Effect of copper (II) ions on morpho-physiological and biochemical variables in *Colobanthus quitensis*

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Abstract

Colobanthus quitensis is a species with a wide geographical distribution, including Antarctica. This species must endure a series of limiting abiotic factors in its habitats, such as, high metal ion concentrations. It has been described to inhabit areas where only metal-tolerant species can live. Therefore, it is postulated that the study of this species may provide information about copper tolerance mechanisms. We evaluated the effect of copper (II) ions (control, 100 and 500 μ M) on *C. quitensis* seedlings *in vitro*, determining morpho-physiological and biochemical variables. Copper showed a significantly negative effect on the development of new shoots (500 μ M) and floral apex appearance (100 μ M). The analyzed Cu concentrations significantly affected leaf and root length and induced a significant increase in guaiacol peroxidase (G-POD) enzyme activity. The highest proline accumulation took place in seedlings subjected to 500 μ M. Furthermore, this concentration induced a significant reduction in chlorophyll a content and exhibited oxidative stress evaluated through an increase in malondialdehyde levels. This is the first study to demonstrate evidence of copper effects on morphological, physiological and biochemical variables in *C. quitensis*.

Keywords: Antarctica, *in vitro*, oxidative stress, proline, tolerance mechanisms

1. Introduction

Metals and metalloids enter the ecosystem due to both anthropogenic and natural processes. High contents of trace elements have been found in many soils, which may become toxic for plants and other components of terrestrial biota (Violante *et al.*, 2010). In Central Chile, environmental problems of metal contamination due to mining activity have required various studies in order to determine the toxicity threshold to plants in soils exposed to copper mining activities (Verdejo *et al.*, 2016). Copper (Cu) has been considered to be an essential micronutrient for plants; it is an element of vital importance in seed production, disease resistance, and regulation of water catchment (Wuana and Okieimen, 2011) and of other essential nutrients, which largely depend on copper solubility in the soil and the type of soil (Joshi *et al.*, 2015). Essential micronutrients are required in low concentrations, so that the plant can develop normally, but are toxic in high concentrations (Wuana and Okieimen, 2011). These can lead to biochemical alterations, which are widely documented (e.g. Sharma *et al.*, 2012; Sreekanth *et al.*, 2013). Cu interferes in several physiological processes and therefore, potentially inhibits plant growth, resulting in a decrease in performance, delays in leaf and root growth, as well as anatomical and ultrastructural alterations that often lead to the formation and accumulation of reactive oxygen species (ROS) (Dey *et al.*, 2014).

Some plant species can withstand the excess of metals present in the environment whether through compartmentalization (Dal corso *et al.*, 2013), chelation, exclusion or accumulation (Thounaojam *et al.*, 2012). This capability is typically described in particular in species that grow or show a frequency and/or high abundance in copper-contaminated soils, allowing soil stabilization through uptake and retaining metals. It has been suggested that this specific compartmentalization of

metals in the cell may be the reason for a plant's development of more complex tolerance mechanisms to certain metals (Dal corso *et al.*, 2013).

Colobanthus quitensis Kunth. (Bartl.) Caryophyllaceae presents a wide distribution from Mexico, the Ecuadorian and Bolivian Andes to the south of Peru, and throughout the Argentinean and Chilean Andes to Western Patagonia. Moreover, its distribution extends to the Falkland Islands, the sub-Antarctic, Antarctic islands (and the western coast of the Antarctic Peninsula to 68°42'S on Tierra Firme (Moore, 1970; Lewis-Smith, 1993).

In general, the distribution of this species is dominated by a series of extreme abiotic factors such as: freezing temperatures (Lewis-Smith, 2003), strong winds, different states of water availability, the presence of metal ions in high concentrations, and low pH in soil (Androsiuk *et al.*, 2015); many of these are considered to be limiting factors for the survival of plant species. The distribution of *C. quitensis* follows the pattern characterized by the presence of Cu throughout the Americas (Borsdorf *et al.*, 2012) and Antarctica (Lu *et al.*, 2012). Thus, it becomes clear that *C. quitensis* has developed tolerance mechanisms that would enable it to survive in the presence of high metal concentrations in the environment. Currently, there are no studies related to the tolerance of *C. quitensis* to Cu; however, there are several other studies that have shown tolerance mechanisms to other extreme abiotic agents in its habitat (Bravo *et al.*, 2007; Bascuñán-Godoy *et al.*, 2012; Cuba-Díaz *et al.*, 2017).

It is proposed that *C. quitensis* may deploy a series of morpho-physiological and biochemical strategies that allow it to mitigate the harmful effects of high Cu ion concentrations. To test this hypothesis, we propose evaluating the effect of the Cu (II) ions on the *in vitro* propagation ability of an Antarctic population of *C. quitensis*.

2. Materials and Methods

2.1. Plant material and treatments

Leaf explants with a root fragment from previously established *in vitro* plantlets of an insular Antarctica population of *C. quitensis* were selected using a laminar flow chamber. 10 explants per flask were placed into Murashige and Skoog (MS) culture medium, supplemented with 3% sucrose, IAA (indoleacetic acid) (0.25 mg L^{-1}), BAP (Benzylaminopurine) (0.5 mg L^{-1}), STS (silver thiosulphate) ($10 \text{ }\mu\text{M}$). The pH (5.7 ± 2) was adjusted and 0.7% agar was added (Cuba-Díaz *et al.*, 2014). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added to the media according to the applied treatments: control ($0 \text{ }\mu\text{M}$), minimum ($100 \text{ }\mu\text{M}$), and maximum ($500 \text{ }\mu\text{M}$). The Cu (II) ion concentrations were established according to a preliminary laboratory test (unpublished data). 20 flasks with 10 plants in each one were used for each treatment, for a total of 60 flasks. The culture flasks were brought to a growth chamber with a 16/8 h light/darkness photoperiod at a temperature of $20 \pm 2^\circ\text{C}$ and a light intensity of $50 \pm 5 \text{ }\mu\text{mol m}^{-2} \text{ s}^{-1}$ for 60 days.

2.2. Morpho-physiological variables

The total number of explants per flask was recorded at 30 and 60 days, exhibiting an increase in the development of new shoots and roots and in the appearance of floral apices and chlorosis; these two last variables are negative indicators of *in vitro* development (Cuba-Díaz *et al.*, 2014). At the end of the assay (day 60), 20 seedlings were selected randomly. Their roots were carefully washed to eliminate traces of the culture medium. Each plant's roots were carefully dried with sterile paper towels, and the fresh weight (FW) and leaf and root length were measured using a caliper (KAMASA®). Subsequently, the seedlings were dried in an oven at $37 \pm 0.2^\circ\text{C}$ until constant weight, the moment in which

the dry weight was recorded. The water content (WC) was determined based on the FW and DW, using the following formula (Cheng *et al.*, 2012):

$$\text{WC} = \frac{(\text{FW} - \text{DW})}{\text{FW}} \times 100$$

FW - fresh weight and DW - dry weight.

Chlorophyll fluorescence (Fv/Fm) was measured in 5 leaves per plantlet, which were darkened for 30 min to maintain all reaction centers open and finally, a saturation pulse of $3500 \text{ }\mu\text{mol m}^{-2} \text{ s}^{-1}$ was applied using a portable fluorometer (Pocket PEA Hansatech).

2.3. Proline content

Proline content was determined according to Bates *et al.* (1973). At the end of the assay (60 days) for each treatment, 250 mg of fresh aerial tissue (leaves and stems, and in triplicate) were taken that were pulverized in the presence of liquid nitrogen and homogenized with 1.25 ml of 3% sulfosalicylic acid, followed by filtration into test tubes. The resulting solution was mixed with glacial acetic acid and ninhydrin reagent in a 1:1:1 proportion. Subsequently, each tube was agitated in vortex for a few seconds. Each covered tube was incubated for 1 h at 100°C in a water bath. After this period, the reaction was stopped in an ice bath 1 ml of toluene was added to the reaction, agitating in vortex for 30 s. After precipitation of the toluene, the proline deposited in the supernatant was taken with a micropipette and its absorbance was measured in a UV-Vis spectrophotometer (Thermo scientific GENESYS 10UV) at 520 nm wavelength using quartz cuvettes. Proline content was expressed as μg of proline per mg of fresh plant material.

2.4. Photosynthetic pigment content

Fresh leaf tissue (200 mg and in triplicate) was pulverized in the presence of liquid nitrogen and the pigments were extracted in 15 ml of 80% acetone. The volume obtained was filtered into volumetric flasks, which were diluted with 80% acetone. The content was homogenized by agitation. The extracts were kept in plugged test tubes and covered with aluminum foil. The absorbance of each one of these was measured in spectrophotometer tubes at 470 (carotenoids), 646 (Chlorophyll a), and 663 nm (Chlorophyll b). The pigment content was expressed as mg of pigments per g of fresh plant material (Steubing *et al.*, 2002).

2.5. Determination of lipid peroxidation

The 200 mg samples of fresh aerial tissue (in triplicate) were macerated in liquid nitrogen and homogenized with 1 ml of 0.5% trichloroacetic acid (TCA). The homogenates were centrifuged at 5 300 rpm for 20 min at room temperature. The supernatant was taken and mixed with 1 ml of 0.5% thiobarbituric acid (TBA) dissolved in 20% TCA. The mixture was incubated at 96 °C for 30 min and cooled in an ice bath. Finally, the absorbance was measured at 532 and 600 nm. Lipid peroxidation was expressed as nmol of malondialdehyde (MDA) per gram of fresh tissue (Dhindsa *et al.*, 1981).

2.6. Enzyme extract and catalase (CAT) and guaiacol peroxidase (G-POD) activities

Enzyme extracts were prepared by pulverizing 100 mg of fresh aerial tissue (in triplicate) in the presence of liquid nitrogen; the obtained powder was homogenized in 1.5 ml potassium phosphate buffer (pH 7.4). Each homogenate was centrifuged at

10 000 g for 20 min at 4 °C and the supernatant was retrieved. To determine CAT activity, 1.78 ml potassium phosphate buffer (pH 7.5), 100 µl H₂O₂ 100 mM, and 120 µl enzyme extract were mixed in a quartz cuvette. The mixture was homogenized by agitation 3 times and the absorbance was measured at 240 nm with intervals of 10 s for 30 min. As a blank, the same reaction mixture was used, replacing the enzyme extract with an identical volume of deionized water. Enzyme activity was expressed as IU min⁻¹ (Ulrich, 1974). G-POD activity was determined in a reaction carried out in a quartz cuvette, where 2.80 ml potassium phosphate buffer (pH 7.0), 50 µl guaiacol 12 mM, 50 µl H₂O₂ 100 mM, and 100 µl enzyme extract were added. The mixture was homogenized by agitation 3 times and the absorbance was measured at 470 nm wavelength with intervals of 10 s for 10 min. As a blank, the same reaction mixture was used, replacing the enzyme extract with an identical volume of deionized water (Kireyko *et al.*, 2006).

2.7. Experimental design and statistical analysis

All assays were randomized, using 20 replicas per treatment, considering an experimental unit of 10 seedlings per flask, where there were a total of 200 seedlings per treatment. Statistical differences between Cu concentrations and the control were evaluated using an analysis of variance (one-way or factorial ANOVA (analysis of variance) (quantification of photosynthetic pigments, and enzyme activity), applying Fisher's LSD multiple comparison test with the STATISTICA software (v 6.0). The graphics were prepared using the mean ± SE with Microsoft Excel 2010 software (v14.0.7116.500). The differences between treatments were considered significant if $p < 0.05$.

3. Results

3.1. Morpho-physiological variables

According to the ANOVA, no effect of assay time was observed (30 and 60 days) on the analyzed variables; therefore, only the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ concentration factor was considered to evaluate the significance of the effect of this on the studied parameters (Table 1). The presence of Cu (II) ions in the culture medium only significantly affected the number of shoots when the concentration was 500 μM , both with respect to the control (0 μM) and the lower concentration

(100 μM), observing a decrease in the number of new shoots. The number of roots showed a significant decrease in the presence of both Cu (II) ion concentrations in comparison with the control, without observing differences between the analyzed concentrations. Only the lowest concentration of Cu (II) ions significantly boosted the development of floral apices. Although the induction of floral apices was also observed in the presence of the highest ion concentration, this did not show significant differences with respect to the control. The studied concentrations showed no significant effect on the appearance of chlorosis symptoms in leaves and stems (Table 1).

Table 1. Quantitative variables in *Colobanthus quitensis* seedlings subjected to three CuSO_4 concentrations for 60 days. Mean and SE (n=20). Differences between treatments (*) were considered significant if $p < 0.05$.

Variable	Concentration (I)	Concentration (J)	Mean difference (I-J)	SE
Shoots (%)	Control	100 μM	5.0	9.35
		500 μM	27.5*	9.35
		100 μM	22.5*	9.35
Roots (%)	Control	100 μM	60.0*	12.91
		500 μM	80.0*	12.91
		100 μM	20.0	12.91
Floral apex (%)	Control	100 μM	-20.0*	8.42
		500 μM	-7.5	8.42
		100 μM	12.5	8.42
Chlorosis (%)	Control	100 μM	-37.5	16.79
		500 μM	-37.5	16.79
		100 μM	0.0	16.79

Of the morphophysiological variables evaluated at the end of the study, only leaf length (Figure 1A) and root length (Figure 1B) showed significant differences in comparison to the control, with a decrease in both variables. Furthermore, although the trend was that this decline was directly associated

with the increase in ion concentration, significant differences were not observed between the studied concentrations (100 μM and 500 μM). The variables, fresh weight, dry weight, water content, and Fv/Fm showed no significant differences in any of the evaluated treatments (data not shown).

Figure 1. Leaf and root length of *Colobanthus quitensis* subjected to CuSO_4 for 60 days. (a) Leaf Length, (b) Root Length. Mean $\pm$ SE (n=20). Differences between treatments were considered significant if $p < 0.05$.

3.2. Biochemical variables

A significant increase was observed in proline content in the plants subjected to the higher concentration of Cu (II) ions ($48.23 \mu\text{g mg}^{-1}$ fresh weight). The proline content between the control and the lower Cu concentration presented very similar values, 25.68 and $27.08 \mu\text{g mg}^{-1}$ fresh weight, respectively (Figure 2A).

Regardless of the fact that the observed trend showed a decrease in pigment content (chlorophyll a, chlorophyll b, and carotenoids) that was directly related to the increase in Cu (II) ions, significant differences were only observed in chlorophyll a content in the plants that were subjected to the highest Cu concentration (500 μM) (Figure 2B).

MDA content was significantly greater in the plants that were in the presence of the highest Cu concentration ($4364.52 \text{ nmol g}^{-1}$), while no differences were observed between the control and the lower concentration of Cu (II) ions (Figure 2C).

Catalase activity (CAT) showed no significant differences for any of the performed treatments, although not the G-POD enzyme, whose activity was not detected in the control treatment, showed a significant increase in the presence of both Cu (II) ion concentrations (Figure 2 D).

Figure 2. Biochemical variables of *Colobanthus quitensis* seedlings subjected to CuSO_4 for 60 days. (a) Proline Content, (b) Photosynthetic Pigments (Black, Chl a; Grey, Chl b; White, Carotenoids), (c) Malondialdehyde (MDA) Content, (d) Enzymatic Activity (White, Catalase (CAT); Black, Guaiaacol peroxidase (G-POD)). Mean $\pm$ SE (n=3). Differences between treatments were considered significant if $p < 0.05$.

4. Discussion

This is the first study to report on the effect of Cu ions in *C. quitensis*, allowing us to obtain new information on tolerance mechanisms that this species has developed to survive in extreme abiotic conditions, such as in the presence of metal ions which high concentrations can be toxic to most plants. According to our results, only high Cu concentration (500 μM) exhibited a decrease in the number of new shoots, thus affecting plant growth and development. On the other hand, the effect on root system development was significantly harmful from the lowest concentration studied (100 μM). This could

indicate that *C. quitensis* showed greater sensitivity to the Cu ions in its root system. According to Marschner (2011) the effect of Cu toxicity is mainly observed in root tissues with a slight translocation towards the shoots. Some plants develop mechanisms such as the reorganization of root architecture in order to adapt to changes in the nutrient availability whether in excess or by default (López-Bucio *et al.*, 2003). In the highest Cu (II) ion concentration, the root development of *C. quitensis* was practically non-existent, however, the plants were able to develop new shoots, although with a significant decrease regarding the lower concentration. This would indicate that, despite of the toxicity in root cells, the plant must have developed some strategy

to continue its growth. Similar effects have been observed in other species such as *Hordeum vulgare* L. (Žaltauskaitė and Šliumpaitė, 2013), *Brassica juncea* L., and *B. napus* L. (Feigl *et al.*, 2013).

In the presence of the lowest Cu concentration, the development of floral apices was evidenced in the first 30 days of the study. This factor has been considered a sign of adaptation in ecotypes that are tolerant to contaminated environments (McNeilly and Antonovics, 1968). Nonetheless, in *in vitro* studies, such as in this case, it is a variable that should be considered with discretion since it is related more to stress than with the events of plant development themselves (Cuba-Díaz *et al.*, 2014).

According to Marschner (2011), the first symptoms of an initial Cu toxicity process are expressed in growth impairment and root elongation, which supports the absence of significant differences in physiological variables (dry weight, water content, and Fv/Fm); these indirectly indicate the proper functioning of photosynthesis. *C. quitensis* exhibited decreases in leaf and root growth that were directly related to the increase in Cu (II) ion concentration. Control plants exhibited differences in comparison to those that were treated with two Cu (II) ion concentration. On the other hand, both concentrations (100 and 500 μM) showed a statistically identical behavior, regarding these two variables. This may provide insight on the species' tolerance range, which, moreover, continued to grow despite the decrease in its root system development. In other species such as *Medicago sativa* L. (Flores-Cáceres *et al.*, 2015) it has been highlighted that the increase in Cu concentration in the substratum negatively affects plant development, being more significant in root development. Other studies, such as in *Hordeum vulgare* L. (Žaltauskaitė and Šliumpaitė, 2013) have been shown that Cu not only inhibits root system development, but also significantly decreases root length.

The amino acid proline is one of the main indicators to assess early metabolic responses to stress (Viehweger, 2014), being deemed a cell osmoprotector. In *C. quitensis*, the proline content only increased significantly in plants exposed to the higher Cu concentration. It has been observed that, in species with ecotypes tolerant and sensitive to metal, like *Silene vulgaris* G., the proline content in the presence of Cu was higher in the first (Schat *et al.*, 1997). One of main protective function of proline is the antioxidant defense that would act through the reduction of ROS, mitigating the damage caused by metals (Viehweger, 2014). In addition, proline provides protection through Cu chelation in the cytoplasm, reduction in metal absorption, and plant osmoregulation, since the excess of this micronutrient collaterally generates an inhibition in water transportation, as well as a change in cell membrane permeability (Hayat *et al.*, 2012). Protection to the water deficit could be confirmed in this study through the positive relationship between the presence of the proline and the water content in plants exposed to 500 μM (data not shown), since these did not express significant differences in water content with regard to the control seedlings.

Membrane fatty acid peroxidation is an indicator of oxidative damage in plants, therefore, is a biomarker of oxidative stress (Žaltauskaitė and Šliumpaitė, 2013). This membrane damage, along with the enzyme inactivation, may occur due to the oxidative stress in the cell in the presence of excess metal ions (Thounaojam *et al.*, 2012). In *C. quitensis*, only the 500 μM concentration cause a significant increase in the malondialdehyde content (MDA), which may be indicative of certain peroxidative damage to the membrane. Lipid peroxidation is described as an extensive degradation factor of intracellular and organelle membranes, particularly in chloroplasts. Although Cu is an essential micronutrient for the plant's metabolism, lipid peroxidation and the destruction of the membranes

caused by the excess of Cu can lead to chlorosis, an indicator of toxicity (Žaltauskaitė and Šliumkaitė, 2013). Although no significant difference was directly observed in plants color (chlorosis) between treatments (Table 1), a decrease was noted in chlorophyll a in the seedlings that were exposed to the higher Cu concentration for 60 days (Figure 2B). High concentration of copper has caused a decrease in chlorophyll a in others species such as tobacco (Khairy *et al.*, 2016) and *Brassica juncea* L. (Singh *et al.*, 2011), which has been associated with both destruction of chloroplast membranes and inhibition of aminolevulinic acid dehydratase (Scarponi and Perucci, 1984).

Catalase (CAT) and peroxidase (POD) are among the main enzymes which constitute the enzyme system catabolizing free radicals and H_2O_2 in plant cells. The activity of these enzymes generally increases in the plants, after the exposure to high concentrations of metals (Gill and Tuteja, 2010). In *C. quitensis*, the CAT did not show significant differences in any of the applied treatments. In contrast, the G-POD had significantly higher activity in the presence of the two Cu (II) ion concentrations evaluated. There is no information available in the literature on the activity of this enzyme in the species; however, in a similar study, where different sodium chloride concentrations were applied to *in vitro* *C. quitensis* plants, G-POD activity was not detected in control plants either (Castel, 2015). Thus, in this case, CAT would not be involved in an eventual defensive response to these Cu (II) concentrations. In *glycine max* L., the application of different arsenic concentrations decreased CAT activity but increased G-POD activity; the latter was associated with a higher participation in antioxidant responses to stress from toxic metal ions (Chandrakar, *et al.* 2016). Although the trend to activity increase observed in both enzymes analyzed in *C. quitensis* is similar to what is described

by Singh *et al.* (2011) in *Brassica juncea* L., it differs considerably from the values reported in those studies, observing for *C. quitensis* that both enzymes show very low activity values.

5. Conclusions

High Cu concentrations (500 μ M) applied in *in vitro* culture on *C. quitensis* seedlings caused a decrease in seedling growth, mainly affecting root development. Other symptoms that may be stress indicators such as an increase in proline content, a decrease in photosynthetic pigments (chlorophyll a), increased oxidative damage to the membrane and to the activity of enzymes related to ROS detoxification also showed significant values regarding the control plants. However, after 60 days, the plants were still growing and did not show symptoms such as chlorosis, decreases in their photosynthetic activity, or a significant decrease in fresh weight and water content with respect to the control plants. Although these results are not sufficient, we can preliminarily propose that, regardless of showing certain symptoms of stress, *C. quitensis* is capable of generating a mechanism that allows it to tolerate the presence of toxic metal ion concentrations, even in the presence of high Cu concentrations.

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