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An efficient antioxidant system and heavy metal exclusion from leaves make *Solanum cheesmaniae* more tolerant to Cu than its cultivated counterpart

Simão Branco-Neves[1,*], Cristiano Soares[1,*], Alexandra de Sousa[1], Viviana Martins[2], Manuel Azenha[3], Hernâni Gerós[2,4,5] & Fernanda Fidalgo[1]

[1]BioISI – Biosystems and Integrative Sciences Institute, Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua Campo Alegre s/n, 4169-007 Porto, Portugal
[2]CITAB-UM – Centre for the Research and Technology of Agro-Environmenal and Biological Sciences, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[3]CIQ-UP, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua Campo Alegre 687, 4169-007 Porto, Portugal
[4]CBMA – Centre of Molecular and Environmental Biology, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[5]CEB – Centre of Biological Engineering, Department of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

Keywords
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Correspondence
Cristiano Soares, Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua Campo Alegre s/n, 4169-007 Porto, Portugal. Tel: +351 220 402 726;
Fax: +351220402709;
E-mail: up201003798@fc.up.pt

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*These authors contributed equally to this work.

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Abstract

Copper (Cu) is an abundant metal in the environment coming from anthropogenic activities and natural sources that, in excess, easily becomes phytotoxic to most species, being its accumulation in plants considered an environmental threat. This study aimed to compare the physiological and molecular responses of *Solanum lycopersicum* and its wild counterpart *Solanum cheesmaniae* to Cu stress. In particular, we wanted to address the hypothesis that *S. cheesmaniae* is more adapted to Cu stress than *S. lycopersicum*, since the former is equipped with a more efficient antioxidant defense system than the latter. Biomarkers of oxidative status (lipid peroxidation, hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) levels) revealed a more pronounced imbalance in the redox homeostasis in shoots of *S. lycopersicum* than in *S. cheesmaniae* in response to Cu. Furthermore, the activity of key antioxidant enzymes clearly differed in both species in response to Cu. Catalase (CAT) activity increased in *S. cheesmaniae* shoots but decreased in the domestic species, as well as ascorbate peroxidase (APX). Both species preferentially accumulated Cu in the radicular system, although a great increase in the aerial parts of *S. lycopersicum* was measured, while in leaves of Cu-treated *S. cheesmaniae*, the levels of Cu were not changed. Overall, results validated the hypothesis that *S. cheesmaniae* is more tolerant to excess Cu than *S. lycopersicum* and the data provided will help the development of breeding strategies toward the improvement of the resistance/tolerance of cultivated tomato species to heavy metal stress.

Introduction

As result of fluctuations in the abiotic environment, agricultural crops are frequently exposed to stress conditions, including drought, salinity, and pollution. Indeed, the steadily global industrialization is greatly increasing the incidence of metals in biosphere, which are already considered as serious environmental pollutants, disturbing the normal physiology of different animal and plant species (Nagajyoti et al. 2010).

Copper (Cu), a transition metal, is one of the oldest known metals and a component of the structure of earth's crust (Alloway 1995). The origin of Cu in soil may arise from both natural and anthropogenic activities. Cu naturally derives from rocks disintegration, parent material, minerals dissolution, and volcanic eruptions. The anthropogenic input of Cu results from livestock production, industrial activities, and intensive agriculture. In fact, in order to protect cultures against fungal diseases, several crops are treated with Cu-containing fungicides (Adrees et al. 2015), leading to an increase of Cu content in soils (Yruela 2005; Micó et al. 2006).

Some heavy metals (HM), like Cu, are essential micronutrients for higher plants and fundamental to different physiological processes, like photosynthesis, respiration, cell wall remodeling, and reactive oxygen metabolism (Burkhead et al. 2009; Marschner & Marschner 2012). However, when above a threshold level, they can easily become phytotoxic, impairing the normal growth and decreasing the nutritional quality and productivity of important crops. Phytotoxicity symptoms driven by Cu include the reduction of root growth prior to shoot growth, since roots are the preferred Cu accumulation site (Burkhead et al. 2009), inhibition of seed germination, anatomic alterations in diverse organs (Adrees et al. 2015), and induction of damages in the photosynthetic apparatus (Yruela 2005). In addition, Cu excess in plant tissues causes overproduction of reactive oxygen species (ROS). In this way, Cu excess often induces oxidative stress (Moller 2001; Yruela 2005), causing inhibition of enzymatic activities at both protein and gene expression level. Therefore, a common response of plants to Cu toxicity is the activation of the enzymatic and non-enzymatic antioxidant system (Fidalgo et al. 2013).

Tomato (*Solanum lycopersicum*) is one of the greatest produced agricultural products in the world being the second most important vegetable, constituting an excellent source of health-promoting compounds (Dorais et al. 2008). The importance of tomato is not restricted to its fresh consumption because ca. 80% of cultivated tomatoes are consumed in the form of processed products like sauce, puree, juice, or ketchup (Kaur et al. 2008). Consistent previous studies have already explored the problem of Cu stress in *S. lycopersicum*, as well as the response of the antioxidant system (Mazhoudi et al. 1997; Liao et al. 2000; Martins and Mourato 2006; Mediouni et al. 2006; Chamseddine et al. 2009; İşeri et al. 2011; Al Khateeb and Al-Qwasemeh 2014; Wang et al. 2015). However, in this study, although we have completed and performed some analyses in this species, the main purpose was to compare the responses of *S. lycopersicum* to its wild counterpart *Solanum cheesmaniae,* potentially more tolerant to Cu because it is considered a salt-tolerant species (Rajasekaran et al. 2000; Peralta and Spooner 2006), and the induction of oxidative stress by metal toxicity is often associated with secondary water stress (Poschenrieder and Barceló 1999).

Solanum cheesmaniae is a wild tomato species, endemic from Galápagos Island (Rick, 1956), where it evolved in segregation from the continental wild tomato species, getting unique morphological characteristics, such as yellow-orange fruits and small-sized seeds. Since it can also be easily crossed with domestic tomato to produce fertile offspring (Rick 1979) and produce edible fruit (Darwin 2009), *S. cheesmaniae* seems to be an appropriate candidate species for tomato breeding.

In this study, *S. lycopersicum* and *S. cheesmaniae* plants were grown in a nutrient solution with up to 250 μmol L^{-1} Cu, and several parameters of both enzymatic and non-enzymatic components of the antioxidant system were analyzed and compared between the two species.

Materials and Methods

Plant material, growth conditions, and treatments

Solanum lycopersicum cv. Ciliegia and *Solanum cheesmaniae* seeds were surface-sterilized with 70% (v/v) ethanol for 5 min and 20% (v/v) commercial bleach (3.5% (v/v) of active chlorine) for 5 min and thoroughly washed with sterilized deionized water. Seeds of both tomato species were hydroponically cultured with a mixture of vermiculite:perlite (2:1) in a growth chamber at 24°C and 16 h-light/8 h-dark photoperiod, with a photosynthetic active radiation (PAR) of 65 μmol m^{-2} sec^{-1}. At the beginning of the experiment, seeds of both species were divided into two sets and allowed to germinate. In control conditions, watering was performed with 25% modified Hoagland solution (HS; Taiz et al. 2015), while under Cu stress, watering solution contained 25% HS supplemented with 250 μmol L^{-1} $CuSO_4$. This Cu concentration was set according to previous reports (Kopittke and Menzies 2006; Zhang et al. 2008; Choudhary et al. 2010; Fidalgo et al. 2013) and preliminary studies (see Supporting Information). For each experimental condition (control and Cu-treated plants from each species), a total of four biological replicates were considered, with five plants in each replicate. After 28 days of growth, plants from each biological replicate were collected and separated into shoots and roots, and the material was carefully processed for different biometric, biochemical, and molecular assays.

Cu concentration

Samples of dried material (0.1 g) from both experimental conditions were digested with a mixture of HCl:HNO3, (1:3)

and then dissolved in a rigorous deionized water volume. Five aliquots of each digested sample were used to prepare solutions for the Cu quantification via multiple standard addition procedure. Cu levels of each sample were measured by flame-atomic absorption spectroscopy (AAnalyst 200 model; Perkin Elmer, Waltham, Massachusetts, USA). A few samples were fortified at the digestion step in order to check for possible losses or contamination during this critical operation. The recovery levels oscillated from 95% to 106%.

Photosynthetic pigments

Photosynthetic pigments were extracted from frozen plant samples (0.2 g) in 80% (v/v) acetone and quantified according to Lichtenthaler (1987), after reading the absorbance at 470, 647, and 663 nm. The results were expressed in mg g^{-1} fresh weight.

Lipid peroxidation

Malondialdehyde (MDA) content was used to quantify lipid peroxidation, as described by Heath and Packer (1968), using frozen aliquots of around 0.250 g. The concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA g^{-1} fresh weight.

H_2O_2 levels

The quantification of hydrogen peroxide (H_2O_2) content was performed in shoots and roots (c.a. 0.250 g) as previously described by Jana and Choudhuri (1982). After measuring the absorbance at 410 nm, the H_2O_2 content was calculated using the extinction coefficient of 0.28 μM^{-1} cm^{-1} and expressed as nmol g^{-1} of fresh weight.

$O_2^{\cdot-}$ levels

Superoxide anion ($O_2^{\cdot-}$) levels were quantified according to Gajewska and Sklodowska (2007). Samples of plant fresh material (c.a. 0.3 g) were cut in small equal pieces of 1 cm^2 width and incubated in a mixture containing 0.01 mol L^{-1} sodium phosphate (pH 7.8), 0.05% (w/v) nitroblue tetrazolium (NBT), and 10 mmol L^{-1} azide (NaN_3). The NBT reducing activity (indicating $O_2^{\cdot-}$ generation) was expressed as the increase in A_{580} h^{-1} g^{-1} fresh weight.

Total phenolics

The total content of phenolic compounds was determined following the method described by Singleton and Rossi (1965). Samples of fresh material (0.3 g) were cut in small equal pieces of 1 cm^2 and were immersed in a mixture of 1% (v/v) methanol in 1% (v/v) HCl. The total content of phenolic compounds was expressed in terms of μg gallic acid equivalents (GAE) g^{-1} fresh weight, calculated from a calibration curve prepared with gallic acid.

Total flavonoids

The total flavonoid content was determined with a colorimetric method, as previously described (Chang et al. 2002). Samples of fresh material (0.3 g) were cut in small equal pieces of 1 cm^2 and immersed in 4.5 mL of reaction solution containing 1.5 mL methanol, 0.1 mL of 10% (w/v) $AlCl_3$, 0.1 mol L^{-1} CH_3COOK, and 2.8 mL of water. After incubation during 30 min in the dark, the absorbance of the reaction mixture was read at 415 nm. The concentration of total flavonoid content was calculated from a calibration curve prepared with up to 100 μg of quercetin per mL of ethanol. The results were expressed as μg g^{-1} fresh weight.

Extraction and activity of the antioxidant enzymes

The extraction of superoxide dismutase (SOD – EC.1.15.1.1), catalase (CAT – EC.1.11.1.16), and ascorbate peroxidase (APX – EC.1.11.1.11) enzymes was performed according to de Sousa et al. (2013), and the soluble protein concentration in the extracts was determined by the method of Bradford (1976). The total activity of SOD was spectrophotometrically assayed by measuring the inhibition of the photochemical reduction of NBT at 560 nm (Donahue et al. (1997). The results were expressed as units of SOD mg^{-1} protein, with one SOD unit being defined as the amount of enzyme that inhibits by 50% the photochemical reduction of NBT at 560 nm. The total activity of CAT was spectrophotometrically assayed according to Rao et al. (1996), by monitoring H_2O_2 degradation at 240 nm over 1 min. The H_2O_2 extinction coefficient of 39.4 mM^{-1} cm^{-1} was used to express the activity of CAT as nmol H_2O_2 min^{-1} mg^{-1} protein. Total activity of APX was spectrophotometrically assayed according to the method described by Amako et al. (1994), based in the oxidation rate of ascorbate at 290 nm. The reaction was initiated by the addition of H_2O_2 and the variation in absorbance at 300 nm was immediately recorded for 30 sec. The total activity of APX was calculated using the ascorbate extinction coefficient of 0.49 mM^{-1} cm^{-1} and expressed as μmol oxidized ascorbate min^{-1} mg^{-1} protein.

Statistical analysis

In all biometric, physiological, and biochemical measurements, at least three biological replicates were used,

each one with three technical replicates and the results expressed as mean ± standard deviation (STDEV) of the mean. The statistical analysis was accomplished by performing a two-way ANOVA, with Cu treatment and species defined as fixed factors and a significance level of 0.05, after checking the homogeneity of variances by using the Levene's test. In cases of significant *P*-values found for Cu treatment, a one-way ANOVA analysis was performed in order to detect the differences between control and Cu-treated plants for each species. When a significant interaction was recorded between both factors tested in the two-way ANOVA, the one-way ANOVA was performed with correction for simple main effects. When the homogeneity of variances was not accomplished, a nonparametric test (Mann–Whitney) was executed in order to discriminate differences between control and Cu-treated plants of each species. All ANOVA results, with respective *F* and *P*-values, can be found in Supporting Information. All statistical data were generated by GraphPad® Prism 6 (GraphPad Software Inc., La Jolla, California, USA).

Results

Accumulation of Cu by roots, stems and leaves

As reported in Materials and Methods, preliminary experiments were performed to find the appropriate Cu concentration in medium to evaluate plant growth and antioxidant response. The concentration of 250 μmol L^{-1} was adopted as it enabled the growth of both plant species, while inducing several toxic symptoms and antioxidant responses (see Supporting Information). Also, this concentration falls in the range of those tested in other reports for several plant species (Choudhary et al. 2010; Fidalgo et al. 2013; Kopittke and Menzies 2006; Zhang et al. 2008a,b).

Table 1 summarizes the values of Cu concentration in leaves, stems and roots measured by flame-atomic absorption spectroscopy in 28-day-old *S. cheesmaniae* and *S. lycopersicum* plants. As can be seen, both species preferentially accumulated Cu in roots. In *S. cheesmaniae,* a 6.3-fold increase was observed over the control, while in *S. lycopersicum*, Cu concentration increased threefold. In Cu-treated plants, Cu levels also increased in stems of both species (1.7- and 3.4-fold increase in *S. cheesmaniae* and *S. lycopersicum,* respectively) and in leaves of *S. lycopersicum* (threefold increase). Thus, leaves of *S. cheesmaniae* seem to be protected from the harmful effects of Cu because no increase in Cu levels was observed in Cu-treated plants in comparison with control plants.

Effects of Cu on growth and photosynthetic pigments of *S. lycopersicum* and *S. cheesmaniae*

Tables 2 and 3 summarize the effects of 250 μmol L^{-1} Cu on different biometric, physiological, and oxidative stress markers of *S. cheesmaniae* and *S. lycopersicum* plants cultivated in hydroponic conditions during 28 days.

Regarding growth-related parameters, root and shoot growth of *S. lycopersicum* was inhibited by 73% and 41%, respectively, while *S. cheesmaniae* revealed to be more tolerant to Cu, because the same growth parameters were inhibited by only 36% and 24%. Regarding biomass production, *S. cheesmaniae* revealed even less sensitive to the harmful effects of Cu than *S. lycopersicum* (Tables 2 and 3). While in *S. cheesmaniae*, the final shoot and root biomass were reduced by 64% and 48%, respectively, over the control, in *S. lycopersicum*, shoot and root biomass suffered a strong reduction higher than 90%. No changes in the final content of both chlorophylls and carotenoids were observed in both species in response to 250 μmol L^{-1} Cu (Table 2).

Based on these results, it can be assumed that Cu effects on the plant growth were dependent on the species, with a more pronounced effect on *S. lycopersicum* than on *S. cheesmaniae.*

Table 1. Cu concentration in leaves, stems and roots of 28-day-old *S. cheesmaniae* and *S. lycopersicum* plants. Cu partition in plants exposed to 250 μmol L^{-1} Cu, expressed in % relative to the total amount of Cu accumulated, is also represented. The concentration is expressed in μg of Cu per g of dry weight.

	S. cheesmaniae			*S. lycopersicum*		
Parameter	Control	250 μmol L^{-1} Cu	Cu partition (%)	Control	250 μmol L^{-1} Cu	Cu partition (%)
Leaves	14 ± 1.2	14 ± 1.2; $P > 0.05$	4.0	16 ± 1.2	42 ± 2.2; **$P < 0.001$**	9.5
Stems	7 ± 1.2	12 ± 2.2; **$P < 0.05$**	3.5	5.3 ± 2.0	18 ± 6.8; **$P < 0.05$**	4.0
Roots	51 ± 1.6	319 ± 3.8; $P < 0.001$	92.5	126 ± 4.0	384 ± 7.8; $P < 0.001$	86.5

Data presented as mean ± STDEV ($n = 4$).
Significant results are bold with the respective *P* value.

Table 2. Effect of 250 μmol L^{-1} Cu on root length, root fresh mass, lipid peroxidation, H_2O_2 and $O_2^{\cdot-}$ contents and total phenols and flavonoids in roots of *S. cheesmaniae and S. lycopersicum* plants.

	S. cheesmaniae		*S. lycopersicum*	
Parameter	Control	250 μmol L^{-1} Cu	Control	250 μmol L^{-1} Cu
Root length (cm)	41.33 ± 8.898	24.68 ± 1.719; $P > 0.05$	45.71 ± 6.346	12.36 ± 0.6843; **$P < 0.05$**
Root fresh mass (g)	1.261 ± 0.1843	0.4562 ± 0.03679; **$P < 0.05$**	1.712 ± 0.3749	0.1141 ± 0.01423; **$P < 0.05$**
MDA (nmol g^{-1} FW)	11.16 ± 0.469	12.82 ± 1.228; $P > 0.05$	14.668 ± 1.383	12.663 ± 0.415; $P > 0.05$
H_2O_2 (nmol g^{-1} FW)	888.9 ± 72.80	1429 ± 4.606; $P > 0.05$	692.757 ± 29.86	967.9 ± 54.06; $P > 0.05$
$O_2^{\cdot-}$ (Abs h^{-1} g^{-1} FW)	1.556 ± 0.07676	0.8707 ± 0.05950; $P > 0.05$	1.648 ± 0.04323	1.03 ± 0.080; **$P < 0.05$**
Total phenols (μg GAE g^{-1} FW)	61.78 ± 1.236	76.28 ± 1.788; $P < 0.05$	61.59 ± 2.446	58.48 ± 2.325; $P < 0.05$
Flavonoids (mg QE g^{-1} FW)	44.27 ± 2.494	91.64 ± 6.095; $P < 0.05$	183.3 ± 19.31	201.3 ± 11.30; $P > 0.05$

Data presented as mean ± STDEV ($n \geq 3$).
Significant results are bold with the respective *P* value.

Oxidative status of *S. lycopersicum* and *S. cheesmaniae* organs

Regarding lipid peroxidation, no differences were found among both all groups of plants. However, in *S. lycopersicum* shoots, MDA levels tended to increase from control to Cu-treated plants, although the differences were not statistically significant. In what concerns ROS levels, Cu treatment induced significant differences in $O_2^{\cdot-}$ and H_2O_2 in both organs, with significant interaction between treatment and species for shoot $O_2^{\cdot-}$ (Supporting Information). The content of $O_2^{\cdot-}$ strongly decreased in roots of both plant species (up to 58%) treated with Cu, but a strong increase of $O_2^{\cdot-}$ content was found in shoots of *S. lycopersicum* (121%), in contrast to *S. cheesmaniae* where $O_2^{\cdot-}$ levels decreased by 37% over the control. Cu treatment did not significantly affect H_2O_2 levels in both organs of *S. cheesmaniae*, but this ROS increased up to 52% in response to Cu in shoots of *S. lycopersicum* (Tables 2 and 3).

Antioxidant response of *S. lycopersicum* and *S. cheesmaniae* in response to Cu

Non-enzymatic component

Statistical data revealed that, in general, Cu concentration differentially affects the non-enzymatic antioxidant system of *S. cheesmaniae* and *S. lycopersicum*. Furthermore, based on the single effects of Cu treatment, differences were found for both phenols and flavonoids (Supporting Information). Thus, results showed that in Cu-treated plants of *S. cheesmaniae*, total phenols content increased by 43% and 24% in shoots and roots, respectively. Also, flavonoids levels were increased by 107% in roots, but a tendency for decreased values was found in shoots. Conversely, in *S. lycopersicum*, the flavonoid content was only increased in shoots (Tables 2 and 3).

Enzymatic component

Regarding the enzymatic component of the antioxidant response, only for CAT activity in shoots was found a significant interaction between Cu treatment and species; however, significant differences for Cu treatment were registered for SOD in roots and CAT both in roots and leaves (Supporting Information). A strong increase of SOD activity (100%) was observed in roots of Cu-treated *S. cheesmaniae,* while a 60% increase was observed in *S. lycopersicum* in response to Cu. In shoots of both species, SOD activity did not change in response to Cu over the controls (Fig. 1).

In shoots of Cu-treated *S. cheesmaniae,* CAT activity was stimulated by 68%, but decreased by 29% in roots, while in *S. lycopersicum* exposed to Cu excess, CAT activity decreased both in roots and shoots (Fig. 2). Regarding APX, although not statistically different, a tendency for an increased activity was observed in shoots of Cu-treated *S. cheesmaniae*, while in *S. lycopersicum* APX activity was not affected in shoots, but decreased by 33% in roots (Fig. 3).

Discussion

The inhibitory effect of Cu on plant growth has been described in different reports for both monocotyledons, including rice (Lin et al. 2013), rye grass (*Lolium perenne*; Verdejo et al. 2015), maize (*Zea mays*; Ali et al. 2002; Aly and Mohamed 2012; Barbosa et al. 2013; Benimeli et al. 2010) and wheat (Gajewska and SkŁodowska 2010; Gang et al. 2013), and dicotyledon species, like *Brassica juncea* (Ansari et al. 2013), and cucumber (İşeri et al. 2011). Different studies have also shown that excess of Cu negatively affects the physiological performance and growth of *S. lycopersicum* (Mazhoudi et al. 1997; Liao et al. 2000; Martins and Mourato 2006; Mediouni et al.

Table 3. Effect of 250 μmol L^{-1} Cu on shoot height, shoot fresh mass, total chlorophyll and carotenoid contents, lipid peroxidation, H_2O_2 and $O_2^{\cdot-}$ contents and total phenols and flavonoids in shoots of *S. cheesmaniae and S. lycopersicum* plants.

	S. cheesmaniae		*S. lycopersicum*	
Parameter	Control	250 μmol L^{-1} Cu	Control	250 μmol L^{-1} Cu
Shoot height (cm)	9.755 ± 0.3895	7.381 ± 0.2797; **$P < 0.001$**	9.716 ± 0.5859	5.698 ± 0.2001; **$P < 0.001$**
Shoot fresh mass (g)	5.315 ± 0.9211	2.792 ± 0.2067; **$P < 0.05$**	7.625 ± 0.9657	0.7546 ± 0.1028; **$P < 0.001$**
Chlorophyll content (mg g^{-1} FW)	0.6473 ± 0.02315	0.8630 ± 0.09390; $P > 0.05$	0.7229 ± 0.02875	0.7645 ± 0.04515; $P > 0.05$
Carotenoids (mg g^{-1} FW)	0.1168 ± 0.004656	0.1460 ± 0.01635; $P > 0.05$	0.1323 ± 0.004102	0.1335 ± 0.008281; $P > 0.05$
MDA (nmol g^{-1} FW)	13.05 ± 1.977	14.373 ± 0.355; $P > 0.05$	14.94 ± 0.482	17.04 ± 0.442; $P > 0.05$
H_2O_2 (nmol g^{-1} FW)	2239 ± 150.2	2453 ± 45.88; $P > 0.05$	1432 ± 105.1	2174 ± 215.6; **$P < 0.05$**
$O_2^{\cdot-}$ (Abs h^{-1} g^{-1} FW)	0.7216 ± 0.008102	0.4576 ± 0.02067; **$P < 0.001$**	1.003 ± 0.1526	2.216 ± 0.1616; **$P < 0.05$**
Total phenols (μg GAE g^{-1} FW)	221.4 ± 29.06	315.5 ± 20.89; $P > 0.05$	174.3 ± 12.63	165.0 ± 10.17; $P > 0.05$
Flavonoids (mg QE g^{-1} FW)	265.0 ± 13.44	193.1 ± 8.434; $P > 0.05$	576.78 ± 7.29	898.2 ± 43.15; $P > 0.05$

Data presented as mean ± STDEV ($n \geq 3$).
Significant results are bold with the respective P value.

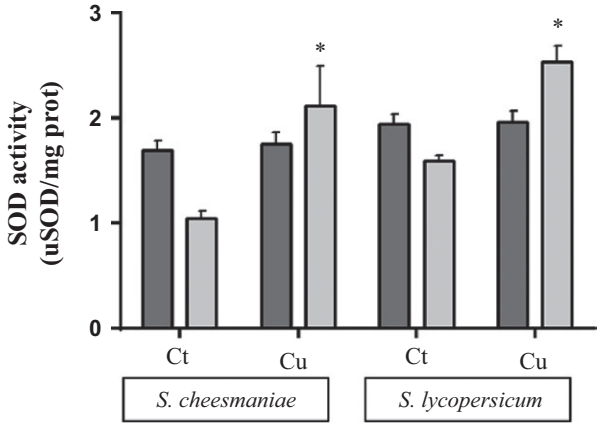

Figure 1. Superoxide dismutase activity in shoots (dark gray) and roots (light gray) of *S. cheesmaniae* and *S. lycopersicum* plants cultivated in nutritional medium supplemented with basal Cu levels (Ct, control) and Cu excess (Cu, 250 μmol L^{-1} Cu). * above bars represent significant differences at $P \leq 0.05$.

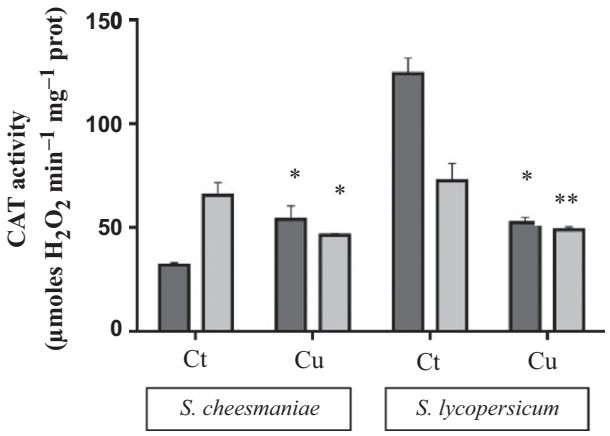

Figure 2. Catalase activity in shoots (dark gray) and roots (light gray) of *S. cheesmaniae* and *S. lycopersicum* plants cultivated in nutritional medium supplemented with basal Cu levels (Ct, control) and Cu excess (Cu, 250 μmol L^{-1} Cu). * and ** above bars represent significant differences at $P \leq 0.05$ and $P \leq 0.001$, respectively.

2006; Chamseddine et al. 2009; İşeri et al. 2011; Al Khateeb and Al-Qwasemeh 2014; Wang et al. 2015). However, an integrative study focused in the interplay of oxidative stress and antioxidant defense in response to Cu was still missing in *S. lycopersicum*, and the effect of Cu on the wild species *S. cheesmaniae* was so far unknown. In this study, we compared the physiological and biochemical mechanisms underlying the antioxidant responses of *S. lycopersicum* and its wild counterpart *S. cheesmaniae* to Cu stress. After 28 days in hydroponic culture, the presence of 250 μmol L^{-1} Cu promoted a decrease in root and shoot length in both *S. cheesmaniae* and *S. lycopersicum* plants and inhibited biomass production, but these effects were more pronounced in *S. lycopersicum* individuals, clearly showing that *S. cheesmaniae* is more tolerant to the toxic effects of the heavy metal.

The more pronounced decrease of biomass and organ length observed in *S. lycopersicum* in response to Cu is likely correlated with the higher levels of Cu found in *S. lycopersicum* organs.

Moreover, excess Cu may interfere with other nutrients' accumulation. Thus, a decrease in the content of calcium (Ca), iron (Fe), and zinc (Zn) in leaves and Mg in roots of tomato plants exposed to Cu was previously reported (Martins and Mourato 2006). The higher accumulation of Cu observed in roots of both plant species than in stems and leaves is in line with previous results in tomato and chicory plants under Cu stress (Liao et al. 2000). Also, this behavior was already found in stone pine (*Pinus pinea*), maritime pine (*Pinus pinaster*), and ash (*Fraxinus angustifolia*) exposed to Cd (Arduini et al. 1996) and Cu in plants of white lupin (*Lupinus albus* L.) growing in

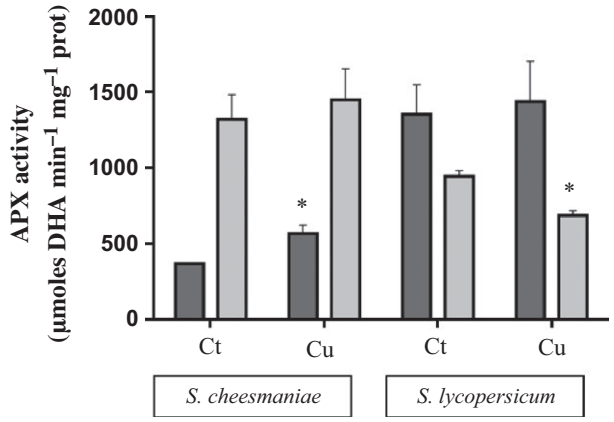

Figure 3. Ascorbate peroxidase activity in shoots (dark gray) and roots (light gray) of *S. cheesmaniae* and *S. lycopersicum* plants cultivated in nutritional medium supplemented with basal Cu levels (Ct, control) and Cu excess (Cu, 250 μmol L^{-1} Cu). * above bars represent significant differences at $P \leq 0.05$.

soils contaminated with Cu, Zn, and nickel (Ni; Fumagalli et al. 2014) and in guava seedlings (*Psidium guajava*) exposed to high concentrations of Ni (Bazihizina et al. 2015). However, the results of this study are particularly relevant, once they clearly suggest that *S. cheesmaniae* is much efficient in preventing Cu translocation from roots to shoots and leaves than *S. lycopersicum,* which support the observed higher capacity of *S. cheesmaniae* to grow under Cu stress than *S. lycopersicum.* These results also validated our hypothesis that being the wild species salt-tolerant (Rush and Epstein 1976; Knapp and Darwin 2006; Peralta and Spooner 2006), it could also be more tolerant to HM than its cultivated counterpart, further supporting that the response to both stresses may share conserved mechanisms.

Being Cu a redox-active transition element, it is able to catalyze the overproduction of ROS (Halliwell and Gutteridge 1984), which in turn can lead to harmful effects in proteins and nucleic acids and the peroxidation of lipids. It is widely accepted that lipid peroxidation occurs as a consequence of oxidative stress, being one of the most damaging process to all organisms (Gill and Tuteja 2010). However, in this study results showed that Cu only induced significant changes in MDA levels in shoots of *S. lycopersicum*, possibly because lipid peroxides were efficiently neutralized or its production avoided by a positive response of the antioxidant (AOX) system. MDA levels also did not change in response to HM stress in some previous reports (Gajewska et al. 2006; Gajewska and Sklodowska 2007; Soares et al. 2016a).

Several studies have reported increased ROS accumulation in several plants species under HM stress (Li et al. 2012; Thounaojam et al. 2012; Lukatkin et al. 2014; Soares et al. 2016b). In this study, we have observed that $O_2^{\cdot-}$ and H_2O_2 levels in response to excess Cu were tissue- and species-dependent: the exposure of *S. cheesmaniae* to Cu led to a decrease of $O_2^{\cdot-}$ levels in roots and shoots, but H_2O_2 levels increased in roots. In contrast, in *S. lycopersicum*, both $O_2^{\cdot-}$ and H_2O_2 content suffered significant increases in consequence of Cu excess, with the exception of $O_2^{\cdot-}$ levels in roots which decreased. In general, *S. cheesmaniae* showed a higher capacity to control and/or to scavenge both $O_2^{\cdot-}$ and H_2O_2 than *S. lycopersicum* in response to Cu.

Plants, as sessile organisms, have developed a complex antioxidant system, to withstand the toxic effects of ROS and oxidative stress (Sharma et al. 2012). In agreement, when the activities of SOD, CAT, and APX were measured in different tissues of both species, our results showed a less efficient AOX system in *S. lycopersicum* than in its wild counterpart. As reported, roots of both species exposed to Cu showed an increased SOD activity and, in accordance to this SOD behavior, roots of Cu-treated plants exhibited a reduction in $O_2^{\cdot-}$ levels and an increase of H_2O_2 content. In shoots, SOD activity remained unaltered in response to Cu in both plant species and, in agreement, the levels of $O_2^{\cdot-}$ suffered a decrease in *S. cheesmaniae* and a marked increase in *S. lycopersicum*, which accumulated more Cu in shoots. Apparently, this reduction of $O_2^{\cdot-}$ in *S. cheesmaniae* is not related to SOD activity. Actually, taking into account that the total Cu accumulated in the shoots of this species did not change between control and Cu-treated plants, we can hypothesize that other AOX mechanism or metabolite is responsible for the decrease of $O_2^{\cdot-}$ levels. Therefore, it seems that SOD showed a differential organ response, with a more pronounced and active protection role in roots. With respect to H_2O_2 detoxifying enzymes, our results showed that CAT activity was downregulated by Cu excess in both species, with decreased activity in all experimental conditions, excepting *S. cheesmaniae* shoots, where a positive response was recorded. In agreement, the inhibition of CAT activity as a consequence of HM stress has been well reported in literature and specifically in what regard Cu stress in several plants species like sunflower (*Helianthus annuus*; Gallego et al. 1996), durum wheat (*Triticum durum*; Sgherri et al. 2001), and in vitro grown plants of Indian ginseng (*Withania somnifera*; Khatun et al. 2008). This decrease in CAT activity can be a consequence of its auto reduction (De Vos et al. 1992; Gallego et al. 1996; Weckx and Clijsters 1996; Mazhoudi et al. 1997; Yamamoto et al. 1997) and/or the autoxidation and Fenton reactions which can also, in turn, cause oxidative injury in defense enzymes (Schutzendubel and Polle 2002). Regarding APX activity, a species- and organ-dependent response was observed. In fact, higher APX activity was

observed in shoots of Cu-treated *S. cheesmaniae* than in control, while it did not change in shoots of *S. lycopersicum*, but decreased in roots. Several studies report a decrease or maintenance in APX activity in response to Cu stress (Mazhoudi et al. 1997; Teisseire and Guy 2000; Bankaji et al. 2015), and a previous work of our group with *S. nigrum* clearly showed that APX was downregulated in response to 100 and 200 μmol L^{-1} Cu in a dose-dependent manner (Fidalgo et al. 2013). Overall, the results of this study showed that the H_2O_2 levels in both plant species in response to excess Cu correlated with the observed APX and CAT activity, which is in accordance with the main role of both enzymes in the cellular detoxification of H_2O_2 (Sharma et al. 2012).

A more effective response to excess Cu of the nonenzymatic AOX system was also observed in *S. cheesmaniae* than in *S. lycopersicum,* when total phenolics and flavonoids were quantified. In what concerns total phenols, Cu exposure led to changes in *S. cheesmaniae*, with a significant increase in both plant organs in plants cultivated with Cu excess, suggesting that they may have a role in the observed higher tolerance of *S. cheesmaniae* to Cu toxicity. Particularly relevant was the observation that flavonoids increased substantially in roots of *S. cheesmaniae* in response to excess Cu and decreased in shoots, data which go in agreement with several references that state that these metabolites can be translocated from one organ to another, specifically from shoots to roots (Saslowsky and Winkel-Shirley 2001; Buer and Muday 2004; Buer et al. 2007). Also, it was also evident from our results that the basal level of flavonoids was higher in the domestic species, although its content did not changed in response to Cu. In this way, and bearing in mind the previously considered hypothesis, it seems that although flavonoids are less produced in *S. cheesmaniae*, their increase in roots may help to prevent oxidative damage and limit the translocation of Cu to the aerial parts of the plants.

Overall, our results validated the hypothesis that *S. cheesmaniae* is more tolerant to excess Cu than its domesticated counterpart, in part, due to its high capacity to limit the translocation of Cu to the aerial parts and its enhanced AOX performance. Studies in progress involving the identification and functional characterization/tissue localization of Cu transporters in both plant species may have important repercussion on the understanding of the molecular basis for the observed capacity of *S. cheesmaniae* in limiting the toxic effects of Cu on leaf tissues and will open new avenues for plant breeding.

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Conflict of Interest

The authors also declare that there is no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/fes3.114/suppinfo

Figure S1. Germination percentage of *S. lycopersicum* in response to different Cu concentrations.

Figure S2. Seedlings of *S. lycopersicum* 7 days after germination in control conditions (a) and in the presence of 250 μM (b), 500 μM (c), 1000 μM (d), and 2500 μM (e) CuSO4.

Table S1. Summary of two-way ANOVA statistical data for leaves, with species and Cu treatment defined as fixed factors.

Table S2. Summary of two-way ANOVA statistical data for roots, with species and Cu treatment defined as fixed factors.

Table S3. Summary of two-way ANOVA statistical data for Cu accumulation in leaves, stems, and roots with species and Cu treatment defined as fixed factors.

Table S4. Summary of one-way ANOVA statistical data performed for Cu treatment in leaves of *Solanum cheesmaniae* and *Solanum lyopersicum* plants.

Table S5. Summary of one-way ANOVA statistical data performed for Cu treatment in roots of *Solanum cheesmaniae* and *Solanum lyopersicum* plants.

