cu	5.67 ± 0.70	126.36 ± 13.90	0.02358 ± 0.00468	0.182 ± 0.032

Table 3. Xylem features for *S. purpurea* saplings inoculated (AMF) and non-inoculated (non-AMF) with *R. irregularis* in response to Cu. Plants were grown in control soil, low Cu or mild Cu. Values represent means ± SD ($n \leq 5$). For D_v and VA, at least 300 vessels were measured for each biological sample. For other traits, at least 50 cells were measured for each biological sample. Different letters indicate that the means were significantly different between treatments. Analysis of variance test was followed by Tukey HSD test for vessel lumen diameters (D_v) and vessel (VDCT) and fibre double cell wall thickness (FDCT) measurements, respectively ($P < 0.05$). Non-parametric test followed by comparison of each pair using Wilcoxon test was used for VAs ($P < 0.01695$).

Mycorrhizal	Cooper	D_v (µm)	VA (µm²)	VDCT (µm)	Fibre lumen (D_f, µm)	FDCT (µm)
Non-AMF	Control Cu	28.03 ± 3.49^{B}	564.76 ± 104.30^{A}	1.22 ± 0.086^{A}	8.52 ± 0.671	1.47 ± 0.252^{C}
Non-AMF	Low Cu	28.20 ± 2.44^{B}	566.03 ± 46.58^{B}	1.42 ± 0.089^{B}	8.28 ± 0.679	1.48 ± 0.228^{C}
Non-AMF	Mild Cu	29.98 ± 2.63^{A}	632.06 ± 35.70^{C}	1.27 ± 0.090^{A}	8.74 ± 0.751	1.44 ± 0.215^{C}
AMF	Control Cu	29.98 ± 1.67^{A}	650.18 ± 60.30^{C}	1.4 ± 0.065^{B}	8.72 ± 0.689	1.38 ± 0.201^{B}
AMF	Low Cu	28.92 ± 2.44^{A}	588.72 ± 66.51^{B}	1.44 ± 0.106^{C}	8.24 ± 0.643	1.38 ± 0.165^{A}
AMF	Mild Cu	28.52 ± 2.26^{B}	559.00 ± 74.12^{A}	1.48 ± 0.070^{C}	8.35 ± 0.841	1.53 ± 0.194^{BC}

Morphological and physiological traits

There was no statistical difference among treatments for basal BSD, plant height, LA and root to shoot biomass ratio measurements (Table 2). However, non-AMF saplings showed an increase in the size of their vessels when exposed to Cu. In contrast, AMF saplings had narrower vessels under Cu (see vessel lumen diameters (D_v) and VA; Table 3). There was no significant difference in fibre diameters (D_f; Table 3) and for fibre areas among treatments (data not shown). Differences in vessel patterns were partially related to trends in K_L (Figure 3a). Non-AMF_mild Cu saplings exhibited the highest K_L compared with saplings of other treatments ($P < 0.05$).

Vessel double cell wall thickness showed an opposite pattern compared with the lumen measurements for AMF saplings, where thicker cell walls were observed in vessels with smaller lumen diameters ($P < 0.05$; Table 3). For fibre cell wall thickness, non-AMF plants exhibited thicker fibre cell walls along with AMF_mild Cu plants when compared with AMF plants in control and low Cu ($P < 0.05$).

The initial L_P values showed different trends among Cu treatments between non-AMF and AMF plants (Figure 3b). Root hydraulic conductance was lower and steady in AMF saplings. For non-AMF saplings, L_P showed a tendency towards lower values with increasing Cu, although the differences were not

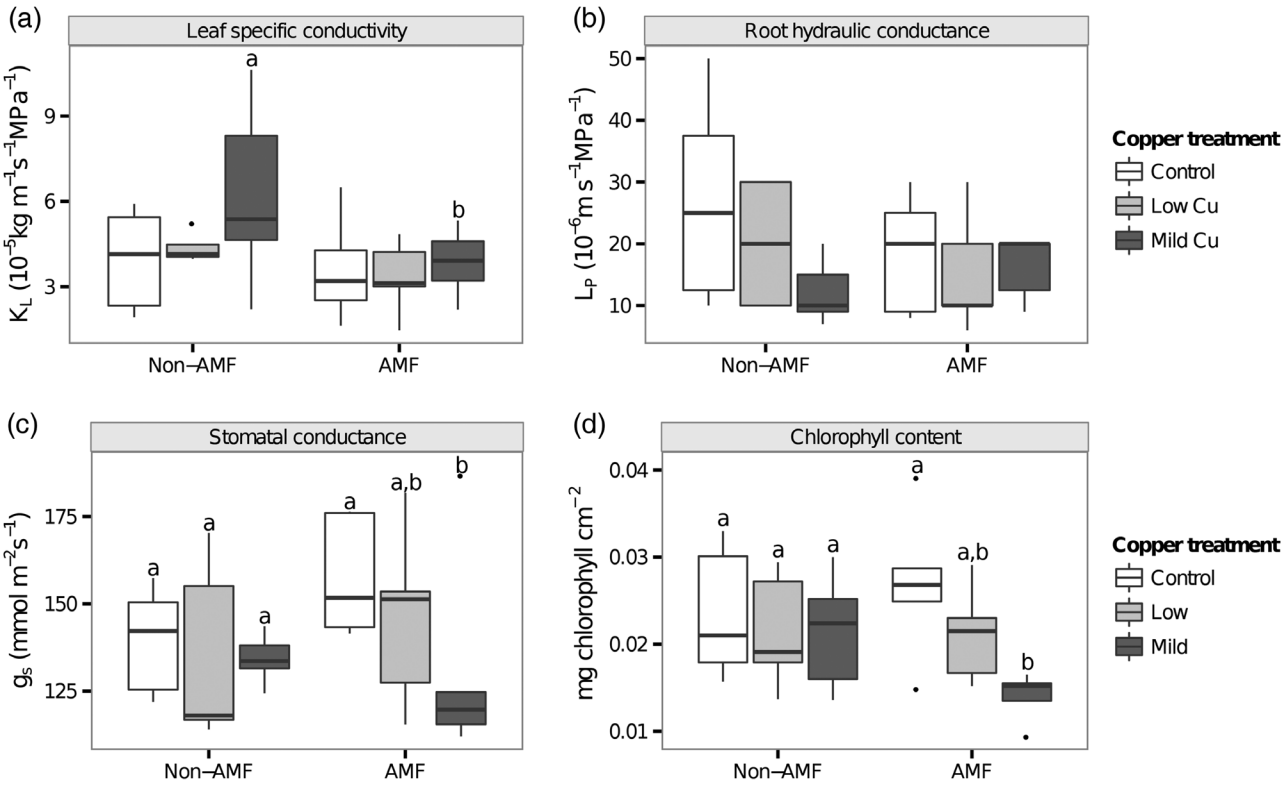

Figure 3. Leaf specific conductivity (K_L) (a), L_P (b), g_s (c) and chlorophyll content (d) of *S. purpurea* saplings inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Box-and-whisker plots. The best transformation of data was selected by Box–Cox transformation for normalizing the data for L_P. Analysis of variance followed by Tukey HSD test for g_s, chlorophyll content and K_L ($P < 0.05$).

Table 4. The relative rate of ETR, the qP, the NPQ and the maximal PSII photochemical yield ($F_vF_m^{-1}$) of fully developed leaves of *S. purpurea*. Saplings were inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Values represent means ± SD ($n \leq 5$). Different letters indicate that the means were significantly different between treatments. Analysis of variance test was followed by Tukey HSD test ($P < 0.05$).

Mycorrhizal	Cooper	ETR	qP	NPQ	$F_vF_m^{-1}$
Non-AMF	Control Cu	102.2 ± 16.51^{A}	0.790 ± 0.089	0.327 ± 0.578^{A}	0.77 ± 0.031
Non-AMF	Low Cu	97.3 ± 10.09^{AB}	0.791 ± 0.061	0.633 ± 0.195^{AB}	0.79 ± 0.009
Non-AMF	Mild Cu	87.6 ± 22.72^{B}	0.756 ± 0.139	0.896 ± 0.639^{B}	0.77 ± 0.013
AMF	Control Cu	113.3 ± 8.84^{A}	0.871 ± 0.044	0.314 ± 0.128^{A}	0.79 ± 0.004
AMF	Low Cu	100.8 ± 5.66^{AB}	0.825 ± 0.028	0.624 ± 0.125^{AB}	0.78 ± 0.001
AMF	Mild Cu	73.0 ± 15.12^{B}	0.680 ± 0.100	1.343 ± 0.397^{B}	0.78 ± 0.006

statistically significant ($P < 0.05$). When roots of non-AMF plants were exposed to the AQP inhibitor ($HgCl_2$), their L_P was reduced to 29.6, 18.7 and 11.43% for control, low Cu and mild Cu, respectively. When AMF plant roots were exposed to $HgCl_2$, L_P was diminished to 7.5, 8.53 and 25.1% for control, low and mild Cu, respectively. Root hydraulic conductance was recovered after $HgCl_2$ was removed in all cases (from 65.2 to 91.43%; data not shown).

Inoculated *S. purpurea* saplings reduced their g_s by 25% when grown under Cu when compared with controls (Figure 3c; $P < 0.05$). Differences in g_s were correlated with the chlorophyll content (Figure 3d), and AMF_mild Cu saplings exhibited a 27% reduction in their chlorophyll content compared with controls ($P < 0.05$).

Saplings exposed to mild Cu showed a decrease in ETR and qP and an increase in NPQ ($P < 0.05$; Table 4). In contrast, no significant differences were found for the $F_vF_m^{-1}$. An increment of SOD and APX activity was observed in *S. purpurea* exposed to mild Cu. Catalase activity did not change between treatments. Similarly, we did not observe differences in H_2O_2 or in MDA (Figure 4).

Salix purpurea AQP family

Thirty-five AQPs were identified in the de novo assembled transcriptome of *S. purpurea* (data not published). The organs included in the transcriptome were buds, leaves, roots and developing xylem from plants growing under control and polluted soil conditions. Twenty-seven recovered AQP sequences had complete coding sequences. Three incomplete sequences were elim-

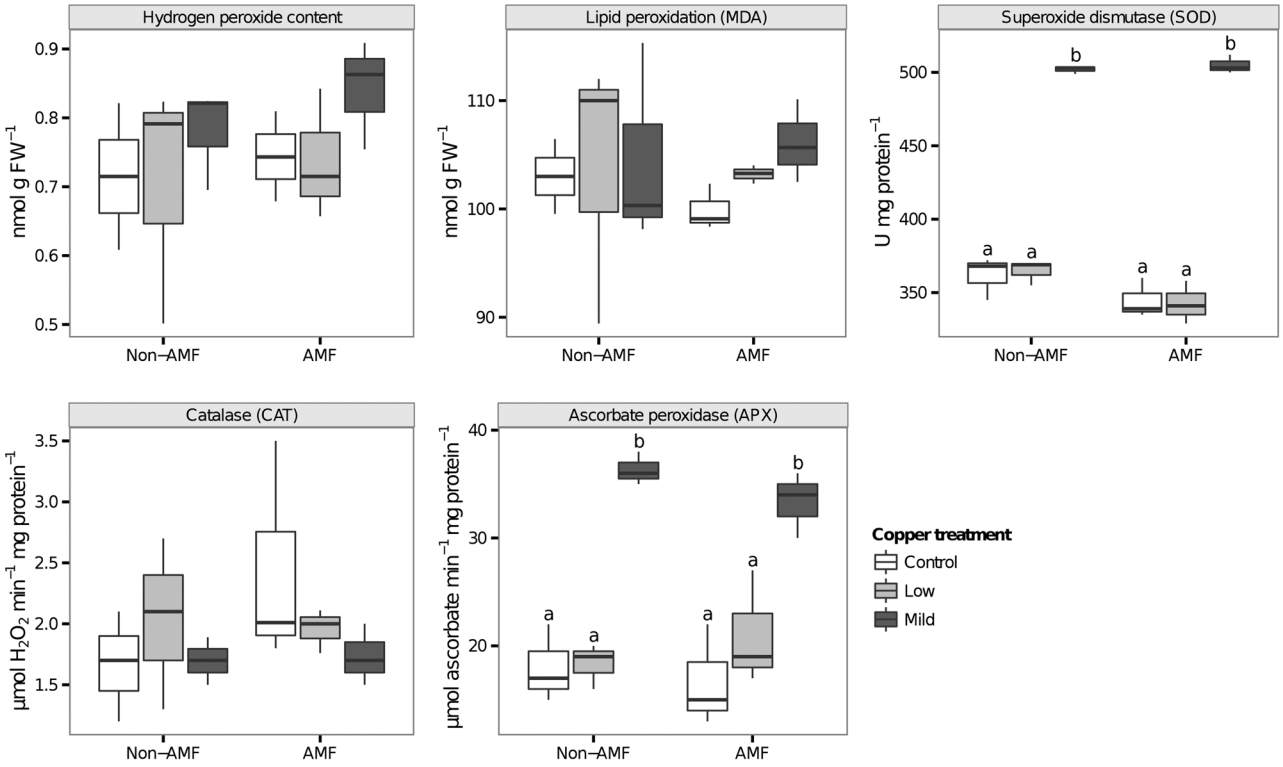

Figure 4. Hydrogen peroxide content (H_2O_2), lipid peroxidation (MDA) and activities of SOD, CAT and APX of *S. purpurea* saplings inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Box-and-whisker plots. Different letters indicate that the means were significantly different between treatments. Analysis of variance followed by Tukey HSD test ($P < 0.05$).

inated because they were too short. Additionally, one full-length sequence was eliminated since it branched outside all AQPs in preliminary phylogenetic analysis. The phylogenetic analysis including *S. purpurea* and *P. trichocarpa* AQPs is shown in Figure 4. *Salix purpurea* AQPs fell into the same five subfamilies as *P. trichocarpa*: PIPs, TIPs, Noduline-like Intrinsic Proteins, Simple Intrinsic Proteins and X-Intrinsic Proteins (Figure 4).

Gene expression of root AQPs

The effect of Cu on AQP transcript abundance of *PIPs* and *TIPs* for non-AMF and AMF *S. purpurea* saplings was estimated (Figure 5). Two *PIP1s* were assayed in this study: *PIP1;1* and *PIP1;2*. The former showed a higher though not significant transcript abundance in AMF saplings. The latter exhibited significant differences among Cu treatments in AMF plants ($P < 0.05$). The expression of *PIP1;2* increased in AMF_low Cu saplings and drops back significantly in AMF_mild Cu plants. Among the *PIP2s* evaluated, *PIP2;1* showed a decrease in expression with increasing Cu concentrations. For non-AMF plants, *PIP2;2* exhibited a slight increase of transcript abundance in Cu treatments compared with non-AMF_control Cu, but the variation was not significant. For AMF saplings, *PIP2;2* transcript abundance was steady among treatments. *Tonoplast Intrinsic Proteins* in AMF saplings growing under control Cu generally showed a tendency for a greater transcript abundance than their non-AMF counterparts, with the exception of *TIP1;5*, although only *TIP2;2* exhibited significant differences between AMF and non-AMF treatments. For non-AMF plants, *TIP2;2* transcript abundance gradually increased in response to Cu up to onefold change under mild Cu. The expression levels for this gene were higher for AMF than for non-AMF plants, and its expression was steady for all AMF plants across Cu treatments, ranging between 1.2- and 1.62-fold change when compared with the non-AMF_control Cu treatment.

Discussion

It is widely accepted that mycorrhizal associations are favoured under certain soil conditions, especially low nutrient or contaminated soils (Lins et al. 2006). However, in this study, AMF plants exposed to Cu showed low arbuscular formation and a reduction of 70% in the *Glomus NAD1* molecules when compared with AMF_control Cu. Inhibition of arbuscule production has been previously observed in *P. alba* cv. Villafranca but not in *Populus nigra* L. cv. Jean Pourtet when exposed to Cu (Todeschini et al. 2007). In addition, reduction in the AMF association under Cu stress has been previously studied in *Oryza sativa* L. (Lin et al. 2014) where Cu was found to inhibit the root colonization by AMF in 60%. A similar reduction in mycorrhizal associations and the presence of spores in the soil has also been observed in roots of plants exposed to other trace metals like Pb (Andrade

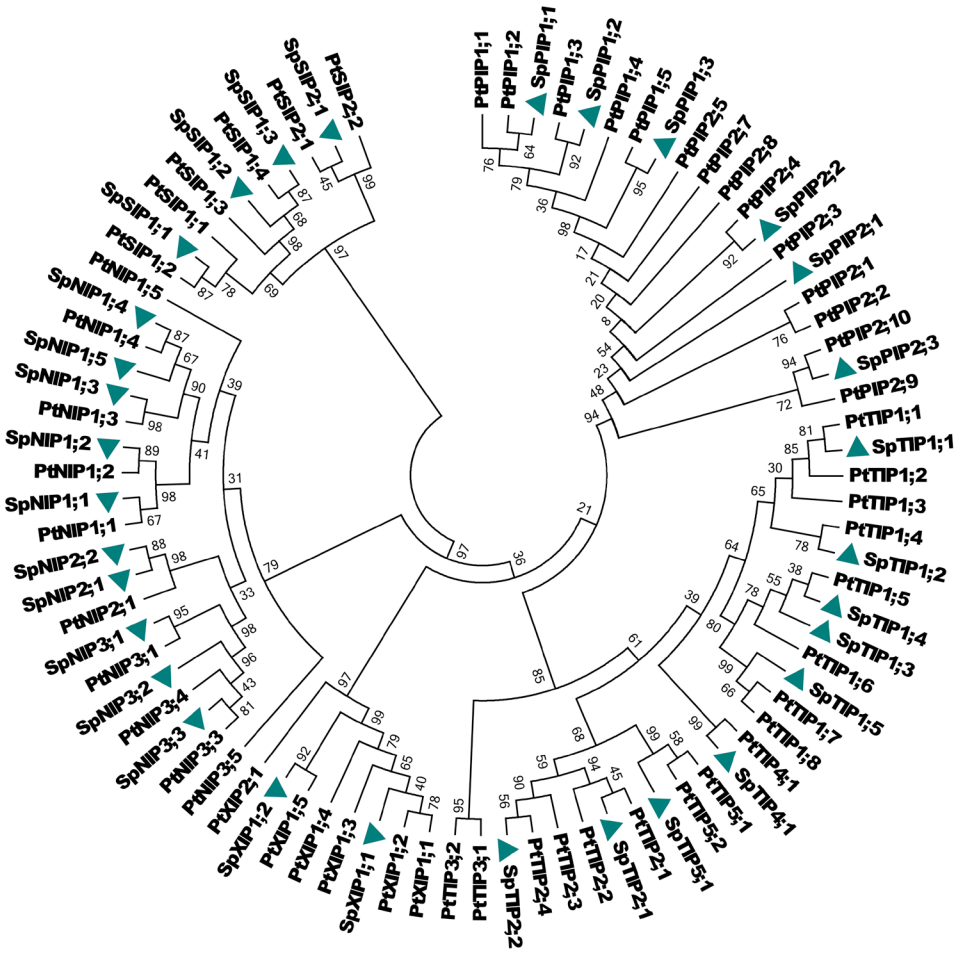

Figure 5. Dendrogram obtained using maximum parsimony showing relationships between members of the AQP family in *S. purpurea*. The AQP family from *P. trichocarpa* (Almeida-Rodriguez et al. 2010) was included for comparison. *Salix purpurea* AQPs are denoted with triangles.

et al. 2004, Lin et al. 2014). Therefore, the formation and establishment of mycorrhizal associations under trace metal stress also depend on the plant and fungus species. For instance, certain plants are more apt than others for developing AMF associations. The *Salix* genus is an example where species are not easily colonized by AMF (Parádi and Baar 2006), which may explain the low root colonization observed. Nevertheless, both intracellular (the Paris-type-like) and intercellular (Arum-type-like) hyphae were observed in the roots of *S. purpurea*. The two types of colonization are not mutually exclusive and have been described for *R. irregularis* (van Aarle et al. 2005). These types of colonization are mainly host dependent (Brundrett and Kendrick 1990) but could be influenced by the fungal species to some extent (Cavagnaro et al. 2001). Nonetheless, we cannot rule out the possibility of colonization of the roots by other fungi spores present in the greenhouse.

In *Salix* and other woody species, Cu accumulation occurs mainly in the roots (Todeschini et al. 2007, Cloutier-Hurteau et al. 2014). The extraction capacity of *S. purpurea* for this trace metal and mobilization to aerial tissues is rapidly attained and plants do not relocate more Cu from the soil at higher Cu soil concentration. As a result, the capacity to phytoremediate a Cu-polluted soil is limited by the number of plants able to grow in a specific area and the amount of Cu in the soil. In this regard, our results are in agreement with previous studies (Smith and Bradshaw 1972, Labrecque et al. 1995, Li et al. 2012). *Salix purpurea* reduces the mobility and bioavailability of Cu by immobilizing it into their roots, acting as a phytostabilizer of this trace metal in the soil. Phytostabilization of Cu in roots was also observed in *P. alba* and *P. nigra* (Todeschini et al. 2007), corroborating the suitability of the Salicaceae family for phytoremediation of soils contaminated with trace metals. It has been shown that the uptake of trace metals by plants could be enhanced by mycorrhizal associations (Hassan et al. 2013, Willis et al. 2013). In this study, however, there was no significant difference in Cu accumulation in organs of *S. purpurea* between AMF and non-AMF plants (Figure 6). Bissonnette et al. (2010) also reported similar results in a field experiment comparing willows (inoculated and/or not inoculated with *R. irregularis*) planted on a brownfield. In contrast, other studies have shown that mycorrhizal associations could instead protect the plant against metal stress via the sequestration of the trace elements

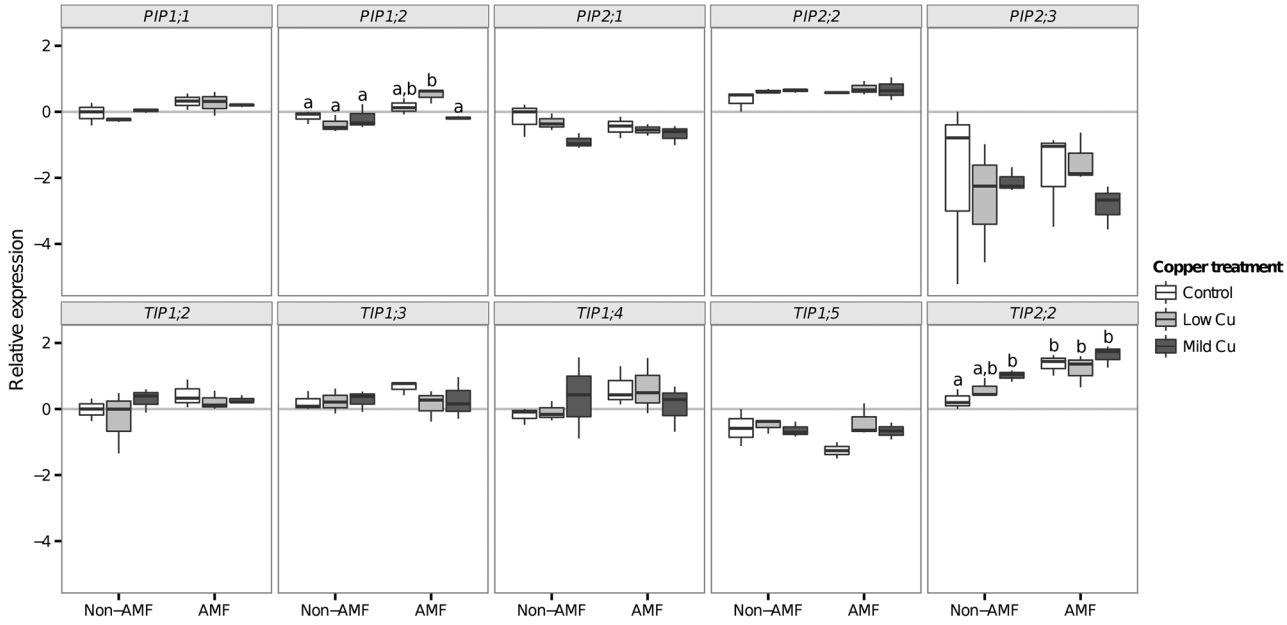

Figure 6. Gene expression of 10 AQPs in fine roots of *S. purpurea* saplings inoculated (AMF) or not (non-AMF) with *R. irregularis*. Plants were grown in control soil, low Cu or mild Cu. Relative transcript abundance for each AQP was normalized with the arithmetic mean of the expression levels of three reference genes (*EF1-α*, *PP2A* and *TIF5A*). Box-and-whisker plots. Different letters indicate that the means were significantly different between treatments. Analysis of variance followed by Tukey HSD test when whole test was significantly different ($P < 0.05$).

in the fungus (Göhre and Paszkowski 2006, Chen et al. 2007). Clearly, the way the mycorrhizal association promotes or protects the plant under trace metal stress depends on the fungus and plant species and their respective tolerances to the metal.

A study comparing the reduction in yield of various crops had set a threshold of 10 mg kg DW^{-1} of Cu accumulated in tissues before symptoms could be detected (Mocquot et al. 1996). In this study, we observed a marginal reduction in saplings height that had accumulated at least 10 mg kg DW^{-1} of Cu in their above-ground tissues (low Cu treatment). We also found that AMF plants showed a bigger reduction in height when compared with non-AMF plants. These results could be explained by the fact that plants provide sugars to the AM fungus, which could result in a reduction in the above-ground plant growth (Citterio et al. 2005).

Different studies have shown how Cu toxicity could have detrimental effects on chloroplast functions by substituting Mg^{2+} in the chlorophyll molecule and disrupting thylakoid membranes and the PSII (Kuper et al. 2004, Bernal et al. 2006). In this study, we observed a reduction in chlorophyll content in AMF saplings exposed to Cu that was very close to the threshold for affecting the efficiency of the PSII (threshold of 0.0128 mg chlorophyll cm^{-2}, according to manufacturer's manual). Decline in chlorophyll contents corresponded with reductions in ETR through the PSII and with g_s, suggesting that Cu affects PSII activity in this species. However, the concentration of Cu in both treatments was not sufficient for inducing oxidative stress in leaves, assessed by H_2O_2 contents along with lipid peroxidation, or for affecting the overall photochemical efficiency of the PSII ($F_vF_m^{-1}$).

When Cu is free in the cytoplasm, the redox cycling of Cu catalyses the production of ROS. Reactive oxygen species are signalling molecules strictly regulated in cells by a number of enzymatic and buffering processes, because their accumulation is chemically toxic and might signal programmed cell death (Liu et al. 2009, Gomes et al. 2014). In membranes, ROS might trigger lipid peroxidation and destabilization (Gunes et al. 2007) while producing the mutagenic compound MDA (Marnett 1999). Enzymatic control of ROS involves the reduction of the oxide ion ($O_2^{\cdot-}$) into H_2O_2 and it is catalysed by the SOD. Hydrogen peroxide is subsequently reduced to H_2O by CAT and peroxidase (Møller et al. 2007). Copper stress up-regulates this enzymatic scavenging, limiting chemical damage caused by high concentrations of ROS. Reactive oxygen species buffering involves the oxidation of glutathione for producing a cysteine-bridged GS–SG dimmer, along with the oxidation of ascorbate by APX for producing monodehydroascorbate. These oxidized buffers are reduced via NADPH, therefore restocking the electron-rich buffer pools (Liu et al. 2009). In this study, AMF_mild Cu and non-AMF_mild Cu *S. purpurea* saplings showed an increase in SOD and APX activity, indicating that oxidative stress and the subsequent enzymatic response in *S. purpurea* was triggered when doses of available Cu in the soil were ~150 p.p.m. and that it was not modulated by the AMF association.

Plants have developed different mechanisms for coping with Cu toxicity. Chelation and exclusion of Cu from the cytosol into the cell wall, the chloroplasts and the tonoplast may help control cytosolic Cu concentrations (Cobbett and Goldsbrough 2002,

Hall 2002, Bernal et al. 2006, Brunner et al. 2008, Konno et al. 2010). Copper sequestered into the cell wall is tightly bound to polysaccharides (Konno et al. 2010), increasing the cell wall thickness in mosses by inducing organizational changes in the cell wall polysaccharides and the morphology of their wall structure (Krzesłowska et al. 2009). In this study, structural changes in the wood anatomy of *S. purpurea* were in agreement with the translocation of Cu into the cell walls. Saplings exposed to Cu developed thicker cell walls in the xylem vessels under low concentrations of Cu. However, only AMF_mild Cu plants showed an increase in their cell wall thickness of the wood fibres, along with thicker cell walls in the xylem vessels, suggesting an AMF modulation effect in this trait under Cu stress. On the other hand, an antagonistic response was observed in D_v and VA, where non-AMF plants increased the size of their xylem vessels, promoting higher K_L, while showing a stabilized g_s in response to Cu. According to the cohesion-tension theory, water is transported from the soil to the atmosphere through the plant organs (roots, xylem and leaves) in a continuous water column triggered by gas exchange (Tyree 1997). Maintaining a steady demand of water via gas exchange while increasing the K_L under an unstable L_P might compromise the water status of non-AMF plants if the water availability becomes a limiting factor. Osmotic changes have been observed also in ice plant (*M. crystallinum*) exposed to high concentrations of Cu, although in that case, ice plants showed a sharp drop in transpiration (Kholodova et al. 2011). Differences in gas exchange between the two studies might be related to the duration of Cu exposure, since gas exchange slowly stabilized below normal levels over time in ice plants. The accumulation of Cu in the cell walls by the AMF plants under both Cu treatments might have an impact in cell elongation as previously reported by Krzesłowska et al. (2011), where the production of pectins along with the accumulation of metals in the cell wall produce an increase in the thickness and stiffness, which might inhibit cell elongation at high levels. Arbuscular mycorrhizal fungi association might modulate hydraulic traits in *S. purpurea* while maintaining a conservative water status by reducing the g_s, thus reducing the risk of hydraulic failure under Cu stress. By maintaining a steady L_P under Cu stress, AMF plants might experience a dynamic regulation of some PIPs and TIPs. Aquaporins might also be involved in the osmoregulation that is required during the translocation and accumulation of Cu in different cellular compartments and organelles.

Aquaporins are a large protein family in plants. Fifty-five AQPs have been previously identified in poplars (Gupta and Sankararamakrishnan 2009, Almeida-Rodriguez et al. 2010). *Populus* and *Salix*, from the Salicaceae family, share a recent whole-genome duplication event (Tuskan et al. 2006) that could have contributed to the large number of AQP genes in these genera. In this study, we identified 35 AQPs in the de novo assembled transcriptome of *S. purpurea* that correspond to five different subfamilies in accordance with the *Populus* genome (Gupta and Sankararamakrishnan 2009). We investigated the gene expression profile of five *PIPs* and five *TIPs* because these subfamilies are involved in cell osmoregulation. A previous study reported AQP inhibition in roots and leaves of ice plants as an immediate response to Cu stress with a recovery of the transcripts over time in two of the AQPs (Kholodova et al. 2011). In our study, *PIP1;2* transcript abundance was modulated by the AMF association, while *TIP2;2* was differentially modulated by both Cu and AMF association. A contrasting response for *TIP2;2* was observed in ice plants in response to Cu. *TIP2;2* was inhibited during the first 24 h of Cu exposure and then reached normal levels after 7 days under Cu stress (Kholodova et al. 2011). The results of our study suggested that *R. irregularis* has a modulation effect in the gene expression patterns of root *PIP1;2* and *TIP2;2* of *S. purpurea* growing under Cu conditions.

Conclusions

Salix purpurea might be considered as an interesting alternative for phytoremediation of trace metals like Cu, because this species accumulated Cu mainly in the roots, acting as a phytostabilizer of this trace metal in the soil. The mycorrhizal association with *R. irregularis* might provide an additional advantage to phytostabilizer species like *S. purpurea* by promoting root growth under Cu stress. In that way, plants could immobilize more metals in their root system. However, we observed a limited AMF association between *R. irregularis* and *S. purpurea*, which might be plant and fungus species dependent and also affected by Cu concentrations. Nevertheless, despite the low colonization rates of AMF, the symbiosis interactions were found to have a significant effect on many physiological properties of willows.

The soil concentrations of Cu used in our study caused a moderate stress to *S. purpurea*, enough for identifying the effects of Cu at different levels in the plants. At the chloroplast level, even though the photochemical efficiency of the PSII was not structurally compromised, we observed that AMF associations might have an adverse influence in the way that *S. purpurea* copes with the effects of Cu in the chloroplast that might be in line with reductions in gas exchange. In the cytoplasm, ROS by-products triggered by Cu stress were regulated by an enzymatic response, especially by SOD and APX, indicating an effective response of *S. purpurea* to oxidative stress. During the exclusion of Cu from the cytoplasm, *R. irreguralis* might modulate the sequestration of Cu into the *S. purpurea* cell walls, generating differential physiological responses of this woody plant to Cu. One of the main effects we observed was in hydraulic traits, like K_L and L_P. Inoculated *S. purpurea* cuttings moderated the water status of the plant, reducing the risk of hydraulic failure under Cu stress. Maintaining a steady L_P under Cu stress might be encompassed by dynamic regulation of *PIP1;2* along with an up-regulation of *TIP2;2* in the roots of *S. purpurea*. This gene expression modulation might also have an overall impact in

the water status, as well as in the osmoregulation required during Cu translocation into different cellular compartments.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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