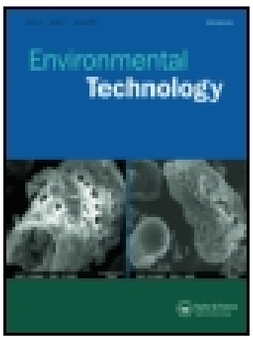


Environmental Technology

ISSN: 0959-3330 (Print) 1479-487X (Online) Journal homepage: http://www.tandfonline.com/loi/tent20

Removal of copper from aqueous solutions by rhizofiltration using genetically modified hairy roots expressing a bacterial Cu-binding protein

Patricia Pérez-Palacios, Elizabeth Agostini, Sabrina G. Ibáñez, Melina A. Talano, Ignacio D. Rodríguez-Llorente, Miguel A. Caviedes & Eloísa Pajuelo

To cite this article: Patricia Pérez-Palacios, Elizabeth Agostini, Sabrina G. Ibáñez, Melina A. Talano, Ignacio D. Rodríguez-Llorente, Miguel A. Caviedes & Eloísa Pajuelo (2017): Removal of copper from aqueous solutions by rhizofiltration using genetically modified hairy roots expressing a bacterial Cu-binding protein, Environmental Technology, DOI: 10.1080/09593330.2017.1281350

To link to this article: http://dx.doi.org/10.1080/09593330.2017.1281350

ENVIRONMENTAL TECHNOLOGY, 2017
http://dx.doi.org/10.1080/09593330.2017.1281350

Taylor & Francis
Taylor & Francis Group

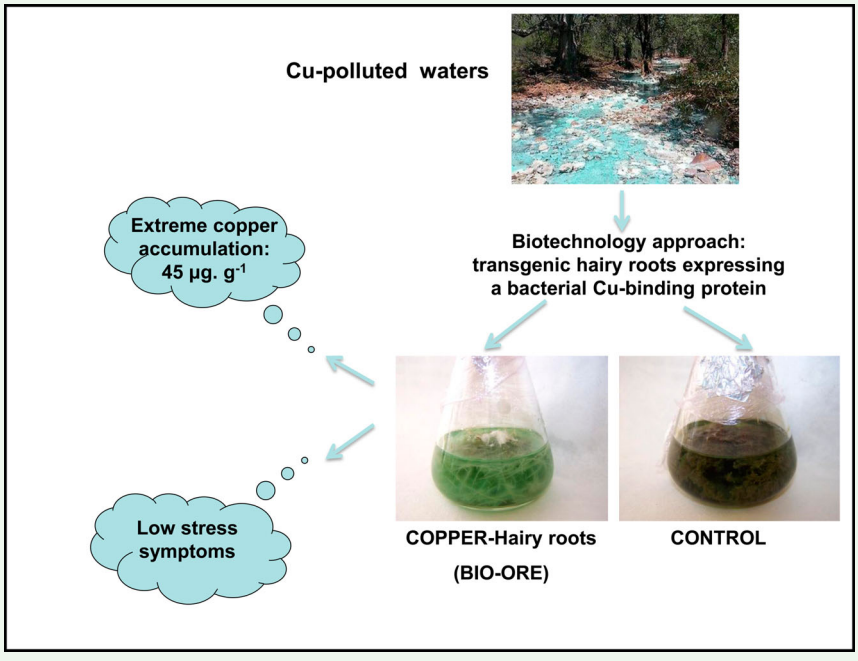
Removal of copper from aqueous solutions by rhizofiltration using genetically modified hairy roots expressing a bacterial Cu-binding protein

Patricia Pérez-Palacios[a], Elizabeth Agostini[b], Sabrina G. Ibáñez[b], Melina A. Talano[b], Ignacio D. Rodríguez-Llorente [a], Miguel A. Caviedes [a] and Eloísa Pajuelo [a]

[a]Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, Sevilla, Spain; [b]Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Córdoba, Argentina

ABSTRACT

The aim of this work was to develop a biotechnological tool to hyperaccumulate high copper (Cu) concentrations from wastewaters. Transgenic tobacco hairy roots were obtained by expressing, either the wild-type version of the gene *copC* from *Pseudomonas fluorescens* in the cytoplasm of plant cells (CuHR), or a modified version targeted to the vacuole (CuHR-V). Control hairy roots transformed with the empty vector (HR) were also generated. The roots were incubated in the presence of solutions containing Cu (from 1 to 50 mM). At 5 mM external copper, transgenic hairy roots accumulated twice the amount of copper accumulated by control hairy roots. However, at 50 mM Cu, accumulation in both transgenic and control roots reached similar values. Maximum Cu accumulation achieved by transgenic hairy roots was 45,000 µg g^{-1} at 50 mM external Cu. Despite the high Cu accumulation, transgenic hairy roots, particularly CuHR-V, showed less toxicity symptoms, in correlation with lower activity of several antioxidant enzymes and lower malondialdehyde (MDA) levels. Moreover, CuHR-V roots displayed low values of the oxidative stress index (OSI) – a global parameter proposed for oxidative stress – indicating that targeting CopC to the vacuole could alleviate the oxidative stress caused by Cu. Our results suggest that expressing *copC* in transgenic hairy roots is a suitable strategy to obtain Cu-hyperaccumulator hairy roots with less toxicity stress symptoms.



Abbreviations: APX: ascorbate peroxidase; ATSDR: Agency for Toxic Substances and Disease Registry (U.S.); BCF: bioconcentration factor; CuHR: *copper-hairy roots*; EDTA: ethylenediamine

ARTICLE HISTORY
Received 16 March 2016
Accepted 5 January 2017

KEYWORDS
Agrobacterium rhizogenes; tobacco; *copC*; oxidative stress; bio-ore

CONTACT Eloísa Pajuelo epajuelo@us.es Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, c/ Profesor García González, 2, 41012, Sevilla, Spain

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/09593330.2017.1281350

tetracetic acid; EPA: Environmental Protection Agency (U.S.); GSH: glutathione; HM: heavy metals; HR: control hairy roots; ICP-OES: Inductively Coupled Plasma/Optical Emission Spectrometry; MDA: malondialdehyde; NBT: nitroblue tetrazolium; OD: optical density; OSI: oxidative stress index; PCR: polymerase chain reaction; PVP: polyvynilpirrolidone; PX: peroxidase; ROS: reactive oxygen species; SOD: superoxide dismutase

1. Introduction

The generation of huge volumes of wastewaters due to increasing industrialization poses a great risk to human health and the environment. Among the contaminants, heavy metals (HM) and metalloids are highly toxic and their presence in water, even in trace amounts, can cause serious health problems to organisms. Moreover, HM bioaccumulation in the food chain can be dangerous to human health [1]. Copper (Cu), one of the most toxic metals, affects plant growth, wildlife and human health. For this reason, the threshold for Cu concentration in drinking water is established as 1.3 mg L^{-1} by the Environmental Protection Agency (U.S.) (EPA), so that special strategies to reduce its quantity are needed [2]. Since HM cannot be degraded neither destroyed, remediation techniques allow separating them from aqueous solutions for convenient disposal [3–4]. In this context, phytoremediation has emerged as a suitable green methodology to clean up contaminated environments, showing a great potential due to its environmental compatibility and cost-effectiveness [5]. In particular, rhizofiltration uses plants to absorb pollutants, such as HM, from water streams. In this sense, sunflower was used for absorption of uranium, cesium and strontium after the Fukushima nuclear disaster [6] and water hyacinth and *Lemna* spp. were widely used for rhizofiltration [7,8]. The success of metal phytoremediation relays on the existence of metal hyperaccumulators, species that have the capability of accumulating metals to a greater concentration than that of the original matrix, such as soil or water [9,10]. Regarding Cu-hyperaccumulation, there are scarce hyperaccumulators species, for example, *Crassula helmsii* hyperaccumulates up to 9000 mg of Cu per kg of dry matter in roots [11]. Besides, some previously reported Cu-hyperaccumulators are now considered as indicators (*Elsholtzia splendens*) [12] or tolerant excluder species, such as the cuprophytes *Crepidorhopalon perennis* and *Haumaniastrum katangense* [13,14].

In plants, Cu is a necessary cofactor of many enzymatic reactions and it is involved in physiological processes such as photosynthesis, mitochondrial respiration, superoxide scavenging, cell wall metabolism, ethylene perception and development of reproductive organs [15]. At high concentrations, Cu inhibits germination and growth, affects the photosynthetic apparatus and induces a strong oxidative stress [16–18]. The oxidative stress is induced by over-production and accumulation of 'reactive oxygen species' (ROS) that react rapidly with lipids, nucleic acids, pigments and proteins, causing lipid peroxidation, membrane damage and inactivation of enzymes, thus affecting cell viability [19]. In order to overcome oxidative stress, plants have developed two main antioxidant defense mechanisms, which can be classified as enzymatic [superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidases (PX), etc.] and non-enzymatic systems [glutathione (GSH), ascorbic acid, carotenoids, tocopherols, etc.]. Among the plant enzymes involved in antioxidant cell-defense responses SOD, APX and PX were found to be specifically activated by exposure to elevated Cu concentrations [20].

In the past decade, an emerging approach to study phytoremediation processes was the development of hairy roots cultures, which are disease manifestations produced upon infection by *Agrobacterium rhizogenes* [21,22]. These cultures show several advantages as a model system compared to whole plants: indefinite propagation, high genotypic and phenotypic stability, fast growth under controlled conditions and the possibility of genetic engineering [22,23]. Besides, their growth *in vitro* on a carbon-rich medium is independent on photosynthesis, which is one of the processes most affected by Cu [24,25]. Hairy roots from metal hyperaccumulators alpine pennycress (*Noccaea (Thlaspi) caerulescens*), *Alysum* sp., Indian mustard (*Brassica juncea*) and sunflower (*Helianthus annuus*) were developed for the accumulation of metals and radionuclides [26–28]. Unfortunately, most metal-hyperaccumulators plant species are recalcitrant to transformation [29,30]. Thus, different model plants, like tobacco could be modified by using genetic engineering tools, to improve extraction, sequestration and/or detoxification of multiple contaminants efficiently [31–33]. In addition, subcellular targeting of proteins in desired cell organelles could enhance metal accumulation [34,35]. Recently, Cu hyperaccumulating *Arabidopsis thaliana* plants were generated by expressing *P. fluorescens* Cu-binding periplasmic protein CopC, which could be targeted to vacuole by fusing with specific signal peptides [36].

The objective of this work was generating transgenic tobacco hairy roots by expressing *copC* either in the cytoplasm of cells or targeted to the vacuole, in an attempt to increase Cu accumulation. Our aims are, on the one hand obtaining a Cu-enriched bio-ore, and on the other hand, to explore the contribution of this prokaryotic protein in alleviating toxicity stress symptoms in transgenic roots.

2. Materials and methods

2.1. Constructs

The *copC* gene from *P. fluorescens* Az13 (acc. Number EF587902.1) was amplified as previously described [36]. To target the CopC protein to the vacuole, specific vacuole sorting determinants were added both at the C-terminus and N-terminus [36]. The polymerase chain reaction (PCR) product of *copC* and the construction with *copC* gene plus vacuole sorting determinants were cloned into the binary vector pCambia1390 (Cambia GPO, Canberra, Australia) driven by a CaMV35S promoter (Suppl. Inf., Fig. S1.A, B). The plasmids with the different two constructs were independently electroporated into electrocompetent *A. rhizogenes* Arqua1. Transformants were selected for kanamycin resistance (25 μg mL^{-1}), and confirmed by plasmid isolation followed by digestion and PCR.

2.2. Obtainment of control and transgenic hairy roots expressing copC

Nicotiana tabacum (cv. Wisconsin) seeds were surface sterilized and germinated in MSRT medium consisting of MS [37] medium plus RT vitamin complex [38] with the addition of 3.0% (w/v) sucrose and 0.9% (w/v) agar. Sterile leaf explants were wounded, inoculated with a 48 h culture of *A. rhizogenes* Arqua1, transferred to 0.9% (w/v) agar-water plates and incubated in the dark at 25°C for 72 h [22] (Suppl. Inf., Fig. S2). Then, they were transferred to plates containing MSRT solid medium with ampicillin (1 g L^{-1}). Transgenic lines were further selected according to morphological and physiological criteria such as color, growth rate and vigorous aspect. [39]. Explants showing de-differentiation, forming *calli* or regenerating plants were discarded (Suppl. Inf., Table S1) as well as those lines showing no growth or poor biomass. Upon appearance of hairy roots, they were cut and transferred to liquid MSRT medium with ampicillin (1 g L^{-1}) and cultivated at 25 ± 2°C in the dark at 70 rpm. Hairy roots were subcultivated every 25–30 d in fresh liquid MSRT medium. The hairy roots lines expressing *copC* in the cytoplasm and in the vacuole were named as *copper-hairy roots* (CuHR) and *copper-hairy roots-vacuole* (CuHR-V), respectively. Cultures obtained by infection with wild-type *A. rhizogenes* Arqua1 were used as control hairy roots (HR). Transformation was confirmed by *rolC* gene amplification [40]. The presence of the *copC* gene was confirmed by PCR using appropriate primers [36].

2.3. RNA expression analysis

Transgenic hairy roots lines (CuHR and CuHR-V) and wild type (HR) were exposed to water (control condition) and 5 mM of $CuSO_4$ for 7 days. RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Three independent RNA samples from each line and condition were obtained. Genomic DNA degradation and reverse transcription were performed using OneStep RT-PCR kit (Qiagen). RT-PCR was carried out using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and an ECO™ termocycler (Illumina). PCR conditions consisted of an initial denaturation at 95°C for 2 min followed by 40 cycles at 95°C for 5 s, 58°C for 10 s, 72°C for 15 s, and a final step at 95°C 15 s, 55°C 15 s, 95°C 15 s. The gene *L25*, encoding a ribosomal protein, considered as the best housekeeping gene for tobacco under abiotic stress, was used for normalization [41]. Expression signals were quantified and normalized using Eco™ Software v4.1.2.0. Results were expressed according to the comparison of the algorithms obtained for the different Ct [42], according to the formula: $2^{-(\Delta CtE-\Delta CtC)} = 2^{-(\Delta\Delta Ct)}$, where ΔCtE is the Ct achieved in experimental samples and ΔCtC is the Ct obtained for the housekeeping gene *L25*.

2.4. Determination of the maximum copper accumulation

Roots *inocula* of 0.2 g were incubated at 25°C and 70 rpm in 50 mL of MSRT medium with ampicillin (1 gr L^{-1}). After 20 days, roots were transferred to MSRT containing different $CuSO_4$ concentrations spanning three orders of magnitude (from 0.5 to 50 mM) and incubated at 28°C in a rotary shaker (70 rpm) for 7 days. After collection, roots were exhaustively washed with water, dried at 80°C for 72 h and used for Cu determination in roots dry weight by inductively coupled plasma/optical emission spectrometry (ICP-OES) [43].

2.5. Determination of the maximum copper accumulation and/or adsorption

Selected lines were exposed to different $CuSO_4$ concentrations (from 1 to 50 mM) and incubated at 28°C in a rotary shaker (70 rpm) for 7 days. After collection, roots

were washed with water, dried at 80°C for 72 h and used for Cu determination by ICP-OES [43].

In order to evaluate Cu accumulation vs. Cu biosorption, hairy roots were exposed to $CuSO_4$ (10, 20 and 50 mM) for 7 days. Then, they were collected and half of the biomass was washed either with water to determine total accumulated Cu (accumulated inside the root and adsorbed onto the surface), or with 100 mM ethylenediamine tetracetic acid (EDTA) to determine Cu accumulated inside roots, since EDTA is a desorption agent [44]. After drying in an oven at 80°C for 72 h, Cu was analyzed by ICP-OES.

2.6. Determination of antioxidant enzymes activity in control and copper-hairy roots

Root *inocula* of 0.2 g were grown for 20 days in MSRT medium with ampicillin (1 gr L^{-1}), and then exposed to increasing Cu concentrations (0, 0.5, 5 and 50 mM). After 7 days, hairy roots were harvested, powdered in a mortar using liquid nitrogen and stored at −80°C until use.

For PX (EC 1.11.1.7) activity, frozen hairy roots were homogenized with 50 mM acetic/sodium acetate buffer, pH 5.0 containing 1 M KCl. The extracts were centrifuged at 5000×*g* for 5 min and supernatants were used for activity determination. PX activity was determined spectrophotometrically at 470 nm using *o*-dianisidine (0.63 mM) and H_2O_2 (0.5 mM) as substrates. One unit of enzyme (U) was defined as the amount of enzyme that generated 1 µmol of product in 1 min in the conditions described and the ε 11.3 M^{-1} cm^{-1} was used [45].

SOD (EC 1.15.1.1) activity was measured by the photochemical inhibition of the nitroblue tetrazolium (NBT) reduction. Roots were homogenized with 50 mM potassium phosphate buffer (pH 7.8), with 5 mM EDTA and 2% polyvinylpyrrolidone (PVP). After adding 13 mM methionine, 75 µM NBT and 20 µL of enzyme extract in 1 mL final volume, the reaction mixture was exposed to light for 7 min with 25-W light tube. Then optical density (OD) at 560 nm was read. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of NBT by 50% [45].

APX (EC 1.11.1.6) activity determination was carried out according to Sosa-Alderete et al. [45]. Enzyme extract was obtained using the same buffer than that used for SOD activity. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 0.4 mM ascorbate, 0.1 mM H_2O_2 and 100 µL enzyme extract. The decrease in absorbance at 290 nm was measured. Activity was calculated according to the extinction coefficient ($\varepsilon = 2.8$ mM^{-1} cm^{-1}).

For specific activity calculation, enzymatic activities were referred to total soluble protein content and it was measured according to Bradford [46].

2.7. Analysis of lipid peroxidation

The degree of lipid peroxidation was estimated following the method described by Sosa-Alderete et al. [45]. Lipid peroxides were expressed as µmol malondialdehyde (MDA) by using the ε 155 mM^{-1} cm^{-1}.

2.8. Estimation of the overall oxidative stress: the oxidative stress index

To globally evaluate the extent of oxidative stress, we propose a new parameter, the oxidative stress index (OSI), calculated as a prorate average of different stress-related magnitudes in relation to their value in the absence of stress. OSI is defined as follows:

$$\mathrm{OSI} = \frac{[\mathrm{PX}]_{Cu}/[\mathrm{PX}]_0 \pm [\mathrm{SOD}]_{Cu}/[\mathrm{SOD}]_0 \pm [\mathrm{APX}]_{Cu}/[\mathrm{APX}]_0 \pm [\mathrm{MDA}]_{Cu}/[\mathrm{MDA}]_0}{4},$$

where $[PX]_{Cu}$, $[SOD]_{Cu}$, $[APX]_{Cu}$ and $[MDA]_{Cu}$ are the values of these activities at a particular Cu concentration, and $[PX]_0$, $[SOD]_0$, $[APX]_0$ and $[MDA]_0$ were the values of the activities in roots incubated in the absence of Cu. Similar indexes have been used, for instance, for calculation of global pollution [47]. A value of 1 corresponds to roots with the same oxidative stress as controls (without Cu). Values above 1 correspond to roots with higher oxidative stress than control hairy roots.

2.9. Statistical analysis

Analytical determinations and enzymatic activities are the means ± standard deviations of three independent measurements. Statistical analysis was performed by ANOVA-One Way, followed by DMS *post hoc* test, using the program IBM® SPSS Statistics.

3. Results

3.1. Obtainment of control and copper-hairy roots expressing copC gene

Initially, 5 independent control HR lines, 8 CuHR and 6 CuHR-V lines were obtained (Suppl. Inf., Table S1). The efficiency of transformation using wild-type *A. rhizogenes* strain (33%) was similar compared with that obtained for the transformants *A. rhizogenes* strains containing *copC* (47%) and *copC*-vacuole sequence (36%). Finally, control and transgenic lines (CuHR and CuHR-V) showing stable morphology, fast growth and high lateral root formation were selected for further studies. The insertion of *rolC,* indicative of hairy roots, was confirmed by the amplification of a 554 bp PCR product. Also, *copC* gene was confirmed by

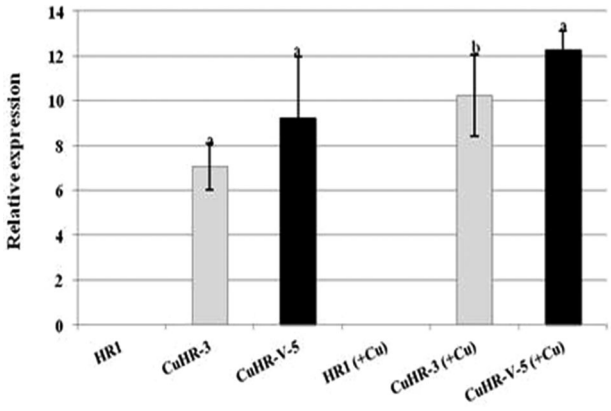


Figure 1. *CopC* expression in *copper-hairy roots*. RT-PCR analysis of *copC* gene expression in *N. tabacum* HR1 or transgenic lines CuHR and CuHR-V with (5 mM Cu) or without Cu exposure. No *copC* transcript was detected in HR. RT-PCR Amplification of the *L25r* transcript was used as a control to normalize expression levels. Results are the means ± SE of three different experiments. Significant differences are indicated by different letters.

an internal 315 bp PCR product present in transgenic lines and absent in control lines (Suppl. Inf. Fig. S3).

3.2. Preliminary Cu accumulation screening in copper-hairy roots

A preliminary Cu accumulation test was developed to select lines with the highest Cu-accumulation potential. Lines CuHR showed a 20–30% increased accumulation compared to the line HR, without significant differences. Moreover, CuHR-V lines accumulated between 40% and 50% more Cu when compared to HR, with no significant differences among them (Suppl. Inf., Table S2). Two transgenic lines showing the highest Cu accumulation (CuHR-3 and CuHR-V-5) and one control line (HR1) were selected for further analysis.

3.3. CopC expression in copper-hairy roots

Expression of *copC* in selected lines (exposed or not to Cu) was studied by RT-PCR (Figure 1). As expected, no expression was found in HR1. Under control conditions (without Cu treatment) CuHR-3 and CuHR-V-5 showed the same levels of *copC* expression, while at 5 mM Cu, only CuHR-3 line showed a significant increase in *copC* gene expression (50%). The subcellular localization of the CopC protein inside the vacuole was previously demonstrated [36].

3.4. Maximum Cu accumulation and/or adsorption in copper-hairy roots

To determine the maximum Cu accumulation capacity, hairy roots were incubated at Cu concentrations ranging from 1 to 50 mM. Under visual comparison of HR1 and *copper-hairy roots* in the absence of Cu, all of them showed the same morphology (Figure 2(B,E,H)). At 1–10 mM Cu treatment, HR1 tissue became brown and necrotic. By contrast, transgenic CuHR-3 and CuHR-

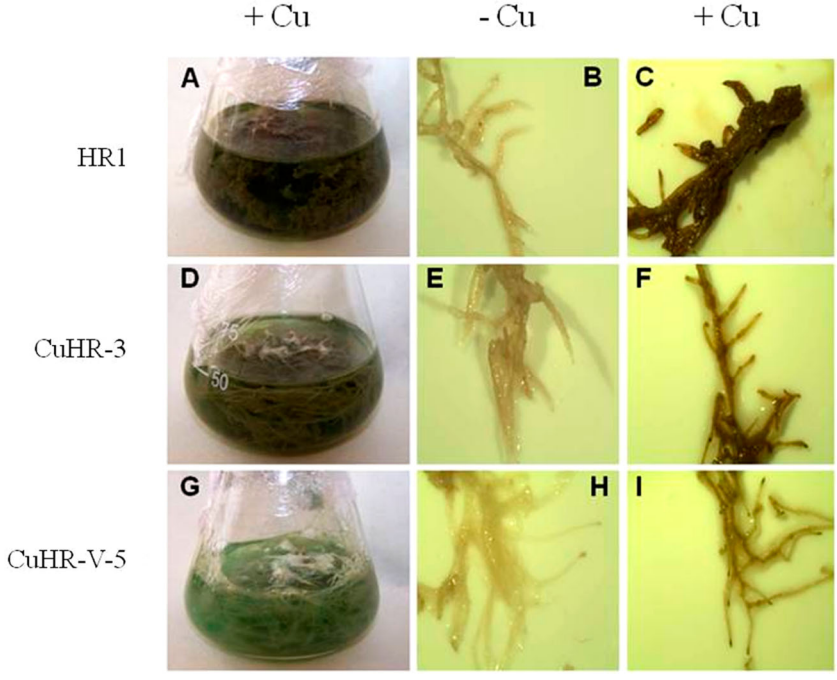


Figure 2. Hairy root morphology in the absence and presence of Cu. HR1 in the presence of 50 mM Cu (A, C) or in the absence of Cu (B). Cu-HR-3 in the presence of 50 mM Cu (D, F) or in the absence of Cu (E). Cu-HR-V-5 in the presence of 50 mM Cu (G, I) or in the absence of Cu (H).

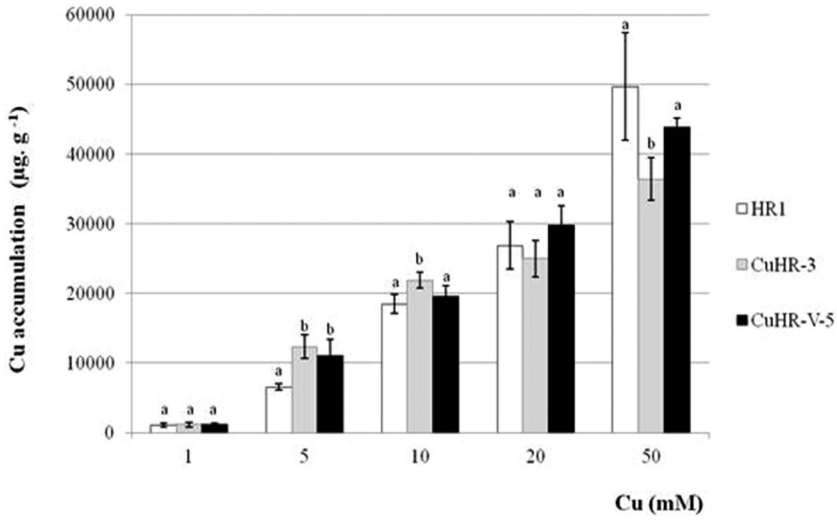


Figure 3. Cu accumulation of HR1 and *copper-hairy roots*. Hairy roots were exposed to increasing Cu concentrations for 7 days. Data of Cu determination are means of three independent measurements and bars indicate standard deviations. Significant differences at $p < .05$ are designated by different letters.

V-5 looked healthy and without macroscopic toxicity symptoms (data not shown). The differences were more accentuated at 50 mM Cu; HR1 were dark and the tissue was necrotic and damaged, and roots were not able to survive (Figure 2(A,C)). Transgenic CuHR-3 showed some toxicity symptoms characterized by browning, especially at the root tips, and slow growth (Figure 2(D,F)). Furthermore, CuHR-V-5 showed lesser toxicity symptoms in the presence of Cu. Roots were light brown except for the root tips, which were dark brown (Figure 2(G, I)).

Cu determination (Figure 3) showed that the highest differences in total accumulation occurred at 5 mM, in which the transgenic lines accumulated twice more Cu than HR1 (around 10,000–12,000 µg g^{-1} versus 6,000 µg g^{-1}). At this Cu concentration, the bioconcentration factor (BCF), which is defined as Cu accumulated into the root tissue with regard to Cu in the external solution [27], was maximal, with values of 35–40 for transgenic hairy roots, whereas the BCF for HR1 was 20. This means that maximum Cu accumulation efficiency was reached when 5 mM of Cu was used. At 10 mM Cu treatment, only CuHR-3 showed statistically higher Cu accumulation. At 20 mM Cu, the accumulation of Cu was high (25,000–30,000 µg g^{-1}) for the analyzed hairy root lines although without significant differences between them. At 50 mM, CuHR-3 and CuHR-V-5 accumulated 39,000 µg g^{-1} and 45,000 µg g^{-1} Cu, respectively (Figure 2(C)). Surprisingly, the determination of Cu in HR1 reached a value of 49,000 µg g^{-1}. This could be due to the high degree of tissue damage, which can expose both external and internal surfaces for Cu biosorption.

In order to evaluate the percentage of Cu that was accumulated inside the roots and adsorbed onto the roots surface, hairy roots previously exposed to Cu were washed either with water or with EDTA, which is a chelator that can remove bioadsorbed Cu linked to the chemical groups on the cellular surface [44]. In HR1, 40–55% of Cu was washed out by EDTA (adsorbed Cu) (Table 1), indicating that Cu was equally accumulated inside the root and adsorbed onto the root surface. By contrast, CuHR-3 and CuHR-V5 accumulated more Cu inside the tissue (between 80% and 96%) compared to control HR1, since only 5–20% Cu was adsorbed. Particularly, up to 96% of Cu was accumulated inside *copper-hairy roots* at 50 mM Cu, showing that the expression of *copC* would contribute to the more efficient intracellular accumulation of Cu either in the cytoplasm or inside the vacuole.

3.5. Determination of antioxidant enzymes activity in wild type and copper-hairy roots

To evaluate oxidative stress caused by Cu, antioxidant enzymes were determined in control and *copper-hairy roots* exposed for 7 days in a wide range of Cu concentrations (0, 0.5, 5 and 50 mM) (Figure 4). Generally, the activities of PX, SOD and APX in HR1, CuHR-3 and CuHR-V-5, increased with increasing Cu concentration. In this sense, HR-1 showed 6-, 32- and 20-fold increases in PX, SOD and APX, respectively, compared to the levels in the absence of Cu. Antioxidant enzymes in CuHR-3 showed moderate increments with increasing concentrations of Cu, reaching the maximum values of PX, SOD and APX at the highest concentration of Cu

Table 1. Determination of Cu accumulated inside and adsorbed to root tissues. Hairy roots were incubated in the presence of 10, 20 and 50 mM Cu for 7 days.

Construction	Cu (mM)	Total Cu ($\mu g\ g^{-1}$)	Accumulated Cu ($\mu g\ g^{-1}$)	Adsorbed Cu ($Cu_{total}-Cu_{accumulated}$) ($\mu g\ g^{-1}$)
HR1	10	18,499 ± 1400	11,034 ± 4756 (59.6%) [(a)]	7465 (40.4%) [(a)]
	20	26,877 ± 3411	12,107 ± 58 (45.0%) [(a)]	14,770 (55.0%) [(a)]
	50	49,709 ± 7710	26,018 ± 5128 (52.3%) [(a)]	23,691 (47.7%) [(a)]
CuHR-3	10	21,907 ± 1128	17,298 ± 4268 (79.0%) [(b)]	4609 (21.0%) [(b)]
	20	24,982 ± 2610	23,819 ± 432 (95.3%) [(c)]	1163 (4.7%) [(c)]
	50	36,441 ± 3055	34,432 ± 3055 (94.5%) [(c)]	2009 (5.5%) [(c)]
CuHR-V-5	10	19,562 ± 1539	15,627 ± 1318 (79.9%) [(b)]	3935 (20.1%) [(b)]
	20	29,746 ± 2814	26,269 ± 1051 (88.3%) [(d)]	3477 (11.7%) [(d)]
	50	43,884 ± 1286	42,107 ± 558 (96.0%) [(c)]	1777 (4.0%) [(b)]

Notes: Hairy roots were washed either with water (in order to determine total Cu, Cu accumulated in the roots plus Cu adsorbed onto the root surface) or with EDTA (in order to wash out Cu adsorbed onto the roots). After drying, Cu was determined by ICP-OES. Data are means ± standard deviations of three independent determinations. Significant differences with regard to the control (a) at $p < .05$ are indicated by different letters.

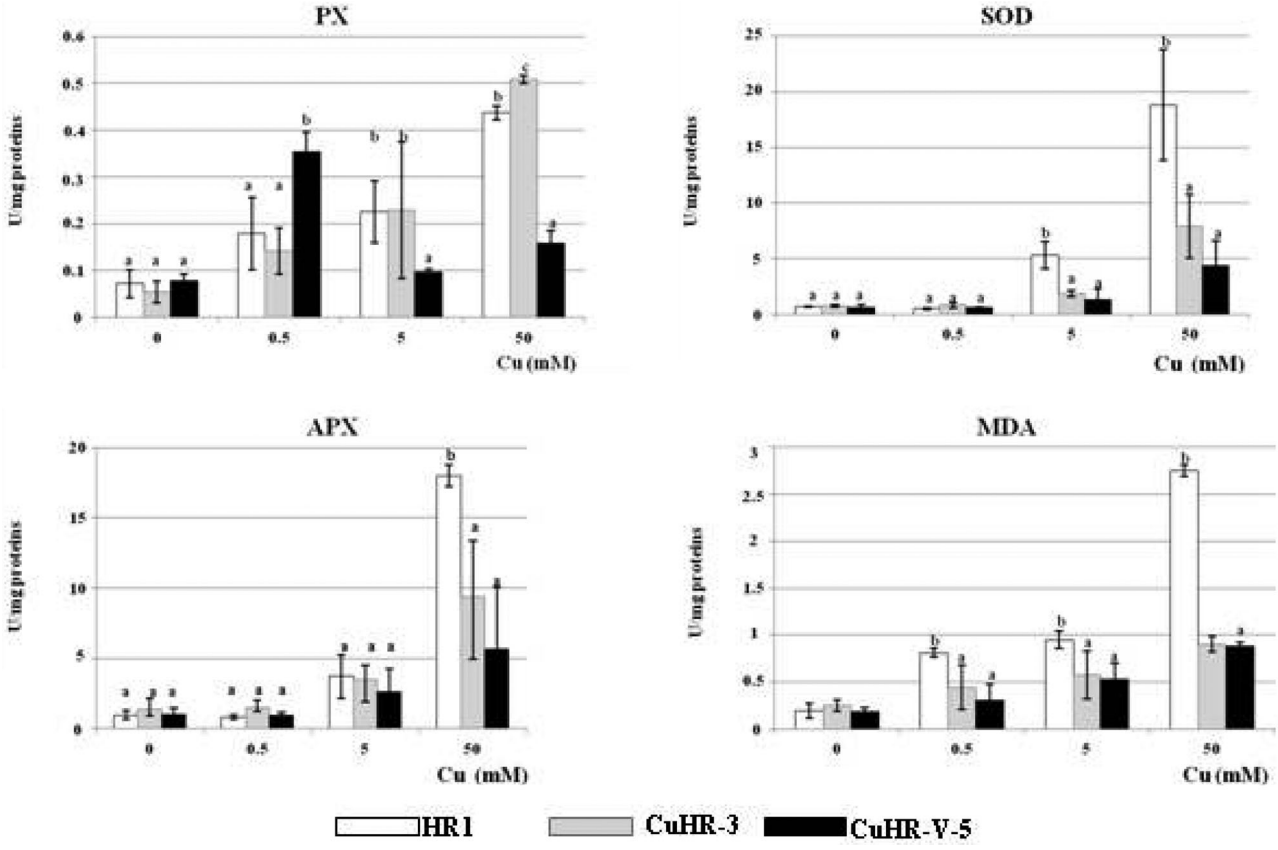


Figure 4. Determination of antioxidant enzymes and MDA in wild-type (HR1) and *copper-hairy roots*. Enzymatic activities of peroxidase (PX), SOD, APX, and levels of MDA were determined in HR1 and *copper-hairy roots* in the absence of Cu and in the presence of three concentrations of Cu (0.5, 5 and 50 mM). Data are means of three independent determinations and bars indicate standard deviations. Significant differences are indicated by different letters.

(50 mM) (increases of 7-, 10- and 15-fold, respectively). In CuHR-V-5, PX activity showed a maximum value at 0.5 mM Cu. Besides, SOD and APX activity showed only mild increases in the presence of pollutant, corresponding to 4- and 5-fold of increase at 50 mM Cu, respectively.

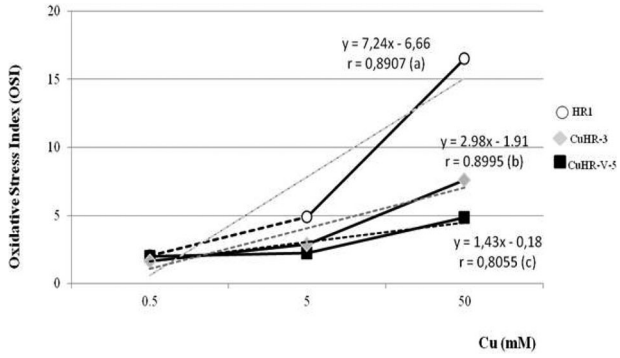


Figure 5. OSI of wild-type (HR1) and *copper-hairy roots* (CuHR-3 and CuHR-V-5). The OSI was defined as an average parameter of oxidative stress based on the levels of antioxidant enzymes and MDA content (see Experimental). This index was measured at three different Cu concentrations (0.5, 5 and 50 mM). The measures were adjusted to the best linear model, and the slopes were calculated. Significant differences are designated by different letters.

The damage produced by Cu to cellular membranes due to lipid peroxidation was estimated by determination of MDA (Figure 4(D)). MDA content was positively correlated with the Cu concentration assayed and reached the highest values at 50 mM Cu. In this sense, HR1 showed the highest increment (14-fold increase respect to untreated HR1) and *copper-hairy roots* showed significantly lesser concentrations of MDA than HR1. These lower levels of cellular damage may be mainly attributed to the beneficial contribution of the presence/expression of *copC* under stress conditions, particularly inside the vacuole.

To globally evaluate the oxidative stress, we propose the use of a stress parameter, the OSI, calculated as an average of different biochemical oxidative stress parameters. A value of 1 indicates a similar level of stress as compared to control hairy roots (non-exposed to Cu). Values above 1 indicate the extent of oxidative stress. The OSI of HR1 (Figure 5) quickly increased, reaching a maximum value of 16.5 at 50 mM Cu. When data were adjusted to a linear model, the slope was 7.24 ($r = 0.9437$), indicating a strong increase in oxidative stress with increasing Cu concentrations. By contrast, CuHR-3 showed lower values of OSI, with a maximum value of 7.6 at 50 mM Cu. The linear model gave a slope of 2.98 ($r = 0.9942$). CuHR-V-5 showed the lowest levels of OSI, with a maximum value of 4.8 with the higher assayed Cu concentration. The linear model showed a slope of 1.43 ($r = 0.8975$). Moreover, this could be related with the sequestration of Cu inside the vacuole, due to the presence of specific vacuole sorting determinants. Thus, Cu compartmentalization may have a beneficial effect that decrease toxicity symptoms and allow cells to continue with their normal metabolism, since vacuoles are compartments to store harmful compounds to allow the normal cell function.

4. Discussion

The ability to exploit the potential of plants for environmental remediation is frequently restricted by limited understanding of plant metabolic pathways, full range of enzymes involved and tolerance mechanisms. In this regard, hairy roots are frequently applied in phytoremediation research, since they allow examining the intrinsic metabolic capabilities of plant cells (uptake, transformation, conjugation and compartmentalization) and their capacities to tolerate, accumulate and/or remove environmental organic and inorganic pollutants [48]. This is in part due to several advantages of this plant model system and the fact that roots have evolved specific mechanisms to deal with pollutants, because they are the first organ having contact with them. Moreover, they were also used for evaluating the contribution of specific proteins through the obtainment of transgenic hairy roots [49,50].

As a result of their highly branched nature, hairy roots have large surface area in comparison with normal roots, and can also been used for rhizofiltration purposes for the remediation of HM such as Cu. One of the important plant strategies to detoxify HM within cells is to synthesize low molecular weight chelators to minimize the binding of metal ions to functionally important proteins and hence limit its toxicity [51]. Other plant strategies include the activity of different transporters, located in plasma membrane or in the tonoplast, which allows accumulating these pollutants in the apoplast or in the vacuoles respectively, reducing their toxic effects [52,53].

In this sense, several attempts were made to obtain hairy roots with high Cu accumulation from Cu indicator species, such as *Hyptis capitata, Polycarpaea longiflora and N. tabacum* [54]. These authors demonstrated that, in short-term experiments (9 h), Cu uptake in *H. capitata* hairy roots reached 4000–6000 μg g^{-1} at 1000 mg L^{-1} added Cu (BCF around 4–6). In long-term studies, at 20 mg L^{-1} Cu added, hairy root cultures accumulated up to 800 ± 73 μg g^{-1} (BCF of 40).

Recently, the generation of Cu hyperaccumulating transgenic *A. thaliana* plants by expressing the prokaryotic *copC* gene was described [36]. This gene codifies a periplasmic Cu-binding protein [55]. In the present work, we describe the generation of transgenic tobacco hairy roots, expressing *copC*, either in its wild-type form or targeted to the vacuole of root cells. Our

Table 2. Comparison of Cu accumulation ad BCFs of transgenic hairy roots with other Cu described (hyper)-accumulators.

Plant	Culture condictions/external Cu conc.	Cu accumulation (ppm)	BFC	Ref.
C. helmsii	Hydroponic (10 µM; 0.6 ppm)	9200	15,333	[11]
E. splendens	Field conditions (77 ppm Cu)	Shoots: 250	3.24	[12]
		Roots: 720–1260	9.3–16.4	
C. perennis	Soil (2161–5300 ppm)	Shoots: 80–1380	0.035	[13]
Crepidorhopalon tenuis Var. Niamumenda	Soil (382–18,144 ppm)	Shoots: 84–2524	0.19	[13]
H. katangense	Hydroponic (100 µM)	Shoots: 2302	354	[14]
		Roots: 7273	1100	
Transgenic *A. thaliana* expressing *copC*)	Soil (watered with 150 µM Cu)	Shoots: 400	42	[36]
		Roots: 2000	210	
H. capitata (hairy roots)	Hydroponic (20 ppm)	800	40	[53]
L. polytropa	Hydroponic (400 µM)	12,992	511	[58]
Transgenic tobacco hairy roots Cu-HR-3	Hydroponic (5 mM)	12,300	38	This work
Transgenic tobacco hairy roots Cu-HR-V-5	Hydroponic (50 mM)	45,000	14	This work

results demonstrated that maximum Cu accumulation was achieved under 50 mM Cu treatments (over 45,000 µg g^{-1}). However, the maximum BCF (35–40) was obtained at 5 mM of Cu treatment, indicating the most efficient Cu uptake under this condition, whereas the BCF of HR1 was 20. To our knowledge, the maximum value of Cu accumulation (4.5% of dry weight) is among the highest Cu concentrations reported in biological samples, even higher than that of several Cu-hyperaccumulator plants [12]. Lichens also showed one of the highest Cu accumulation capacities (revised in [56]), for instance *Acarospora rugulosa* (16% Cu on a dry mass basis) [57], *Lecidea lactea* (5%) [58] and *Lecanora polytropa* (1.3%) [59]. Table 2 compares the accumulation and BCFs of transgenic hairy roots expressing *copC* with those of other copper hyperaccumulators (lichens, plants, transgenic plants and hairy root cultures). For instance, cells of *Ralstonia picketii* were able to accumulate up to 38.19 mg copper per g dry weight of cells [60]. On its side, fungi have been also used for metal biosorption [61].

High Cu content was also found in HR1 when exposed to 50 mM Cu (up to 49,000 µg g^{-1}). However, it is important to notice two facts: (1) roots were not viable, and (2) in this case, half of the Cu was accumulated inside the root and half was adsorbed to the root surface. This could be attributed to the membrane damage produced by Cu exposure, as determined from the MDA levels. If membrane stability is compromised, both external and internal surfaces could contribute to Cu biosorption, thus reaching high Cu contents.

Previous studies in hairy roots have demonstrated that Cu uptake was biphasic, showing a rapid accumulation in the initial phases followed by a steady increase [27,54]. The first step of biosorption is passive and occurs by processes such as ion exchange, coordination, physical adsorption and microprecipitation. [62]. Groups such as carboxyl, phosphate, amine, hydroxyl and imidazol, are involved in metal binding. The second phase is active and depends on the specific metal uptake by hairy roots. While the sorption of metals was independent of plant species, being or not a hyperaccumulator, the total uptake in long-term experiments was higher in hairy roots from hyperaccumulators [54,63].

Owing to its redox properties, Cu causes oxidative stress mainly by catalyzing the formation of hydroxyl radicals through the Haber–Weiss reaction [64]. Since ROS accumulation induces oxidative damage of membrane lipids, nucleic acids and proteins, a tight control of the steady-state concentration of ROS seems to be necessary to avoid oxidative damage at subcellular levels [65]. Furthermore, the increase in the activities of antioxidant enzymes indicates that they play an important role to attenuate Cu stress [66,67]. In this sense, positive correlations between SOD and APX levels and Cu exposure were found in roots and shoots of *Brassica juncea* and *Trigonella foenum-graecum* [68,69]. Similarly, the results obtained in this work also showed a positive correlation between Cu concentration and antioxidant activity. Moreover, the values of PX, SOD and APX activities in HR1 seem to suggest that wild-type hairy roots are more affected by the presence of Cu, due to the higher increments in the antioxidant activities, especially at 50 mM of Cu. It must be also considered that measurements were done after long-term (7 days) exposure to Cu. It cannot be ruled out that transient increases in antioxidant enzymes could occur upon transferring the roots to Cu [70].

ROS over-production under Cu stress is also highly destructive to the cell membrane, leading to the oxidation of membrane lipids [71]. The results obtained with HR1 and *copper-hairy roots* indicate that the presence of Cu increases lipid peroxidation, in accordance with previous findings showing that MDA accumulated due to Cu treatment and that the cell membrane is the primary site affected by Cu toxicity [64,66,70]. Besides, it is important to point out, that *copper-hairy roots* have lesser values of MDA, which could be indicative of lesser membrane damage in the presence of the heavy metal.

The OSI was introduced as a new parameter to evaluate oxidative stress, being defined as an integrative parameter. The roots with the highest OSI were HR1, whereas *copper-hairy roots* had the lower values. Interestingly, CuHR-V-5 where the CopC protein was targeted to the vacuole of root cells had the lowest value of OSI. Our results could mean that the expression of *copC* targeted to vacuole could have beneficial effects and could alleviate the adverse effects of Cu on plant tissues.

5. Conclusions

Transgenic tobacco hairy roots expressing CopC in the cytoplasm and vacuole reached maximum efficiency for Cu accumulation at external 5 mM Cu (BCF = 35–40), and were able to accumulate up to 4.5% Cu in dry weight. In contrast to control hairy roots, for which half of Cu was accumulated inside the roots and the other half was adsorbed onto the root surface, up to 96% of Cu was accumulated inside the transgenic roots expressing CopC. Moreover, Cu compartmentalization inside the vacuole could avoid the toxic effects of copper, leading to less membrane damage. Oxidative stress of copper-hairy roots was 2–6 times lower than in control. In conclusion, tobacco hairy roots expressing *copC* are a suitable biotechnological tool to hyperaccumulate Cu from polluted solutions. Our findings also demonstrate that Cu compartmentalization/sequestration inside the vacuole could avoid the deleterious effects produced by ROS accumulation, allowing hairy roots to continue growing and accumulating Cu, being this a key fact in rhizofiltration.

Acknowledgements

Thanks are given to the Microanalysis Service (CITIUS, Univ. Sevilla).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

P.P.P. acknowledges grants from the University of Sevilla and Asociación Universitaria Iberoamericana de Postgrado [mobility grant]. E.A., M.A.T. and S.G.I. acknowledge Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) [mobility grant] for financial support; This project was supported by Ministerio de Economía y Competitividad and FEDER [project BIO-2009-7766].

ORCID

Ignacio D. Rodríguez-Llorente http://orcid.org/0000-0001-7621-8303
Miguel A. Caviedes http://orcid.org/0000-0001-8184-0455
Eloísa Pajuelo http://orcid.org/0000-0002-1084-5301

References

[1] Davis MA, Gilbert-Diamond D, Karagas MR, et al. A dietary-wide association study (DWAS) of environmental metal exposure in US children and adults. PLoS One. 2010;9:e104768.
[2] Luo Y, Sander S, Hunter KA. Removal of copper and copper-binding organic ligands during potable water treatment. Environ Technol. 2005;26(1):75–83.
[3] Cao X, Cao H, Sheng Y, et al. Mechanisms of Cu^{2+} migration, recovery and detoxification in Cu^{2+}-, SO^{2-}_4-containing wastewater treatment process with anaerobic granular sludge. Environ Technol. 2014;35(14):1956–1961.
[4] Fu F, Wang Q. Removal of heavy metal ions from wastewaters: a review. J Environ Manag. 2011;92(3):407–418.
[5] Vangronsveld J, Herzig R, Weyens N, et al. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ Sci Pollut Res Int. 2009;16(7):765–794.
[6] Lee M, Yang M. Rhizofiltration using sunflower (*Helianthus annuus* L.) and bean (*Phaseolus vulgaris* L. var. vulgaris) to remediate uranium contaminated groundwater. J Haz Mat. 2010;173:589–596.
[7] Mkandawire M, Dudel EG. Are *Lemna* spp. effective phytoremediation agents? Biorem. Biodiv. Bioav. 2007;1(1):56–71.
[8] Xia H, Ma X. Phytoremediation of ethion by water hyacinth (*Eichhornia crassipes*) from water, Biores. Technol. 2006;97(8):1050–1054.
[9] Jaffré T, Pillon Y, Thomine S, et al. The metal hyperaccumulators from New Caledonia can broaden our understanding of nickel accumulation in plants. Front Plant Sci. 2013;4:279.
[10] Krämer U. Metal hyperaccumulation in plants. Ann Rev Plant Biol. 2010;61:517–534.
[11] Küpper H, Gotz G, Mijovilovich A, et al. Complexation and toxicity of copper in higher plants. I. characterization of copper accumulation, speciation and toxicity in *Crassula helmsii* as a new copper accumulator. Plant Physiol. 2009;151(2):702–714.
[12] Jiang LY, Yang XE, He ZL. Growth response and phytoextraction of copper at different levels in soils by *Elsholtzia splendens*. Chemosphere. 2004;55:1179–1187.

[13] Faucon MP, Colinet G, Mahy G, et al. Soil influence on Cu and Co uptake and plant size in the cuprophytes *Crepidorhopalon perennis* and *C. tenuis* (*Scrophulariaceae*) in SC Africa. Plant Soil. 2009;317:201–212.
[14] Chipeng FK, Hermans C, Colinet G, et al. Copper tolerance in the cuprophyte *Haumaniastrum katangense* (S. Moore) PA Duvign & Plancke. Plant Soil. 2010;328:235–244.
[15] Festa RA, Thiele DJ. Copper: an essential metal in biology. Curr. Biol. 2011;21:R877–R883.
[16] Chen LM, Lin CC, Kao CH. Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H_2O_2 level, and cell wall peroxidase activity in roots. Bot Bull Acad Sin. 2000;41:99–103.
[17] Drążkiewicz M, Skórzyńska-Polit E, Krupa Z. Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. BioMetals. 2004;17:379–387.
[18] Sharma S, Dietz KJ. The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci. 2009;14(1):43–50.
[19] Demidchik V. Mechanisms of oxidative stress in plants: from classical chemistry to cell biology. Environ Exp Bot. 2015;109:212–228.
[20] Lukatkin A, Egorova I, Michailova I, et al. Effect of copper on pro-and antioxidative reactions in radish (*Raphanus sativus* L.) in vitro and in vivo. J Trace Elem Med Biol. 2014;28(1):80–86.
[21] Christey MC, Braun RH. Production of hairy root cultures and transgenic plants by *Agrobacterium Rhizogenes*-mediated transformation. Transgenic Plants: Methods and Protocols. Chapter 3, p. 47–60. Springer. 2004.
[22] Georgiev MI, Agostini E, Ludwig-Müller J, et al. Genetically transformed roots: from plant disease to biotechnological resource. Trends Biotechnol. 2012;30(10):528–537.
[23] Zhou ML, Tang YX, Wu YM. P lant hairy roots for remediation of aqueous pollutants. Plant Mol Biol Rep. 2013;31:1–8.
[24] Cambrollé J, Mateos-Naranjo E, Redondo-Gómez S, et al. Growth, reproductive and photosynthetic responses to copper in the yellow-horned poppy, *Glaucium Flavum*. Crantz Environ Exp Bot. 2011;71:57–64.
[25] Mateos-Naranjo E, Redondo-Gómez S, Cambrollé J, et al. Growth and photosynthetic responses to copper stress of an invasive cordgrass, *Spartina densiflora*. Marine Environ Res. 2008;66(4):459–465.
[26] Eapen S, Suseelan KN, Tivarekar S, et al. Potential for rhizofiltration of uranium using hairy root cultures of *Brassica juncea* and *Chenopodium amaranticolor*. Environ Res. 2003;91:127–133.
[27] Nedelkoska TV, Doran PM. Hyperaccumulation of cadmium by hairy roots of *Thlaspi caerulescens*. Biotechnol Bioeng. 2000;67:607–615.
[28] Nedelkoska TV, Doran PM. Hyperaccumulation of nickel by hairy roots of *Alyssum* species: comparison with whole regenerated plants. Biotechnol Prog. 2001;17:752–759.
[29] Assunção AGL, Schat H, Aarts MGM. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytol. 2003;159:351–360.
[30] Peer WA, Mamoudian M, Lahner B, et al. Identifying model metal hyperaccumulating plants: germplasm analysis of 20 *Brassicaceae* accessions from a wide geographical area. New Phytol. 2003;159:421–430.
[31] Campos VM, Merino I, Casado R, et al. Phytoremediation of organic pollutants. Spanish J Agric Res. 2008;6:38–47.
[32] Eapen S, Souza SFD. Prospects of genetic engineering of plants for phytoremediation of toxic metals. Biotechnol Adv. 2005;23:97–114.
[33] Pilon-Smits EA, Pilon M. Phytoremediation of metals using transgenic plants. Crit Rev Plant Sci. 2002; 21: 439–456.
[34] Bizily SP, Kim T, Kandasamy MK, et al. Subcellular targeting of methylmercury lyase enhances its specific activity for organic mercury detoxification in plants. Plant Physiol. 2003;131(2):463–471.
[35] Hussein H, Ruiz ON, Terry N, et al. Phytoremediation of mercury and organomercurials in chloroplast transgenic plants: enhanced root uptake, translocation to shoots and volatilization. Environ Sci Technol. 2007;41:8439–8446.
[36] Rodríguez-Llorente ID, Lafuente A, Doukkali B, et al. Engineering copper hyperaccumulation in plants by expressing a prokaryotic *copC* gene. Environ Sci Technol. 2012;46(21):12088–12097.
[37] Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiol. 1962;15:473–497.
[38] Flocco CG, Giulietti AM. *In vitro* hairy root cultures as a tool for phytoremediation research. In: Willey N, editor. Phytoremediation: methods and reviews. Totowa, NJ: Humana Press; 2006. p. 161–170.
[39] Bandyopadhyay M, Jha S, Tepfer D. Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *Withania somnifer*a. Plant Cell Rep. 2007;26:599–609.
[40] Medina-Bolivar F, Condori J, Rimando AM, et al. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochem. 2007;68:1992–2003.
[41] Schmidt GW, Delaney SK. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. Mol Gen Genom. 2010;283(3):233–241.
[42] Pfaffl MW. Relative quantification. In: Tevfik Dorak M, editor. *Real-time PCR*. New Castle-upon-Tyne (UK): New Castle University; 2006. p. 63–82.
[43] Margui E, Queralt I, Cravalho ML, et al. Comparison of EDXRF and ICP-OES after microwave digestion for elements determination in plant specimens from abandoned miming area. Anal Chim Acta. 2005;549:197–204.
[44] Hammaini A, González F, Ballester A, et al. Biosorption of heavy metals by activated sludge and their desorption characteristics. J Environ Manag. 2007;84(4):419–426.
[45] Sosa-Alderete LG, Agostini E, Medina MI. Antioxidant response of tobacco (*Nicotiana tabacum*) hairy roots after phenol treatment. Plant Physiology and Biochemistry. 2011;49:1020–1028.
[46] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72:248–254.
[47] Dary M, Chamber-Pérez MA, Palomares AJ, et al. "in situ" phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. J Haz Mat. 2010;177(1):323–330.
[48] Doran PM. Application of plant tissue cultures in phytoremediation research: incentives and limitations. Biotechnol Bioeng. 2009;103:60–76.

[49] Wevar-Oller AL, Agostini E, Talano MA, et al. Overexpression of a basic peroxidase in transgenic tomato (*Lycopersicon esculentum* Mill. cv. Pera) hairy roots increases phytoremediation of phenol. Plant Sci. 2005;169(6):1102–1111.
[50] Singh S, Sherkhane PD, Kale SP, et al. Expression of a human cytochrome P4502E1 in *Nicotiana tabacum* enhances tolerance and remediation of γ-hexachlorocyclohexane. New Biotechnol. 2011;28(4):423–429.
[51] Mejáre M, Bülow L. Metal-binding proteins and peptides in bioremediation and phytoremediation of heavy metals. Trends Biotechnol. 2001;19(2):67–73.
[52] Colangelo EP, Guerinot ML. Put the metal to the petal: metal uptake and transport throughout plants. Curr Opin Plant Biol. 2006;9:322–330.
[53] Hall JL, Williams LE. Transition metal transporters in plants. J Exp Bot. 2003;54(393):2601–2613.
[54] Nedelkoska TV, Doran PM. Characteristics of heavy metal uptake by plant species with potential for phytoremediation and phytomining. Minerals Eng. 2000b;13(5):549–561.
[55] Arnesano F, Banci L, Bertini I, et al. A redox switch in CopC: An intriguing copper trafficking protein that binds copper (I) and copper (II) at different sites. Proc Natl Acad Sci. 2003;100(7):3814–3819.
[56] Bačkor M, Loppi S. Interactions of lichens with heavy metals. Biol Plantarum. 2009;53(2):214–222.
[57] Chisholm JE, Jones GC, Purvis OW. Hydrated copper oxalate, moolooite, in lichens. Mineral Mag. 1987;51: 715–718.
[58] Purvis OW. The occurrence of copper oxalate in lichens growing on copper sulphide – bearing rocks in Scandinavia. The Lichenologist. 1984;16:197–204.
[59] Pawlik-Slowronuska B, Purvis OW, Pirszel J, et al. Cellular mechanisms of Cu-tolerance in the epilithic lichen *Lecanora polytropa* growing at a copper mine. The Lichenologist. 2006;38(3):267–275.
[60] Yang F, Pecina DA, Kelly SD, et al. Biosequestration via cooperative binding of copper by Ralstonia pickettii. Environ Technol. 2010;31:1045–1060.
[61] Dhankhar R, Hooda A. Fungal biosorption – an alternative to meet the challenges of heavy metal pollution in aqueous solutions. EnvironTechnol. 2011;32:467–491.
[62] Das N. Recovery of precious metals through biosorption – a review. Hydrometallurgy. 2010;103(1):180–189.
[63] Devani MA, Munshi B, Oubagaranadin JUK, et al. Remediation of Hg(II) from solutions using *Cajanus cajan* husk as a new sorbent 2016. Environ Technol. DOI:10.1080/21622515.2016.1240240
[64] Elleuch A, Chaâbene Z, Grubb DC, et al. Morphological and biochemical behavior of fenugreek (*Trigonella foenum-graecum*) under copper stress. Ecotoxicol Environ Saf. 2013;98:46–53.
[65] Karuppanapandian T, Moon JC, Kim C, et al. Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. Austr J Crop Sci. 2011;5(6):709–725.
[66] Hossain MA, Piyatida P, Teixeira da Silva J, Fujita M. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. J Bot. 2012; DOI:10.1155/2012/872875
[67] Ravet K, Pilon M. Copper and iron homeostasis in plants: the challenges of oxidative stress. Antioxid Redox Signal. 2013;19(9):919–932.
[68] Singh M, Singh L, Srivastava SBL. Combining ability analysis in Indian mustard (*Brassica juncea* L. Czern & Coss). J. Oilseed Brassica. 2010;1(1):23–27.
[69] Mediouni C, Ben Ammar W, Houlné G, et al. Cadmium and copper induction of oxidative stress and antioxidative response in tomato (*Solanum lycopersicon*) leaves. Plant Growth Regul. 2009;57:89–99.
[70] Feigl G, Kumar D, Lehotai N, et al. Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress. Ecotoxicol Environ Saf. 2013;94:179–189.
[71] Panda SK. Impact of copper on reactive oxygen species, lipid peroxidation and antioxidants in *Lemna minor*. Biol Plant. 2008;52:561–564.