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RESEARCH ARTICLE

Evaluating the potential use of Cu-contaminated soils for giant reed (*Arundo donax*, L.) cultivation as a biomass crop

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Abstract

Over the past decades, the important topic of environmental sustainability, impact, and security of the fossil fuel supply has stimulated interest in using lignocellulosic feedstocks as biofuel to partially cover energy demands. Among energy no-food crops, giant reed (*Arundo donax*, L.), a perennial rhizomatous grass has been identified as a leading candidate crop for lignocellulosic feedstock, due to its positive energy balance, and low ecological/agro-management demands. The aim of the present study was to characterize the physiological response of *Arundo donax* (L.) to artificial soil contamination with three different Cu levels (200, 400, and 800 ppm), and to assess the relationship between plant Cu tolerance and S assimilation rate. The present study not only confirms the ability of *Arundo donax* L. to cope with Cu stress and therefore to grow in marginal, degraded lands abandoned by mainstream agricultural, but also shows that plant performance might be likely ascribed to a modulation of sulfate metabolism resulting in increased thiols content.

Keywords *Arundo donax* L. · Bioenergy · Copper · Energy crops · Sulfur

Introduction

Well-established and widespread agricultural practices, such as the excessive use of fertilizers, the agronomic use of farm effluents, urban sewage sludge, and the uncontrolled distribution of pesticides have worsened the quality of soils. The pollution caused by the presence of heavy metals in soil poses a threat to agricultural productivity, but also to food security and human health (Toth et al. 2016a). The presence of contaminated areas embraces most European regions, differently affected by the specific contaminant and the level of contamination (Lado et al. 2008; Toth et al. 2016a, b).

Inorganic copper (Cu) is used as a broad-spectrum fungicide and bactericide as it combines efficiency and low cost. The widespread use of copper-based plant protection products in vineyards, orchards, and also organic farming has led to a progressive and worrisome accumulation of this metal in the upper layer (0–20 cm) of the soils of the Mediterranean countries (France, Italy, Portugal and Spain). Copper has a low mobility in soil and relatively small variations in the total content of the soil profiles. In Italy, the total Cu concentration of the soil was reported between 2 and 375 mg kg^{-1} (Toselli et al. 2009; La Torre et al. 2018), but Cicchella et al. (2015) observed values above 800 mg kg^{-1} in the Vesuvian area.

Currently, EC legislation 473/2002 limits the annual dose of Cu applied to 6 kg Cu ha^{-1}, which corresponds to an annual accumulation of about 5 mg of Cu per kilogram of soil in the first 10 cm (Ruyters et al. 2013). For copper, a regulatory review process is underway as the substance has been included among the candidates for substitution due to the long persistence in the soil and toxicity on aquatic and telluric organisms (Armentano 2017).

Cu is an essential micronutrient for plants, playing a very important role in their growth and development (Brunetto

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et al. 2016). It acts as an acceptor of electrons in numerous enzymatic systems, some of which are involved in antioxidative processes. Cu, as a component of plastocyanin, involved in electron transport, both at mitochondrial level and in chloroplasts (Wintz and Vulpe 2002). However, at high concentrations, Cu negatively influences plant growth and development (La Torre et al. 2018). Its toxic effects affect different biochemical and physiological processes producing oxidative stress, leading to profound alterations in plant metabolism associated with growth reductions, nutrient uptake, chlorophyll content, and leaf expansion (Miotto et al. 2014; Shiyab 2018; Marzilli et al. 2018). However, the copper concentration causing a damage to plants cannot be easily established because it depends on the bioavailability of the element (Ruyters et al. 2013; Brunetto et al. 2016; La Torre et al. 2018).

The use of energy crops for the removal of heavy metals in contaminated sites (phytoremediation) has been proposed as a sustainable technology able to couple the environmental concerns with the sustain of income farmer (Marchiol and Fellet 2011; Nsanganwimana et al. 2014; Fernando et al. 2016). To be suitable for phytoremediation, beside the ability to detoxify the pollutant the plant species must possess some other characteristics associated to biomass production (high amount in short time), its handling (adoption of known farming practices, ease of biomass collection, and clear destination), and the possibility to produce a feedstock for no-food uses (Marchiol and Fellet 2011; Fernando et al. 2016; Tripathi et al. 2016). Among the perennial crops, the giant reed (*Arundo donax*, L.) is deemed to match the ideotype requested to assure a cost-effective removal of heavy metals in soil (Nsanganwimana et al. 2014; Fernando et al. 2016). In the last 10 years, the studies appeared about its ability in phytoremediation have concerns the growth responses, the accumulation capacity, and the uptake of nutrients when grown in the presence of different elements such as As, Cd, Cr, Cu, Ni, Pb, Se, and Zn (Papazoglou et al. 2007; Mirza et al. 2011; Kausar et al. 2012; Barbosa et al. 2015; Elhawat et al. 2015; Domokos-Szabolcsy et al. 2018; Shaheen et al. 2018). A study on *Arundo* tolerance to copper has been carried out by Elhawat et al. (2014, 2015) using plantlets regenerated by somatic-embryos of two genotypes grown on increasing amount of Cu (0–26 mg L^{-1}) in the medium. The plantlets showed an appreciable level of tolerance growing on Cu-contaminated soil up to 10 mg L^{-1} without significant foliar symptoms. Moreover, high levels of bioaccumulation and translocation factor revealed the great potential of the species for Cu removal.

The aim of the present work was to evaluate the adaptive capacity of *Arundo* in soils artificially contaminated with different concentrations of Cu (200, 400, and 800 ppm), higher than the thresholds set in the Italian legislation for residential (120 mg kg^{-1}) and industrial (600 mg kg^{-1}) areas. The work linked the effect on plant growth with specific physiological response, associated with the enzymatic machinery activated during the stress.

Materials and methods

Plant growth

The *Arundo* cuttings were prepared from a 6-year-old plantation grown in the experimental farm of CREA-IT in Monterotondo (42° 03′ N 12° 37′ E) accordingly to Castelli and Ceotto (2016) with minor modifications. Briefly, the auxiliary stems growing on culms of 2 years were immerged in a plastic tank of 0.46 m^3 (0.80 × 0.50 × 1.16 m) filled up with two-thirds of water (about 300 L) and containing a structure of 120 plastic tubes (80 cm high). One cutting per hole was inserted and left immersed up to 20–25 cm. The tank has been placed inside a not heated protected structure. After 3 months, the cuttings had abundantly rooted.

In May, 28 rooted plants were transplanted in plastic wells of 0.125 m^3 (0.5 × 0.5 × 0.5 m) containing 40 kg of soil. The soil was a clay-loam soil (USDA classification), with sub-alkaline pH and low organic matter content (Table 1). The substrate had a medium-high content of nutrients and a good CeC. From July to October, the plants were watered two times a week with an amount required to fill soil up to 15% of field capacity in the 0–40-cm soil depth, where most of the roots are expected to grow in *Arundo*. During the winter, the irrigation was suspended, relying on the natural precipitations.

Treatment with Cu

In mid-July, the soil was contaminated with copper. The experimental design was a completely randomized design. Treatments included four concentrations of Cu (0, 200, 400, and 800 ppm of Cu ion) with 6 replicates (single plant) for

Table 1 Chemical and physical characterization of the soil used as substrate for the growth of the plants

Parameter	U.M	Valore
Sand	%	24.4
Silt	%	47.6
Clay	%	28.0
pH		7.6
Organic matter	%	1.79
Nitrogen	%	0.12
P Olsen	mg kg^{-1}	25.20
P2O5	mg kg^{-1}	58.40
K2O	mg kg^{-1}	598.14
Cation-exchange capacity (CEC)	meq 100 g^{-1}	29.51
Field capacity	wt%	
Ca	meq 100 g^{-1}	24.33
Mg	meq 100 g^{-1}	3.21
K	meq 100 g^{-1}	1.27
Na	meq 100 g^{-1}	0.70

each treatment. The remaining four plastic wells were left without plants but filled with soil and treated with the scheduled doses of copper.

The hardening of rooted cuttings lasted for 1 month. Then, they were contaminated using a commercial product (Manica S.p.a.) containing 99% of $CuSO_4$ $5H_2O$. The doses of the product were calculated considering the concentration of copper ion presents into the salt, dissolved in water, and applied to the plants.

On each plant, the following traits were measured at 30 (August), 90 (October), and 120 (January) days after the contamination (DAC): height of the main stem, number of stems, chlorophyll content. The latter was measured using a SPAD-502 Plus (Konica Minolta, Japan) on the penultimate leaf of each plant. On each leaf were taken three readings.

Leaf samples collected after 6 months from contamination (January) at the end of growth period were used for ICP-MS analysis, and biochemical and enzymatic assays.

ICP-MS analysis

The Cu content of macro and micro element was analyzed at the laboratory LAS-ER-B of CREA-IT (Monterotondo). Leaf samples were dried at 105 °C for 24 h and homogenized to have a uniform distribution of the elements. At the end, about 0.5 g of each sample was weighed and placed into a microwave Milestone START D with the addition of 6 ± 0.1 ml of HNO_3 65% and 3 ± 0.1 ml of H_2O_2 30%. The digestion was accomplished at a 180 °C, 650 W for 42 min. At the end, the samples were filtered and diluted with deionized water. Every sample was analyzed in triplicate and with a blank. The content of microelements was measured with ICP-MS (Agilent 7700).

Determination of malondialdehyde content

The level of lipid peroxidation was expressed as determination of malondialdehyde (MDA) content and was determined as TBA reactive metabolites according to (Astolfi et al. 2005). Briefly, fresh apical leaf tissues (0.2 g) were homogenized in 10 ml of 0.25% TBA made in 10% TCA. Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10,000×*g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as μmol g^{-1} fresh weight by using an extinction coefficient of 155 mM cm^{-1}.

Enzyme extraction and assay

Apical leaves (ca. 1 g FW) of plants grown in control condition or treated with four concentrations of Cu (0, 200, 400, and 800 ppm of Cu ion) were powdered in a pre-chilled mortar under liquid nitrogen and then homogenized in 3 mL of a cold extraction buffer (pH 7.4), prepared with 50 mM HEPES-KOH, 5 mM $MgCl_2$, 1 mM EDTA, 10% (*v*/*v*) glycerol, 0.1% (*v*/*v*) Triton X-100, 5 mM DTT, 1 mM PMSF, and 1% (*w*/*v*) PVPP. The homogenate was filtered and then centrifuged at 4 °C for 5 min at 1000*g*. The supernatant was desalted at 4 °C on a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) pre-equilibrated with the extraction buffer without Triton X-100. The desalted extract was centrifuged at 4 °C for 5 min at 30,000*g*, and the resulting supernatant was frozen in liquid nitrogen and stored by freezing (− 80 °C) until use for in vitro enzyme assays.

The extractable ATPS (EC 2.7.7.4) activity was determined by the bioluminescence technique, as described in Celletti et al. (2016). Briefly, the ATP production during the enzyme reaction is coupled to the light producing reaction catalyzed by firefly luciferase (E.C. 1.13.12.7). The reaction mixture contained in a total volume of 0.25 ml: 16 mM Tris-Acetate buffer pH 7.75, 8 mM APS, 68 mM $Na_4P_2O_7$, 40 ml of firefly luciferase (ATP Monitoring Reagent, ThermoLabSystems), and 5 ml of sample. Light emission was measured with LKB 1250 luminometer.

OASTL (EC 4.2.99.8) activity was assayed by detecting cysteine production (Celletti et al. 2016). Reaction medium (pH 7.5) contained 50 mM Tris-HCl, 1 mM DTT, 10 mM *O*-acetyl-Ser, and 2.5 mM Na_2S.

Quantitative protein determination of the root extracts was estimated by the protein-binding Coomassie brilliant blue G-250 dye method, using bovine serum albumin (BSA) as standard (Bradford 1976).

Statistics

For the morphological traits, the differences among treatments were analyzed by one-way ANOVA of the PAST software version 2.15 (https://folk.uio.no/ohammer/past/). The analyses were carried out after checking the normality. Different means were separated by Tuckey's HSD test.

For biochemical measurements, each reported value represents the mean ± SD of measurements carried out in triplicate and obtained from four independent experiments. All the data were statistically analyzed using ANOVA with the GraphPad InStat Program (version 3.06). Significant differences were established by posthoc comparisons (Tukey test) at $P < 0.01$. The Past 3.25 statistical software package was used for principal component analysis (PCA).

Results

Plant growth as a function of Cu application

Increasing the Cu concentration in soil from 200 to 800 ppm did not significantly affect the plant growth, in terms of both height of the main stem and number of shoots during the first 6 months (Table 2). A significant reduction in chlorophyll content was observed just 30 days after the treatment with the highest Cu concentration (Table 2). In particular, plants grown with 800 ppm Cu showed 29% less chlorophyll level than the control (Table 2). However, in the following months, the leaves of the plants grown on 800 ppm recovered gradually and the chlorophyll content increased from 71 to 92% of the control (Table 2).

Cu, Fe, and Zn concentration as a function of Cu application

The increase in Cu concentration supplied to the soil was followed by a significant increase in Cu accumulation in *Arundo* leaves, which can be described by the following linear function (Fig. 1):

$$y = 0.1458x + 1.4463 \left(R^2 = 0.9833\right).$$

In particular, the leaves of plants treated with 200, 400, and 800 ppm contained 5-fold, 10-fold, and 22-fold higher Cu, respectively, than the control (Figs. 1 and 2).

In the present study, we found that exposure of *Arundo* plants to increasing concentrations of Cu did not significantly affect Zn content in leaves, but the content of Fe in the same plants was enhanced when challenged with both 400 and 800 ppm (twofold and 30% higher than the control, respectively (Fig. 2). On the other hand, the lowest Cu concentration treatment level (200 ppm) did not cause a significant change in leaf Fe content (Fig. 2).

Table 2 Main morphological traits of the plants after 30, 90, and 180 days after the treatment. When present, different letters represent significant differences between the treatments ($P < 0.01$) within each column

Treatment (ppm)	Days after contamination (n)		
	30	90	180
Height of the main stem (cm)			
Control	73.3	101.7	93.0
200	76.5	102.5	97.2
400	76.3	105.7	98.5
800	76.0	111.2	105.3
Number of stems (*n*)			
Control	9.7	9.7	9.2
200	8.7	9.3	8.8
400	8.2	8.2	7.5
800	7.8	8.5	7.5
Chlorophyll content (SPAD units)[1]			
Control	39.7 a	32.4	33.4
200	40.4 a	31.4	27.1
400	38.2 a	31.7	30.0
800	28.4 b	30.0	31.0

[1] Measured at 30, 90, and 150 days from contamination

Lipid peroxidation as a function of Cu application

Plants suffering from Cu toxicity often exhibit symptoms associated with oxidative stress and membrane peroxidation as evidenced by enhanced accumulation of malondialdehyde (MDA), a highly toxic by-product generated by lipid peroxidation and commonly used as biomarkers for the assessment of membrane damage (Pätsikkä et al. 2002; Miotto et al. 2014; Adrees et al. 2015).

In the present study, leaf MDA content was not significantly affected by the treatment with the lowest Cu concentration (200 ppm). By contrast, MDA levels in leaves of plants exposed to the higher Cu concentration showed a significant increase relative to control leaves (28 and 51%, respectively) (Fig. 3).

Thiol pools as a function of Cu application

Changes in thiols accumulation in plant tissues can be explained by their role in the protection against oxidative damage (Foyer and Noctor 2011; Brunetto et al. 2016).

In plants that had been exposed to 200 ppm Cu, a 32% depletion in leaf thiols concentration was observed (Fig. 4). On the other hand, when grown with 400 and 800 ppm Cu, plants exhibited a 46% and 54% increase in thiols accumulation, respectively, relative to the control (Fig. 4).

Protein concentration as a function of Cu application

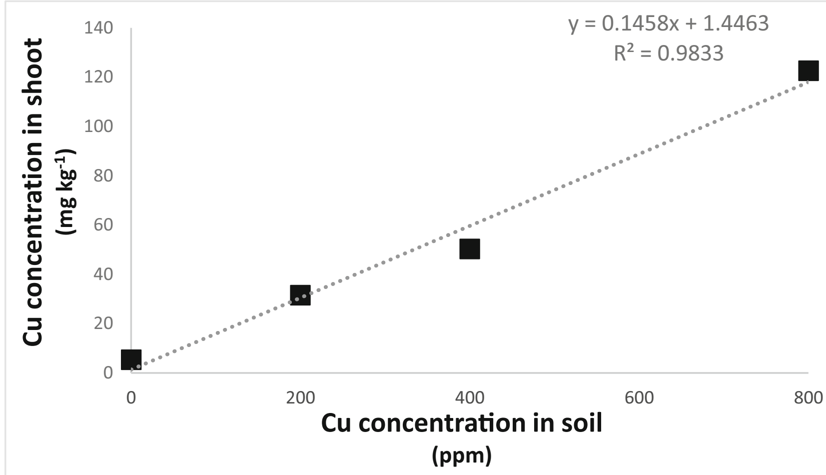
In leaves of exposed plants, the concentration of protein was slightly higher than that of control plants, with increases ranging from 7 to 10% (Fig. 5).

Sulfur assimilation rate as a function of Cu application

Changes in thiols accumulation in response to Cu exposure would be expected to modulate sulfate assimilation through the adjustment of the activity of enzymes involved in the S metabolism. Therefore, changes of ATPS and OASTL activity, the first and the last enzyme of the S assimilatory pathway, respectively, were evaluated in leaves of *Arundo* plants in response to increasing levels of Cu application (Figs. 6 and 7).

Results showed that Cu application stimulated the foliar ATPS activity and the metal effects on ATPS activity were greater when plants were exposed to the lowest and the highest Cu concentration (+ 163 and + 170% with respect to the control) (Fig. 6). Application of 400 ppm Cu also induced ATPS activity, but to a lesser extent (+ 65% with respect to the control) (Fig. 6).

Fig. 1 Copper concentration measured in *Arundo* shoots according to the concentration of Cu in the external medium. Shoot content of Cu can be modeled by the function: [Cu] in shoots (mg kg^{-1} DW) = 0.1458 [ppm Cu] in soil + 1.4463 (R^2 = 0.9833)



On the other hand, OASTL activity in leaf of treated plants was similar to that of control plants when Cu was added at 200 and 400 ppm, whereas the application of the highest concentration (800 ppm) resulted in a significant increase of the enzyme activity (+ 46% with respect to the control) (Fig. 7).

Principal component analysis

Following detailed point-by-point comparison of the different treatments, a more detailed statistical comparison of the combined data sets (herein, micronutrients, MDA levels, thiol compounds, and enzyme activities) was performed to verify which variables contributed to the separation of treatment. The principal component analysis (PCA) was used to achieve this goal, and all treatments were clearly separated (Fig. 7).

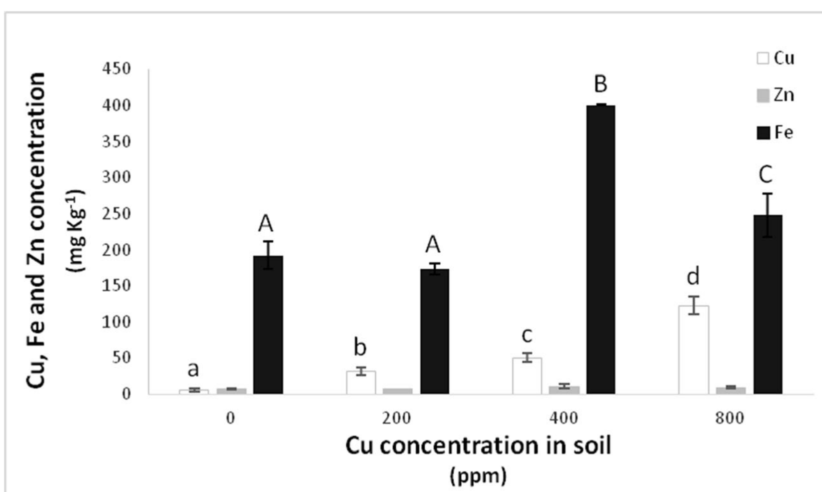
The PCA carried out on the dataset previously described, extracted three components having an eigenvalue higher than 1 (4.09, 2.04, and 1.05, respectively) and describing 79.74% of the total variance. The first two components used to build both the scatter and loading plots (Fig. 8a–c respectively) accounted for 68.11% of the total variance.

The first principal component (PC1) explained 45.45% of the variation separating the treatments on the basis of the Cu treatment level (Fig. 8a). The respective PCA loading displays that the main variables driving this separation are Cu, MDA level, thiols content, and OASTL activity (Fig. 8b).

The second principal component (PC2) explained 22.66% of the total variation, with a high positive loading for ATPS activity and a negative loading for Fe (Fig. 8c). Along PC1, treatments are separated based on Cu treatment level concentration, with the highest treatments, such as 800 and 400 ppm, clustering on the upper and lower right side of the plot, respectively, and the lowest treatments, such as 0 (control) and 200 ppm, clustering in the upper and lower left quadrant, respectively (Fig. 8a).

The PC2 differentiated treatments with high and low Fe content and ATPS activity (Fig. 8a). Treatments showing higher ATPS activity and lower Fe content (200 and 800 ppm) clustered in the upper part of the PCA (left and right

Fig. 2 Cu, Fe, and Zn concentration in leaves of *Arundo* plants grown being exposed to different Cu levels (from 0 to 800 ppm). Data are means ± SD of three independent replications run in triplicate. The statistical significance was tested by means of ANOVA with Tukey post-test. Different letters indicate statistically different values ($P < 0.01$)



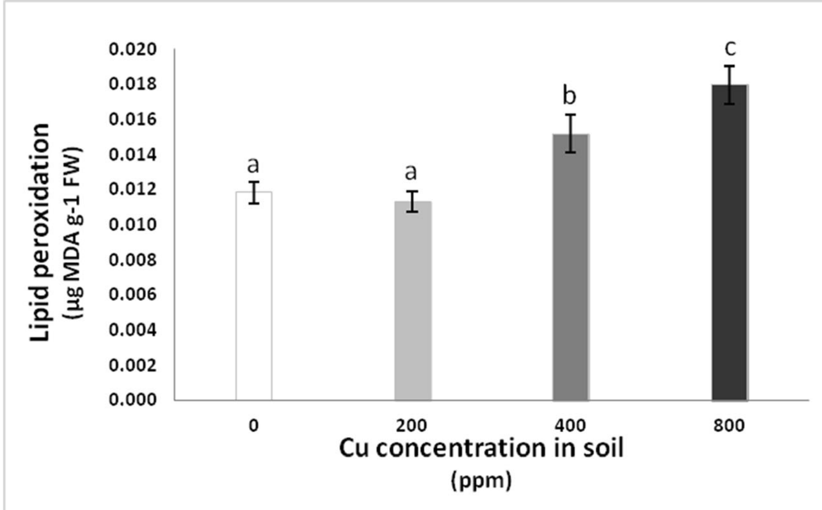


Fig. 3 MDA levels in shoots of *Arundo donax* plants. Treatments and statistics as in Fig. 2

quadrant, respectively), while treatments showing lower ATPS activity and higher Fe content (control and 400 ppm), clustered in the lower part (left and right quadrant, respectively) (Fig. 8a).

Discussion

Arundo donax (L.) possesses some key attributes required to energy crops (Ceotto and Di Candilo 2010). It has a perennial habit, a fast growth, a high biomass production, a low susceptibility to pests and diseases, and it is adaptable to a wide range of ecological conditions that range from the most fertile soils to the saline up to the marginal soils. Such characteristics make *Arundo* a valid candidate for phytoremediation systems.

This study describes a short-term Cu treatment to investigate the phytoextraction potential of *Arundo*. Plants were exposed to Cu concentrations from 200 to 800 ppm. Such values comprised and overcome the limits set by the Italian law (DL 152/06) for Cu contamination for both residential (120 mg kg^{-1}) and industrial (600 mg kg^{-1}) soils. Furthermore, the Cu treatments included the wide range of concentrations (from 200 to above 800 mg kg^{-1}) observed in the Italian soils with particular reference to vineyard or volcanic soils (Deluisa et al. 2007; Ruyters et al. 2013; Cicchella et al. 2015; La Torre et al. 2018).

Plants exposed to heavy metal stress often exhibit symptoms associated with growth reduction of both root and shoot (Wang and Zhou 2005). In the present study, we found that exposure of *Arundo* plants to Cu concentrations from 200 to 800 ppm did not limit stem development (Tab. 2). Our finding is consistent with that reported in the literature. For instance, Papazoglou et al. (2007) demonstrated that in *Arundo*, the height of the plants and the average number of branches were not significantly affected by high dosages of cadmium and nickel.

This observation can be explained by the fact that different factors can affect the degree of Cu toxicity, including plant species, metal concentration, exposure time, and soil properties

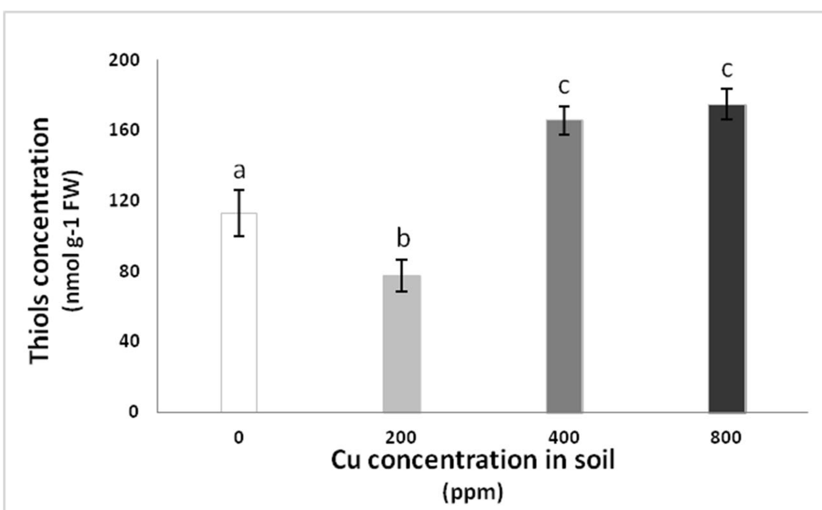


Fig. 4 Thiol concentration in shoots of *Arundo donax* plants. Treatments and statistics as in Fig. 2

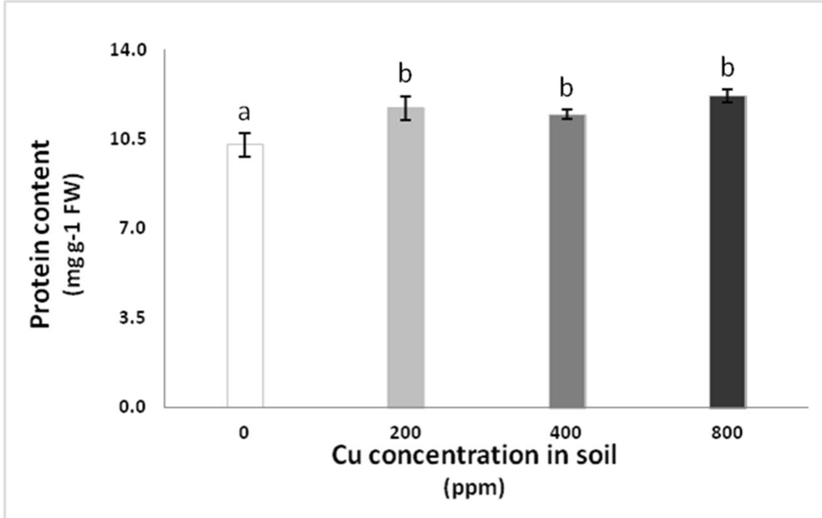


Fig. 5 Protein content in shoots of *Arundo donax* plants. Treatments and statistics as in Fig. 2

(Yruela 2009). A reduced toxicity may result from the reduction of Cu uptake, its partitioning in less-sensitive tissues, systems of chelation or binding to cell walls (Adrees et al. 2015), and the activation of an efficient antioxidant machinery.

Chlorophyll concentration rapidly dropped to 71% of control during the first month when Cu was applied at highest concentration (800 ppm) but increased again to control values in the following 2 months as indicated by chlorophyll measurements reported in Table 2. Previous studies have shown that young grapevine plantlets exposed to Cu concentration ranging from 25 to 165 mg kg^{-1} showed a decrease of leaf chlorophyll *a* and *b* by 32 and 44%, respectively, between the lowest and the highest Cu dose (Ambrosini et al. 2018). However, the study lasted 53 days, and no indications are available about a recovery of chlorophyll content. In our study, the dramatic recovery of chlorophyll concentration of *Arundo* indicates a high capability to tolerate high Cu levels. Furthermore, analysis of protein data evidenced that the protein content in leaves was increased by increasing Cu concentration in soil (Fig. 5). This unexpected result might be explained considering that the oxidative stress imposed to the plants upon copper exposure requires the stimulation of the synthesis of proteins having a protective role (antioxidant enzymes).

Leaf Cu accumulation by *Arundo* plants appeared to be dose dependent, with a positive relationship clearly existing between the Cu concentrations in the soil solution and in plant tissues (Fig. 1).

On the basis of metal uptake rate in relation to the substrate concentrations, higher plants are classified into three basic groups: excluder, indicator, and accumulator (Baker and Walker 1990). Results of the present study showing that Cu concentration in the leaves were enhanced by increasing Cu application to soil, suggest that *Arundo* performance is more consistent with the indicator group (Baker and Walker 1990).

It is well known that the first negative effect caused by presence of heavy metals, such as Cu, is related to nutrient

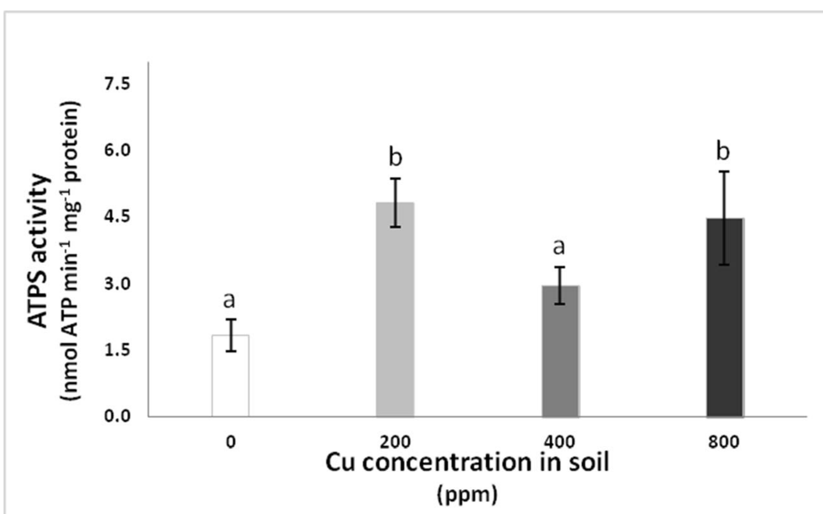
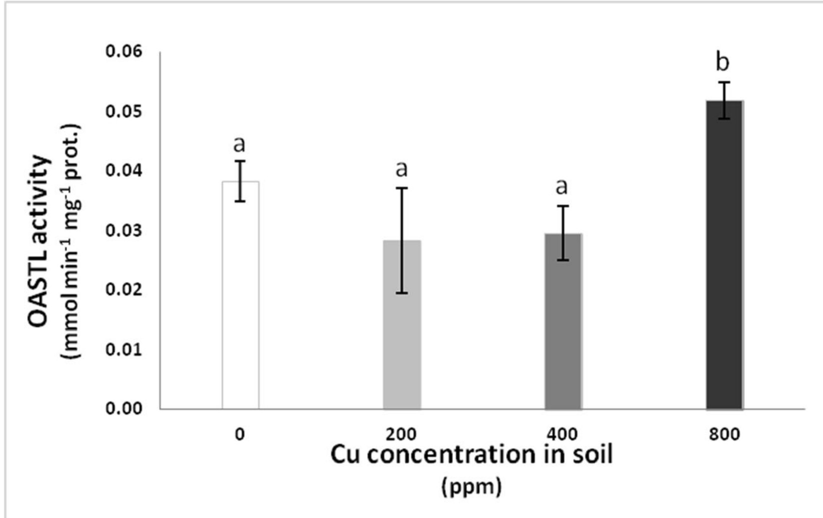


Fig. 6 ATPS activity in shoots of *Arundo donax* plants. Treatments and statistics as in Fig. 2

Fig. 7 OASTL activity in shoots of *Arundo donax* plants. Treatments and statistics as in Fig. 2



uptake, since these metals compete for the same nutrient uptake transporters as Fe, Mn, and Zn (Clemens 2006). The alteration of the ion balance generally leads to the so-called "inducible deficiency" (Astolfi et al. 2005; Astolfi et al. 2014) which, together with the metal toxicity effects, can seriously inhibit the development of plants and then compromise the phytoextraction of metals.

The absorption of Cu by the roots is essentially mediated by the COPT1 transporter (Sancenón et al. 2003), even if transporters belonging to the ZIP family, such as the iron transporter (IRT1) (Korshunova et al. 1999), also play an important role in Cu homeostasis in plants (Wintz et al. 2003). Accordingly, it cannot be ruled out a direct competition of Cu with other essential elements, such as Fe and Zn during uptake (Monni et al. 2000; Astolfi et al. 2014).

However, in *Arundo* plants, high external concentrations of Cu did not cause a decrease in the tissue concentrations of both Zn and Fe (Fig. 2). Rather, it might be proposed that the better nutritional status of Cu-treated plants can alleviate the effects of Cu toxicity thus contributing to the plant tolerance to the metal (Yang et al. 2002). This finding is consistent with that reported in the literature. For instance, Marzilli et al. (2018) demonstrated that tissue concentration of Fe was increased by two- and fourfold after exposure of micropropagated poplar plantlets to 250 and 500 mM Cu, respectively.

An increased production and accumulation of reactive oxygen species (ROS), such as superoxide radical (O_2–) and hydrogen peroxide (H_2O_2), is commonly observed on exposure of plants to Cu (Noriega et al. 2012). Cells generate ROS as part of their metabolism, but the increase in ROS levels in response to various external stress factors can potentially result toxic by causing significant damage to biomolecules and cell structures. In particular, excessive ROS production can lead to lipid peroxidation evidenced by enhanced accumulation of malondialdehyde (MDA), used as biomarkers for the assessment of membrane damage.

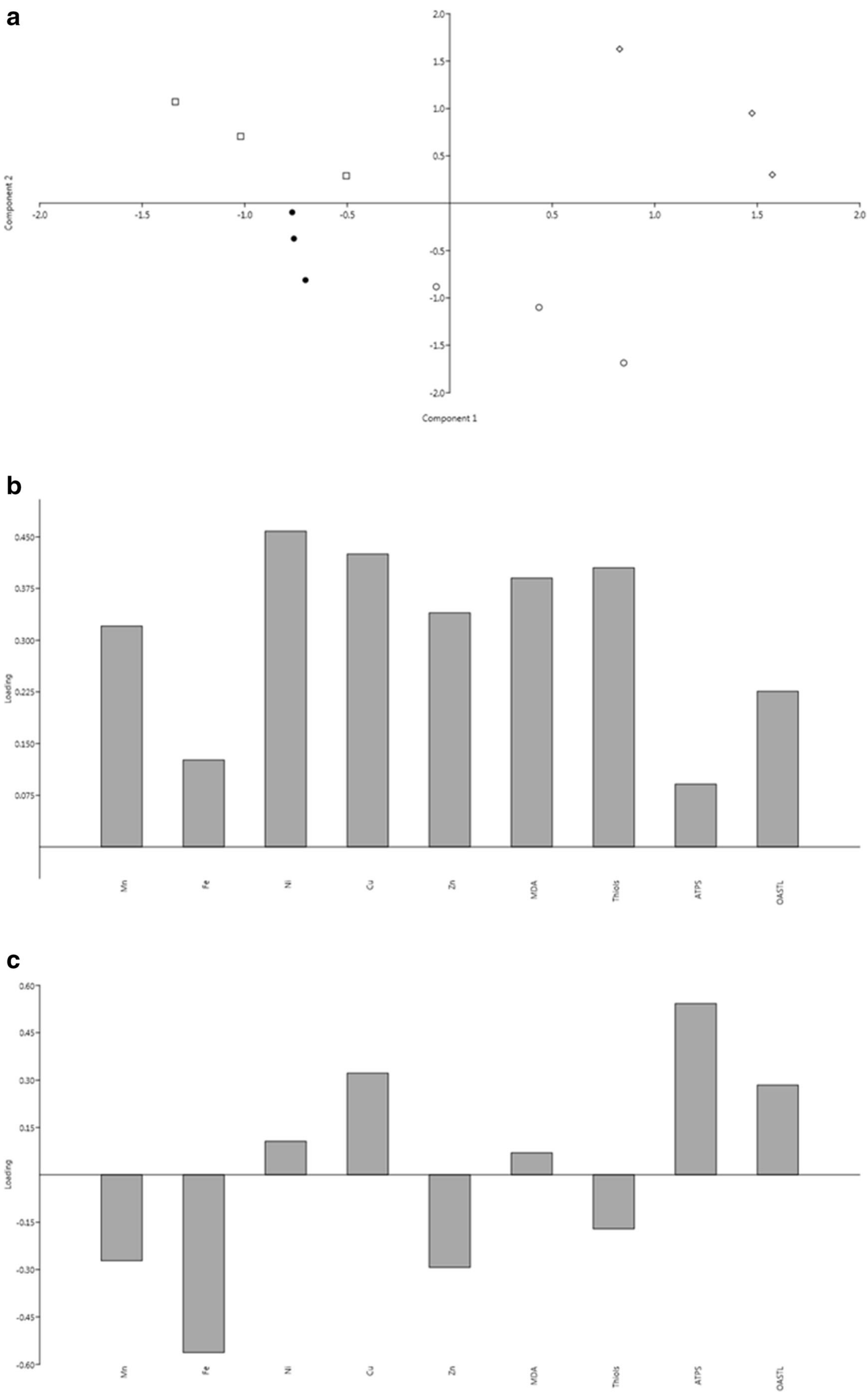
In the present study, we found that exposure of *Arundo* plants to 400 and 800 ppm Cu significantly increased MDA contents in shoots by 28 and 51%, respectively (Fig. 3).

This pattern is consistent with previous studies showing increased MDA levels in *Arundo* plants grown in hydroponic system in presence of cadmium (Shaheen et al. 2018). In particular, the production of MDA, as well as of some enzymatic antioxidants such as superoxide dismutases and catalases, followed a dose-dependent pattern and, at the highest Cd concentration the MDA level was about tenfold higher than that found in control plants. Interestingly, our results showed as at the lowest dose of Cu (200 ppm), the level of MDA was comparable with the control thus indicating that the specie could cope with a mild oxidative stress.

Besides enzymatic antioxidants, also antioxidant compounds, such as glutathione (GSH), play an important role in alleviating heavy metals-induced oxidative damage (Hernández et al. 2015). The tripeptide glutathione is the most abundant soluble S-containing metabolite and not only plays a role as major antioxidant and redox buffer in plant metabolism (Foyer and Noctor 2011), but also as precursor of phytochelatins (PCs), cysteine-rich peptides peptides that limit the free ion cellular concentration of heavy metals (Cobbett and Goldsbrough 2002).

Therefore, suppressing ROS production or enhancing the capacity for ROS scavenging it is expected to result in increased rate of synthesis of total non-protein thiols by tightly

Fig. 8 **a** Scatterplot representing the four clusters as a function of Cu application levels (● = control, = 200 ppm, o = 400 ppm, ∘ = 800 ppm). The analysis was performed as defined in the "Materials and methods" section. Growth conditions as in Fig. 2. Principle Component 1 (PC1) contributed with 45.45% and Principle Component 2 (PC2) with 22.66% to the separation. **b** Major PC1 loadings were Cu content, MDA level, thiol content, and OASTL activity. **c** Major PC2 loadings were ATPS activity (positive loading) and Fe (negative loading) ▶


a
Component 1
Component 2
-2.0
-1.5
-1.0
-0.5
0.5
1.0
1.5
2.0
b
Loading
0.450
0.375
0.300
0.225
0.150
0.075
Mn
Fe
Ni
Cu
Zn
MDA
Thiols
ATPS
OASTL
c
Loading
0.60
0.45
0.30
0.15
-0.15
-0.30
-0.45
-0.60
Mn
Fe
Ni
Cu
Zn
MDA
Thiols
ATPS
OASTL

regulation of the sulfur assimilation pathway (Rausch and Wachter 2005).

Our results support this hypothesis, as the significant increase in MDA levels found in plants under Cu stress was significantly positively correlated ($R = 0.928$***) with total non-protein thiols (Fig. 4). Furthermore, this study evidenced the modulation of the activity of ATPS and OASTL, the first and the last enzyme of S assimilation pathway in plants upon Cu exposure (Figs. 6 and 7). In particular, the pattern of thiols production correlated well with the OASTL activity ($R = 0.585$*), suggesting that both contribute toward the activation of the plant defense response to cope with oxidative stress induced by Cu uptake. It is interesting to note that OASTL directly catalyzes the cysteine biosynthesis (Wirtz and Hell 2006), and some investigations have already demonstrated the responsiveness of OASTL activity in plants exposed to toxic metals (Astolfi et al. 2004; Pajuelo et al. 2007).

Summarizing all these findings by the PCA analysis computed with the complete dataset comprising the leaf micronutrients content, MDA and thiols levels, and enzyme activities (Fig. 8) appeared a clear clustering along the first component (describing 45.45% of the total variance) on the basis of Cu dose application and along the second one (describing 22.66% of the total variance), with a high positive loading for ATPS activity and a negative loading for Fe. This distribution indicates that in *Arundo* the activation of defense response against Cu toxicity is not only dose dependent, but also rely on the regulation of plant nutritional status and, more importantly, on the modulation of S assimilation pathway.

In conclusion, this study allowed for the characterization of the Cu phytoextraction potential of *Arundo donax* and importantly demonstrated that plants responded to Cu application by the induction of defense responses having different impact in terms of plant metabolic costs, but without detrimental consequences on biomass accumulation.

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