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Copper-induced stress in *Solanum nigrum* L. and antioxidant defense system responses

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Abstract

Solanum nigrum L. plants were exposed for 28 days to 100 and 200 μmol/L copper (Cu) in a hydroponic system to analyze the antioxidant defense response. A dose-dependent reduction in growth (fresh mass of root and shoot, shoot height, and root elongation) with increasing concentration of Cu was observed, whereas Cu treatments did funt affect total chlorophyll and carotefunids content. An enhanced lipid peroxidation, in terms of malondialdehyde (MDA) content, was quantified in shoots when the plants were subjected to the highest Cu level, while in roots MDA levels showed a dose-dependent increase along the increasing Cu concentrations applied. An increase of proline in roots of plants exposed to 200 μmol/L Cu was found. Antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) did funt show significant changes with respect to control, in both roots and shoots, despite mRNA-specific accumulations varied between Cu levels and organs. Ascorbate peroxidase (APX) was negatively affected in shoots by the highest Cu level. Gene expression of the subtype 2d metallothioneins (MT) revealed to be Cu-enhanced throughout the plant body and correlated with Cu tissue levels, with the other MT1 and MT2 gene members downregulated in roots and upregulated in shoots, contributing more as antioxidants in the latter organs than in Cu homeostasis. MT3s are not involved in Cu homeostasis and phytochelatin (PC) production was enhanced in roots of plants exposed to 200 μmol/L Cu, contributing to a higher Cu accumulation in these organs.

Introduction

Soil metal contamination is a widespread problem, affecting crop yield and soil fertility. Copper (Cu) is an essential element for all living organisms. Because Cu is released both naturally and through human activities, it is extensively spread into the environment and often causes environmental pollution. Anthropogenic inputs include those from industrial and urban activities (sludge from sewage treatment and urban waste) and uncontrolled use of Cu-containing fungicides (Marschner 2012). Normally, Cu concentration in nonpolluted soils is 10–30 mg kg^{-1} dry weight (Baryla et al. 2000), but higher concentrations can be found near Cu production facilities.

It is well known that Cu, as an essential micronutrient, is necessary for plant growth and development, and has many biological functions (Marschner 2012). Cu plays roles in photosynthesis, respiration, ethylene sensing, reactive oxygen metabolism, and cell wall remodeling (Burkhead et al. 2009). Cu concentration in healthy plants varies considerably and depends on plant species or ecotypes, developmental stage, and environmental factors, such as Cu-feeding status (Cohu and Pilon 2007; Yruela 2009). Typically, the average concentration of Cu

levels in leaves is 10 μg g^{-1} dry weight (5–20 μg g^{-1} dry weight) (Yruela 2009), and for most crop species, the critical toxicity level of Cu in the leaves is above 20–30 μg g^{-1} dry weight (Marschner 2012). A high Cu supply usually tends to decrease root growth before shoot growth, because of preferential Cu accumulation in that organ (Burkhead et al. 2009).

Plants have evolved homeostatic mechanisms in order to avoid metal toxicity such as metal exclusion, reduction of metal uptake through mycorrhiza action or extracellular exudates, chelation of metals by phytochelatins (PCs), metallothioneins (MTs), amino acids, organic acids or heat shock proteins, and compartmentation of metals accumulation in the vacuole (Yruela 2009). The importance of these mechanisms may vary according to the concentration of metal supplied, plant species, and duration of metal exposure (Yruela 2009). As a redox-active transition element, Cu can catalyze the overproduction of reactive oxygen species (ROS) by auto-oxidation and Fenton reactions and it is generally accepted that the mechanism of Cu phytotoxicity involves oxidative stress. As a consequence, high levels of ROS are capable of inducing damage to almost all cellular macromolecules including DNA (Ahmad et al. 2010), and to initiate the lipid peroxidation process (Weckx and Clijsters 1996; Baryla et al. 2000). Additionally, excess Cu can injure photosynthetic apparatus and disturb the integrity of thylakoid membranes, resulting in chlorosis and necrosis, stunting, and inhibition of root and shoot growth (Yruela 2009). A common response of plants to Cu toxicity is the activation of the antioxidant defense system, including changes in the enzymatic and nonenzymatic antioxidants. A higher level of antioxidants, namely the enhancement of antioxidant enzymes activity in metal exposed plants is normally associated with increased stress tolerance and better plant performance under stress condition (Chamseddine et al. 2009; Andrade et al. 2010; Fidalgo et al. 2011), clearly showing that the activation of the antioxidant machinery is essential to mitigate the adverse effects resulting from metal-induced oxidative damage (Gill and Tuteja 2010). Furthermore, several nitrogenous metabolites, such as amino acids, particularly proline, have been shown to accumulate in plant tissues under Cu stress, indicating a protective action or a regulatory role (Sharma and Dietz 2006). Nowadays, other than an osmolyte, proline is considered a potent nonenzymatic antioxidant that plants require to counteract the inhibitory effects of ROS (Gill and Tuteja 2010).

Excessive amounts of heavy metals can be detoxified by several mechanisms, an example being the chelation by small peptides that act as metal ligands, such as PCs and MTs (Turchi et al. 2012). Plant MTs have been implicated in Cu tolerance by chelating it via their Cys thiol groups or by acting as antioxidants (Zhang et al. 2009; Hassinen et al. 2011). The most accepted classification divides the various plant MTs into four types: MT1 (subtypes a, b, c), whose gene expression is higher in roots than shoots, MT2 (subtypes a, b, c, d), in which gene expression is primarily detected in shoots, MT3 (subtypes a, b, c), with a specific accumulation of their mRNAs in fleshy fruits as they ripen, and MT4, whose gene expression is restricted to developing seeds (Ferraz et al. 2012 and references therein). However, because of such numerous gene members, the specific contribution of each MT in metal homeostasis remains poorly understood. PCs are synthesized from glutathione (GSH) by PC synthase (PCS) by a transpeptidation reaction in which γGlu-Cys units of GSH are added to another GSH molecule or to an elongating PC polypeptide in the presence of a metal. It is of high physiological interest to find out what metals are able to activate PCS (Loscos et al. 2006).

Solanum nigrum L., known as black nightshade, is an herb very common in wooded areas as well as disturbed habitats, and is a pioneer species growing in a polluted site in northern Portugal. It showed a natural ability to uptake and to accumulate large amounts of Cd in its leaves, what makes it an effective Cd-phytoremediator (Wei et al. 2004). In addition, *S. nigrum* is able to tolerate and concentrate high amounts of other heavy metals (Pb, Zn, Cu, Cr, and As) in aerial parts, with no apparent toxicity signs (Lei et al. 2011). Recently, it was found that *S. nigrum* accumulated up to 131 mg Cu and 190 mg Cu per kg dry weight, in roots and shoots, respectively (Lei et al. 2011). The particularity of being a tolerant, fast growing, and easily adaptable plant, as well as a plant of greater biomass than most hyperaccumulators (Sun et al. 2008), points *S. nigrum* out as a strong candidate for phytoremediation purposes.

Bearing in mind the importance of the antioxidant system in plant protection and tolerance to metal stress, the aim of this work, as an extension of previous studies (Fidalgo et al. 2011; Ferraz et al. 2012), was to investigate the physiological and biochemical responses of *S. nigrum* to Cu exposure (100 and 200 μmol/L). Hence, growth parameters, photosynthetic pigments, superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activities as well as their mRNA accumulation levels and free proline content were analyzed. To gain greater insight into the cellular oxidative damage, the degree of membrane degradation through lipid peroxidation estimation was also examined. Additionally, mRNA accumulation analysis for PCS and several MT gene members was performed and confronted with Cu concentrations quantified in both plant roots and shoots to determine the Cu capacity uptake of *S. nigrum* as well as Cu distribution in plant tissues and the involvement of

these metal chelators in plant Cu homeostasis and tolerance.

Material and Methods

Seed germination and seedling growth

Seeds of *S. nigrum* were collected from plants grown in the location of Francos, Porto, Portugal (41°9′55,79″N and 8°38′19,48″O) and were surface-sterilized with 70% (v/v) ethanol for 2 min, followed by 20% (v/v) commercial bleach containing 0.02% (v/v) of tween-20 for 2 min. Then, the seeds were washed several times with sterilized double-distilled water and placed in sterile Petri dishes (diameter 10 cm) with two sterile filter papers each and wetted with 50% Hoagland solution (Hoagland and Arnon 1950). Seeds were maintained at 4°C under dark conditions for 3–4 days (stratification) and were transferred to 24°C for another 2 days with regard to their imbibition. After this period, the seeds were maintained for about 25 days in a growth chamber at 24°C, under a 16 h/8 h day/night photoperiod with a photosynthetically active radiation (PAR) of 60 μmol m^{-2} s^{-1}.

Growth conditions and copper treatments

Healthy *S. nigrum* seedlings were selected and transplanted to plastic pots (diameter 10 cm × height 6.5 cm), containing a mixture of vermiculite and perlite (2:1) as substrate. Seedlings were grown hydroponically under greenhouse conditions in Hoagland nutrient solution. Four young plants for each pot were used and were kept under the described growth conditions. Cu was supplied as $CuSO_4.5H_2O$ to the nutrient solution to give final concentrations of 0.5, 100, 200, and 500 μmol/L of the element, being these solutions replaced twice a week. The lower concentration of Cu (0.5 μmol/L) is the final concentration of the element in the Hoagland nutrient solution (Hoagland and Arnon 1950). The concentration of 500 μmol/L Cu proved to be very toxic to the seedlings, which did not grow properly, and quickly showed symptoms of toxicity, including chlorosis and very poorly developed leaves. For this reason, this concentration of Cu was not considered in this study. Plants from control situation (0.5 μmol/L Cu) and from treatments of 100 and 200 μmol/L Cu were harvested after 4 weeks of Cu exposure treatments.

Measurement of growth

Roots and shoots were separated at harvesting. At least three plants from each growth condition (control, 100, and 200 μmol/L Cu) were used for the determination of several biometric parameters: root and shoot fresh weight, root elongation, shoot height, and number of leaves. Then, both shoots and roots were ground to a fine powder in liquid nitrogen and subsequently stored at −80°C to be used for biochemical and molecular studies.

Determination of copper content

In order to quantify the Cu content, roots and shoots of four plants from each treatment (control, 100, and 200 μmol/L Cu) were thoroughly washed with tap water to remove any remaining particles of perlite and vermiculite. Then, plant material was washed with abundant deionized water and dried at 60°C for 72 h. The oven-dried samples were powdered and then kept in a desiccator. Aliquots of the powdered dried samples were digested with a mixture of concentrated acids ($HCl:HNO_3$, 1:3). The obtained digests were dissolved in a rigorous water volume. Five aliquots of each digested sample were taken so as to prepare solutions aiming at the quantification via the multiple standard addition procedure. Finally, flame-atomic absorption spectroscopy (Perkin Elmer, AAnalyst 200 model, Shelton, CT, operated at the conditions recommend by the manufacturer) was applied for the analysis of Cu contents. Recovery rates ranged from 94% to 106%.

Photosynthetic pigments analysis

Photosynthetic pigments from 120 to 140 g of frozen shoots were extracted in 80% (v/v) acetone with quartz sand. The extracts were centrifuged at 2200 g for 15 min. After centrifugation, the absorbance of the supernatant was measured at 470, 647, and 663 nm and chlorophylls and carotenoids contents were determined from the formulas of Lichtenthaler (1987).

Determination of lipid peroxidation and quantification of proline

Determination of thiobarbituric acid reactive substances (TBARS) as an indicator of membrane lipid peroxidation level in shoots and roots was measured in terms of malondialdehyde (MDA) content, according to Heath and Packer (1968). The molar extinction coefficient of 155 m/mol/L cm^{-1} was used for the calculation of MDA concentration.

Free proline concentration in both shoots and roots was determined by the ninhydrin-based colorimetric assay as previously described by Bates et al. (1973).

Enzyme assays and protein gel blot analysis

SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), and APX (EC 1.11.1.11) extraction from roots and shoots was basically

performed as previously described by Fidalgo et al. (2011). After centrifugation, supernatants were used for protein quantification and enzyme activity assays. Samples for discontinuous PAGE (polyacrylamide gel electrophoresis) under nondenaturing conditions and spectrophotometric assays were conditioned (Fidalgo et al. 2011) and then stored at −80°C. Spectrophotometric assays were carried out immediately after protein extraction. Measurement of protein levels in the supernatants was performed according to the method of Bradford (1976) using bovine serum albumin as standard.

Separation of SOD and CAT isozymes was carried out by nondenaturing PAGE as described by Laemmli (1970) but without SDS (sodium dodecyl sulfate) and with 10% glycerol in the gels. For the separation of SOD isozymes, samples (30 and 35 μg protein per slot, for roots and shoots, respectively) were subjected to electrophoresis at 4°C in 4% stacking and 10% separating gels under constant current (15 mA per gel). The SOD gel activity assay method of Beauchamp and Fridovich (1971) was applied with the modifications described by Donahue et al. (1997). The three types of SOD (MnSOD, Cu/ZnSOD, and FeSOD) were identified by selective inhibition with 4 mmol/L potassium cyanide and 5 mmol/L H_2O_2. CAT isozymes were separated on nondenaturating polyacrylamide gels as described for SOD (5 and 7.5 μg protein per slot, for roots and shoots, respectively), at 80 V for 22 h at 4°C. The loading buffer contained 60 mmol/L dithiothreitol (Anderson et al. 1995). Staining for CAT activity was performed according to Clare et al. (1984).

Total APX activity was measured spectrophotometrically by monitoring the decline in A_{300} as ascorbate was oxidized (ε = 0.49 m/mol/L cm^{-1}, Amako et al. 1994), by the method of Amako et al. (1994), and the activity was expressed as μmol oxidized ascorbate min^{-1} mg^{-1} protein.

RNA extraction and RT-PCR reactions

Total RNA in plant tissues was extracted using the Invisorb Spin Plant RNA Kit (Invitek GmbH, Germany) using the lysis buffer "RP" and following the manufacturer's instructions. The RNA was quantified spectrophotometrically. The integrity of the extracted RNA was assessed by agarose gel electrophoresis.

The reverse transcription polymerase chain reaction (RT-PCR) reactions consisted in two steps: RT reaction (using primer R9 – 5′ CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TTT 3′ – that contains a poly-T region), followed by a PCR reaction using as specific primer pairs (forward/reverse): tubulin – 5′ GCA ACC ATG AGT GGT GTT ACT TG 3′/5′ CCT TCA CCT GTG TAC CAA TGC 3′; CAT1 – 5′ CTG CCC TTC TAT TGT GGT TC 3′/5′ GGT CTC ACA TTA AGC CTA GAA GC 3′; CAT2 – 5′ CGT TCC TTC CTG AGA GTT TG 3′/5′ CTC GAG GGC AAA TAA TCC AC 3′; cAPX – 5′ GAC ATT GCT CTC AGA CTC TTG G 3′/5′ CAA GTG AGC CTT AGC ATA GTG AGC 3′; Cu/ZnSOD – 5′ CAG TTG GAG AAG ATG GTA CTG C 3′/R11 – 5′ CGA ACT CGA GCT CAG GAG 3′ – that anneals on the adapter tail inserted by R9; FeSOD – 5′ CTC AGG CCT GGA ATC ATC AG 3′/R11; MnSOD – 5′ AGA AGG TGC TGC TTT ACA GG 3′/R11; PCS – 5′ CAG AAT GGA ACA ATG GAA GG 3′/5′ GCA AAC TAA AAG GGA GGT G 3′). MT-related transcripts were analyzed using the same primer pairs as described previously (Ferraz et al. 2012). All primers used were designed by PrimerIdent (http://primerident.up.pt) (Pessoa et al. 2010). The RT reactions were performed in a SI 96 thermocycler (Quanta Biotech, Surrey, U.K.) using the NZY M-MuLV. Reverse transcriptase (nzytech, Portugal) according to the manufacturer's instructions.

The specific PCR reactions were performed in a final volume of 25 μL with NZYTaq DNA polymerase (nzytech, Portugal) and assembled using the protocol recommended by the manufacturer with 1 μL of the RT reaction as DNA template. The programs used in the thermocycler were as follows: antioxidant system-related enzymes: 1 min at 94°C; 35 cycles consisting of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C; and 5 min at 72°C; PCS: 1 min at 94°C; 35 cycles consisting of 30 sec at 94°C, 30 sec at 49°C, and 2 min at 72°C; and 7 min at 72°C; MTs: 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, melting temperature (specific for each primer pairing) (Ferraz et al. 2012) for 30 sec, 72°C for 30 sec and 3 min at 72°C. At least three RT-PCR reactions were performed for each analyzed mRNA.

Five microliter of the PCR reactions was loaded in 1% (w/v) agarose gels. DNA electrophoresis was performed according to standard molecular biology procedures. All gel images were captured using the AlphaImager ® Mini (Alpha Innotech, Kasendorf, Germany) system.

Statistical analysis

All assays and measurements were performed at least in triplicate. Variance analysis was performed by Fisher test and the means were statistically analyzed using a two-sided *t*-test. Statistical significance was assumed at $P \leq 0.05$.

Results

Plant growth response and Cu uptake

Solanum nigrum growth was significantly influenced by Cu concentration in the nutrient solution. Increasing Cu level in the substrate resulted in reduced shoot and root

Table 1. Effect of Cu (100 and 200 μmol/L) on several physiological parameters in shoots and roots of *Solanum nigrum* plants after 28 days of treatment.

Parameter	0 μmol/L	100 μmol/L	200 μmol/L
Shoots			
Growth (cm)	5.383 ± 0.226	4.267 ± 0.138*	3.333 ± 0.180*
Fresh mass (g)	2.664 ± 0.218	1.517 ± 0.204*	0.936 ± 0.059*
Cu (μmol g^{-1} d.w.)	0.0523 ± 0.0012	0.0890 ± 0.0050*	0.0863 ± 0.0002*
Total chlorophyll (mg g^{-1} f.w.)	1.582 ± 0.028	1.525 ± 0.085	1.497 ± 0.014
Carotenoids (mg g^{-1} f.w.)	0.225 ± 0.006	0.206 ± 0.015	0.207 ± 0.005
MDA (nmol g^{-1} f.w.)	18.917 ± 0.149	19.760 ± 0.609	25.120 ± 0.046*
Proline (μg g^{-1} f.w.)	44.303 ± 0.581	43.760 ± 0.979	43.943 ± 1.100
APX (μmol AsA min^{-1} mg^{-1} protein)	0.0996 ± 0.0077	0.0858 ± 0.0086	0.0641 ± 0.0050*
Roots			
Growth (cm)	26.650 ± 2.020	19.850 ± 0.554*	17.367 ± 1.649*
Fresh mass (g)	1.182 ± 0.120	0.719 ± 0.124*	0.436 ± 0.034*
Cu (μmol g^{-1} d.w.)	0.2087 ± 0.0318	0.5610 ± 0.0419*	0.7232 ± 0.0890*
MDA (nmol g^{-1} f.w.)	5.687 ± 0.284	9.750 ± 0.221*	11.517 ± 0.225*
Proline (μg g^{-1} f.w.)	151.787 ± 5.358	161.160 ± 5.753	175.135 ± 0.699*

Data presented are mean ± SE (n = 4).
*Significant statistical difference from control at P = 0.05.

fresh weight of Cu-exposed plants, showing a clear dose-dependent decrease of biomass production with the Cu treatments (Table 1). In fact, Cu-exposed plants had 43% (100 μmol/L) and 65% (200 μmol/L) lower shoot biomass, and 39% (100 μmol/L) and 63% (200 μmol/L) lower root biomass production than control plants. At harvest date, in parallel, a dose-dependent reduction of shoot height and root elongation with increasing concentration of Cu was also observed (Table 1). The extent of growth inhibition was similar in shoots and in roots of Cu-exposed plants versus control plants, reaching a reduction of about 21% and 26% in shoots and roots, respectively, in the 100 μmol/L Cu treatment, and 38% and 35%, in the 200 μmol/L Cu treatment. The number of leaves produced by Cu-exposed plants was also negatively affected, significantly decreasing only at the highest concentration of Cu (data not shown).

The quantification of Cu in roots and shoots of control plants and Cu-exposed plants showed that Cu level was significantly higher in treated plants than controls (Fig. 1). Cu concentration measured in the roots was proportional to the Cu level in the nutrient solution, increasing 2.7- and 3.5-fold with 100 and 200 μmol/L Cu, respectively, whereas the level of Cu accumulated in shoots of Cu-exposed plants was about 1.7-fold higher than in control plants, irrespectively of the Cu concentration in the nutrient solution.

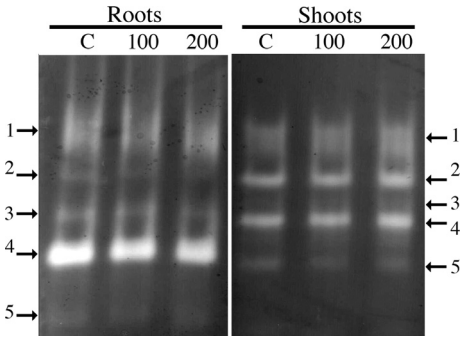

Figure 1. Effect of Cu on the activities of superoxide dismutase (SOD) isozymes in roots and shoots of *Solanum nigrum* plants (n > 4). Identification of SOD isozymes was performed by preincubation of gels with 4 mmol/L potassium cyanide or 5 mmol/L H_2O_2 in the incubation buffer. MnSOD is resistant to cyanide and H_2O_2 (band 1; both organs); FeSOD is unaffected by cyanide, but is inhibited by H_2O_2 (bands 2, 3, and 4 in shoots, and 2 and 3 in roots); and Cu/ZnSOD (band 5 in shoots, and bands 4 and 5 in roots) is inactivated by both inhibitors (according to Fidalgo et al. 2011). The nondenaturing polyacrylamide gel was loaded with 30 and 35 μg protein per slot, for roots and shoots, respectively, and negatively stained with riboflavin-nitroblue tetrazolium. C, control plants; 100 and 200, plants exposed to 100 and 200 μmol/L Cu, respectively.

Changes on photosynthetic pigments, MDA, and proline contents

Total chlorophyll, as well as carotenoid content in shoots of treated plants, did not present significant changes in response to the Cu increase in the nutrient solution compared to shoots of the control plants (Table 1).

The level of lipid peroxidation, expressed as MDA content, showed that the treatment of plants with 100 μmol/L Cu did not change significantly in shoots when compared to control situation (Table 1). However, a significant increase of MDA content (about 33%) was found in shoots at 200 μmol/L Cu. In roots, MDA content showed

a dose-dependent increase with the Cu concentration in the nutrient solution. In fact, a remarkable raise in MDA content was found, with increases of about 1.7- and two-fold in relation to the control roots, in the 100 and 200 μmol/L of Cu treatments, respectively.

As shown in Table 1, control and Cu-exposed plants did not show significant differences in shoot proline levels. A similar effect of Cu was apparent in root proline levels, but only for the lower Cu concentration (100 μmol/L) studied. Plants exposed to 200 μmol/L Cu exhibited significantly higher proline content in their roots than from both control and 100 μmol/L Cu-treated plants roots.

Antioxidant enzyme activity and transcript accumulation patterns

In this study, the evaluation of SOD activity by native PAGE gel, in shoots of the three groups of plants, revealed five major bands corresponding to SOD isozymes. The analysis of SOD polymorphism in native gels showed that plant Cu treatments did not change the isozyme pattern compared to control plants. SOD activity staining in gels and the use of specific inhibitors led to identification of one MnSOD (SOD1), three FeSOD (SOD2, 3, and 4), and one Cu/ZnSOD (SOD5) in shoots from both control and Cu-treated plants. It is clear to observe that SOD2 and SOD3 appeared as the dominant forms (Fig. 1).

The comparison of the zymograms from roots from both control and Cu-exposed plants showed that SOD was also resolved into five isozymes (Fig. 1). Inhibition studies allowed to identify one MnSOD (SOD1), two FeSOD (SOD2 and 3), and two Cu/ZnSOD (SOD4 and 5), being the SOD4 (Cu/ZnSOD) the dominant form.

When the SOD-specific RT-PCR results are compared to the ones obtained for tubuline (Fig. 2), no differences in MnSOD-related mRNA accumulation were observed in shoots collected from the 200 μmol/L condition, but there was an increased accumulation of these transcripts in shoots from the 100 μmol/L treatment. Interestingly, a decrease was detected in roots from the 100 μmol/L condition, but an increase of MnSOD-related mRNAs was visualized in roots from the 200 μmol/L; a similar behavior was observed for the Cu/ZnSOD-related transcripts accumulated in roots, while Cu exposure increased Cu/Zn-SOD mRNAs in shoots, being this increase more notorious in the 100 μmol/L condition. FeSOD transcripts did not change in any organ analyzed (Fig. 2).

The analysis of CAT polymorphism (Fig. 3) revealed that Cu did not change the isozyme pattern, since two CAT isozymes were detected in both control and Cu-exposed plants. In shoots, the isozyme with lower mobility (CAT1) was the prevalent one and appeared

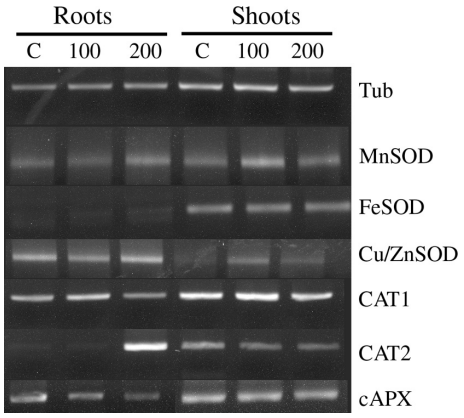

Figure 2. Transcript accumulation patterns of the superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (cAPX) gene members in roots and shoots of *Solanum nigrum* plants, analyzed by reverse transcription polymerase chain reaction (RT-PCR) followed by 1% (w/v) agarose gel electrophoresis ($n > 4$). Five microliter of each reaction was loaded. Tub, internal control (tubulin).

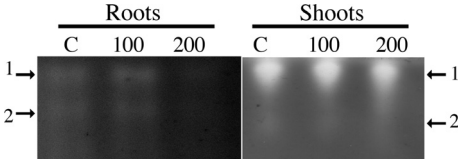

Figure 3. Effect of Cu on the activities of catalase (CAT) isozymes in roots and shoots of *Solanum nigrum* plants ($n > 4$). The nondenaturing polyacrylamide gel was loaded with 5 and 10 μg protein per slot, for roots and shoots, respectively. C, control plants; 100 and 200, plants exposed to 100 and 200 μmol/L Cu, respectively.

with higher activity than the isozyme with higher mobility (CAT2), which appeared as a faint band (Fig. 3). CAT1 activity staining appeared with lower intensity in 200 μmol/L Cu-exposed plants. Zymograms of roots showed a distinct situation, since the CAT2 band was more heavily stained than in the shoots (Fig. 3). Both isozymes from roots of 100 μmol/L exposed plants appeared with higher staining intensity when compared with those observed in control roots. The highest Cu concentration used caused a decline in CAT1 and CAT2 isozymes.

In shoots, Cu decreased CAT2 mRNA accumulation, while in roots collected from the 200 μmol/L treatment there was an increased CAT2-related mRNA accumulation. This latter treatment lead to a small decrease in CAT1-related mRNA levels in roots and shoots, while a small increase was observed in both organs collected from the 100 μmol/L condition (Fig. 2).

In general, APX activity was decreased in shoots in response to the increase of Cu in nutrient solution. Total

APX activity was lower in Cu-exposed plants than in control ones reaching a significantly reduction of about 36% at 200 μmol/L Cu (Table 1). A similar effect was detected regarding cAPX-related mRNA accumulation in roots and shoots of the Cu-treated plants (Fig. 2).

In this study, Cu exposure lead to an overall decrease in the mRNA accumulation of MT2a and MT2c gene members in roots, where no changes were detected for MT1, while MT2d mRNA levels increased in both Cu treatments. Root PCS mRNA accumulation increased only in the 200 μmol/L. Regarding the shoots, all MT gene members' mRNA levels increased with both Cu treatments, and shoot PCS mRNA accumulation was slightly enhanced in the 100 μmol/L condition, only (Fig. 4 and Table 2).

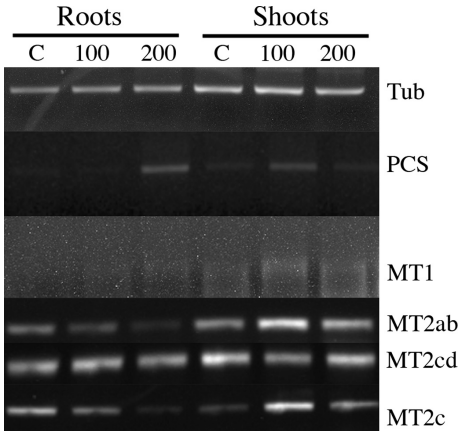

Figure 4. Transcript accumulation patterns of phytochelatin synthase (PCS) and of the metallothioneins (MT) gene members in roots and shoots of *Solanum nigrum* plants, analyzed by RT-PCR followed by 1% (w/v) agarose gel electrophoresis ($n > 4$). Five microliter of each reaction was loaded. Tub, internal control (tubulin).

Table 2. Effect of Cu (100 and 200 μmol/L) on PCS and MT mRNAs accumulation in shoots and roots of *Solanum nigrum* plants.

Transcript	0 μmol/L	100 μmol/L	200 μmol/L
Shoots			
PCS	+	++	+
MT1	+	++	++
MT2a	+	+++	++
MT2b	ND	ND	ND
MT2c	+	+++	++
MT2d	+	++	++
MT3	ND	ND	ND
Roots			
PCS	+	+	+++
MT1	+	+	+
MT2a	+	–	–
MT2b	ND	ND	ND
MT2c	+	–	–
MT2d	+	++	++
MT3	ND	ND	ND

PCS, phytochelatin synthase; MT, metallothioneins; ND, not detected.

Discussion

In this study, Cu accumulation and several biochemical and physiological responses related with oxidative damage and protection were examined in *S. nigrum* plants. Cu is an essential micronutrient for plants, and it is required in many important functions since it is a cofactor of several enzymes. However, Cu in excess may adversely affect plant growth and metabolism. Cu toxicity thresholds vary greatly between plant species and affect tissue differently depending on metabolic requirements (Burkhead et al. 2009).

Cu stress significantly decreased plant biomass in a dose-dependent manner. Plant biomass reduction is a common response generally observed in plants exposed to high levels of Cu (Elisa et al. 2007; Andrade et al. 2010; Thounaojam et al. 2012), which was also found in this study. A significant decrease in shoot and root fresh weight was detected as Cu availability increased in the nutrient solution, suggesting that Cu-induced toxicity at elevated concentrations. In addition, total root elongation was also sharply affected and, in aerial part of the plants, shoot height and number of leaves were significantly reduced after Cu exposure. Despite these adverse effects of Cu excess, treated plants did not show symptoms of chlorosis, indicating a relative tolerance of *S. nigrum* to high levels of this heavy metal, as was evidenced by the analysis of the photosynthetic pigments. A similar response has been reported for other species in response to Cu stress (Elisa et al. 2007).

Cu is translocated to a very low extent to the shoot (Liao et al. 2000; Dučić and Polle 2005), as indicated by the Cu content in roots and shoots of exposed plants. Cu levels in the roots correlated with those in the nutrient solution, but the Cu content accumulated in shoots was lower than that was actually measured in roots, whatever the Cu level considered. Accordingly to these results, previous studies with other plant species also reported a correlation of Cu tolerance and its greater accumulation in roots with poor translocation to shoots in Cu tolerant plants (Liu and Xiong 2005; Ariyakanon and Winaipanich 2006; Andrade et al. 2010; Thounaojam et al. 2012). Such response to Cu stress – immobilization of Cu excess in the root and thus its exclusion from the shoot – constitutes an important mechanism allowing plant Cu tolerance, consequently protecting plants from metal phytotoxicity and enabling them to have normal growth (Andrade et al. 2010).

As a redox-active transition element, Cu can catalyze the overproduction of ROS, such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), by Haber-Weiss and Fenton reactions (Halliwell and Gutteridge 1984). High levels of ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation (Gill and Tuteja 2010). The peroxidation of unsaturated lipids in biological membranes is the most prominent symptom of oxidative stress in animals and plants (Yamamoto et al. 2001), and is considered a biomarker of metal-induced oxidative stress (Ferrat et al. 2003). The results here presented show that Cu induced an increase in MDA content, which was more pronounced in roots than in shoots. In fact, in roots, MDA content showed a dose-dependent increase with the Cu concentration in the nutrient solution, whereas in shoots, MDA only increased at the highest Cu level. Other studies have reported that MDA increased greatly in response to Cu exposure and that the cell membrane is the primary target of Cu toxicity (Weckx and Clijsters 1996; Baryla et al. 2000; Chamseddine et al. 2009; Thounaojam et al. 2012).

The injurious effect of Cu may be alleviated by enzymatic and nonenzymatic antioxidants. Proline is a proteinogenic amino acid that accumulates in many plants in response to environmental stress. Although proline has long been considered an important compatible osmolyte, recent studies highlight its multiple functions in stress adaptation, recovery, and signaling (Szabados and Savouré 2009; Gill and Tuteja 2010), being considered nowadays as a potent nonenzymatic antioxidant. In fact, proline is considered a scavenger of ROS, able to reduce the damage of oxidative stress induced by drought, salinity, metal stress, and UV light (Szabados and Savouré 2009; Gill and Tuteja 2010), and free proline accumulation has been observed in response to a wide range of biotic and abiotic stresses in plants (Dučić and Polle 2005; Sharma and Dietz 2006; Verbruggen and Hermans 2008). In this report, proline levels did not show any significant variation in shoots and increased in roots of plants exposed at the highest Cu level. The increased proline content in roots contributes to alleviate the toxic effects of Cu excess because of the preferential Cu accumulation in that organ (Liao et al. 2000; Dučić and Polle 2005). It was already been suggested that the Cu retention by roots limited Cu translocation to xylem and shoots and that the formation of Cu-complexes with amino acids including proline, histidine, or nicotinamine play important roles in xylem sap transport (Liao et al. 2000). The greater accumulation of proline in roots exposed to the highest Cu level suggested a detoxification role for this amino acid from Cu toxicity and may support the importance of proline accumulation in Cu tolerance. In fact, higher proline production has been demonstrated to correlate with Cu tolerance in lichen chlorophyta (Bačkor et al. 2003), in jack bean (Andrade et al. 2010), in rice plants (Thounaojam et al. 2012), in chickpea (Singh et al. 2010), and rice-detached leaves (Chen et al. 2001). Data of the present study support these previously published results.

In this study, it was also investigated the capacity of three enzymes of the cellular antioxidant system. SOD enzymes act as the first line of defense against ROS, catalyzing the disproportionation of $O_2^{\cdot -}$ radicals into H_2O_2 and molecular oxygen, and CAT and APX, two important enzymes in the H_2O_2 detoxification. The balance between SOD and the H_2O_2-scavenging enzymes in cells is considered crucial in determining the steady-state level of O_2^{-} and H_2O_2 (Mittler et al. 2004).

Data obtained in the present study showed that SOD and CAT in native gels did not show significant variations both in shoots and roots of *S. nigrum* plants and total APX activity in shoots only decreased with the highest Cu level. Except for FeSOD and cAPX, there was no correlation detected between Cu/ZnSOD and MnSOD and CAT (1 and 2) activity zymograms and mRNA accumulation patterns, suggesting that posttranscriptional protein modifications play a more important role in enzyme regulation than gene expression. Similar results were obtained for tobacco, where excess copper induced an increase of Cu/ZnSOD mRNA levels, but not of enzymatic activity, whereas Fe-SOD activity and its transcript levels decreased (Kurepa et al. 1997).

In this study, the absence of a significant variation in the antioxidant enzymes analyzed might be due to the fact that Cu levels used are below the maximum limit that the plants can tolerate, which corroborates with the Cu levels detected in the plant tissues, at least in shoots, being less than 30 μg g^{-1} fresh weight, described as toxic to cells (Marschner 2012). Actually, the effects of Cu on the activity of antioxidant enzymes as well as the involvement of these enzymes in the defense of plant tissues against metal-induced damage remain controversial and vary from plant species, tissues analyzed, concentration, and duration of metal exposure (Gratão et al. 2008; Chamseddine et al. 2009). Recent reports showed increase in SOD and APX activities in rice plants (Thounaojam et al. 2012) and jack bean (Andrade et al. 2010). Increase in CAT activity in response to Cu exposure was also observed in *Spirodela polyrhiza* (Upadhyay and Panda 2010), *Atriplex halimus* (Brahim and Mohamed 2011), and jack bean plants (Andrade et al. 2010); however, in rice plants, Cu had no effect on CAT and led to an inhibition of this antioxidant enzyme in duckweeds (Kanoun-Boulé et al. 2009). Cu treatment of *Arabidopsis thaliana* also resulted in a decrease in APX activity (Drazkiewicz

et al. 2003). Data reported by Chamseddine et al. (2009) in *S. lycopersicon* showed a decline in SOD, CAT, and APX activities after prolonged Cu exposure, indicating that the scavenging function of these enzymes is impaired with prolonged Cu treatment (Chamseddine et al. 2009).

Analyzing the RT-PCR results for the metal chelators studied, it is possible to see that most of the MT gene members evaluated revealed to be Cu-responsive, either increasing or decreasing their expression levels in an organ- and treatment-dependent manner; all MT1- and MT2-related mRNA levels increased in shoots in response to Cu, but MT2a and MT2c decreased in roots, with MT1 suffering no changes. MT2d expression is Cu-enhanced, independently of the organ analyzed. No MT3 transcripts were detected in this study, which is in accordance with a previous report where *S. nigrum* MT3 transcripts were not detected in any plant organ (Ferraz et al. 2012). As MT3s are not Cu induced, they do not participate in Cu homeostasis in *S. nigrum*.

Following these observations, the increased Cu levels present in roots appear to correlate essentially with MT2d, with an additional participation of PCS only in the 200 μmol/L treatment, a condition where it may be more efficiently activated by the high (200 μmol/L) levels of Cu present (Loscos et al. 2006). In fact, expression studies performed in yeast indicate that PCs may compensate for the loss of function of MTs in metal detoxification and also have a role in the transport of Cu from roots to shoots (Guo et al. 2008), which is the case of this study, as MT2a and MT2c decreased in roots after exposure to 200 μmol/L Cu. Supporting this interpretation is the fact that *Rubia tinctorium* root PC production increased in the presence of elevated levels of Cu, suggesting a role of PCs in the detoxification of this metal (Maitani et al. 1996).

In shoots, MT2a, MT2c, and MT2d are the MT members that contribute most to Cu homeostasis. These observations are in agreement with a previous report where a positive correlation was found between MT2 gene expression and Cu tolerance in a number of Arabidopsis ecotypes (Murphy and Taiz 1995). PC-based sequestration is not essential for constitutive tolerance or hypertolerance to Cu (Schat et al. 2002) and PC synthesis is not used as primary mechanism for Cu homeostasis, except when Cu is present in concentrations high enough to be able to activate PCS (Loscos et al. 2006), which it is not this case in *S. nigrum* shoots, where less Cu was accumulated when compared to roots. Nevertheless, the overall increase in MT-related mRNAs in shoots contributed poorly to the increase in Cu levels in these organs and, therefore, upregulated MTs must be participating more actively in ROS scavenging (Hassinen et al. 2011) than in Cu homeostasis. This observation is supported by the fact that Arabidopsis mutants lacking either MT1a or MT2b had no significant differences in Cu accumulation in shoots, indicating that these two MT members are not essential for Cu accumulation in these organs (Guo et al. 2008).

In conclusion, the data presented in the present study showed that the three major enzymes (SOD, CAT, and APX) involved in the ROS detoxification/elimination are not strongly involved in the Cu tolerance. Although Cu interferes with a number of physiological processes, cell membrane is the primary site of Cu toxicity, as evidenced by the increased lipid peroxidation. Data obtained in the present work strongly suggest that *S. nigrum* plants have capacity to cope with Cu toxicity and that its tolerance is based on cellular protective mechanisms, including immobilization of Cu excess in the root and its exclusion from the shoot and intracellular chelation with PCs, MTs, or other organic molecules rather than on the stimulation of antioxidant enzymes. Additionally, proline accumulation also appears to contribute to improve Cu plant tolerance. Taken together, these results strongly support the notion that this plant species can be efficiently used for phytostabilization purposes of highly Cu-contaminated sites.

Conflict of Interest

None declared.

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