Chapter

Phytoextraction of Zn(II) and Cu(II) by *Canna indica*: Related Physiological Effects

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Abstract

Phytoremediation is a technique for treatment areas with medium or low heavy metals concentrations. A pot experiment was carried out to determine the usefulness of *Canna indica* L. as phytoremediator species. The plants were treated with three increasing Zn(II) and Cu(II) solutions. 21 days later, dry weight, relative membrane conductivity, chlorophyll, carotene, malondialdehyde, soluble proteins, proline, and Zn(II) and Cu(II) contents were measured. Zn(II) and Cu (II) treatments caused a decline in the dry weight, chlorophyll, carotene, and soluble proteins content, whereas the relative conductivity, malondialdehyde, and proline content showed the opposite pattern. The bioaccumulation reached values approximately 48 and 15 times higher (5293 mg kg^{-1} and 1425 mg kg^{-1}), compared with the control, for Zn(II) and Cu(II), respectively. Our results suggest that this species can be used for the phytoremediation of polluted soils with moderate concentrations of Zn(II) and Cu(II).

Keywords: phytoremediation, *Canna indica*, copper, zinc, physiological response

1. Introduction

Heavy metal pollution of soil and water is a worldwide concern because of its harmful effect on human health. The constant accumulation of heavy metals in soil can pose a serious risk to living organisms including plants, animals, and microorganisms [1, 2]. To date, phytoremediation is confirmed to be the most environmentally friendly and cost-effective strategy. Types of phytoremediation include phytoextraction, phytovolatilization, phytostabilization, phytodegradation, and rhizosphere. The advantages of phytoremediation compared with traditional physical surface and chemical remediation methods are low cost and simplicity [3]. Phytoremediation is linked with the morphological, biochemical, and physiological effects on plant growth. During the phytoremediation process, some morphophysiological growth parameters have to be evaluated such as root growth, net biomass weight, leaf area, the net rate of photosynthesis, the effects on the plasma membrane of plants, reactive oxygen species (ROS) generation, hydrogen peroxide (H_2O_2) content, and malondialdehyde (MDA) level, linked to genotoxicity. Plants try to elude

their harmful effects by adopting various defense mechanisms, which include antioxidant activation and other mechanisms of metal homeostasis. In response, plants have developed enzymatic and nonenzymatic antioxidant mechanisms and increased activities of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POD) [4].

In literature, *C. indica* was investigated by different authors as phytoremediation species in pot, hydroponic, and wetlands systems [5–7]. Most of these works focused on the efficiency of the plant to accumulate heavy metals but did not evaluate the effect of these metals on the physiology of the plant.

This study aimed to evaluate the impact of Zn(II) and Cu(II) excess on the growth and metabolism of *C. indica* through the determination of physiological parameters and Zn(II) and Cu(II) bioaccumulation to establish the strategies used by the plant to overcome the stress and determine the correlation between metal accumulation and physiological parameters modification. Results obtained were compared with parameters measured and published in the scientific literature to provide information for future phytoremediation research.

2. Material and methods

2.1 Growth conditions

The test was carried out in a greenhouse with natural light, forced ventilation, and controlled temperature in La Plata city (Argentina) (34°54′45.5″ S–57°55′51.5″ W) from April to July (2019).

C. indica L. (achira) seeds were superficially disinfected with NaClO (10%) for 5 min, flushed with sterilized water, and placed in Petri dishes with filter paper moistened with water for their germination. Previously, they were subjected to a mechanical scarification treatment to break their dormancy.

Once germination had occurred, the seedlings were transferred to 0.5 L pots and then to 5 L pots with a substrate composed of soil and sand (2:1 v/v). After 45 days, when the plants were approximately 50 cm tall, metal solutions were applied by immersion for 24 h. Cu(II) was added in the form of $SO_4Cu{\cdot}5H_2O$ in three concentrations (500, 1000, and 1500 ppm) and Zn(II) in the form of $SO_4Zn{\cdot}7H_2O$ in three concentrations (1000, 2000, and 3000 ppm).

After 21 days of the application, plants were harvested to perform the different physiological and biochemical determinations.

2.2 Measurements performed

2.2.1 Biomass and leaf area

At harvest, the dry weight per plant (DW) was determined for all treatments by oven-drying them at 80°C until constant weight, distinguishing the shoot from roots.

2.2.2 Chlorophyll and carotene content

For all treatments, the contents of chlorophyll and carotene were determined from a 1 cm diameter leaf disk. Pigment content calculation was performed using Wellburn

technique [8] with a Shimadzu UV 160-A spectrophotometer (Kyoto, Japan). The results were expressed in μg of chlorophyll cm^{-2} and μg of carotenoids cm^{-2}.

$$\text{Chlorophylla } \left(\mu g\ cm^{-2}\right) = 12 \times A_{643.8} - 3.11 \times A_{646} \quad (1)$$

$$\text{Chlorophyllb}\left(\mu g\ cm^{-2}\right) = 20.78 \times A_{646} - 488 \times A_{663.8} \quad (2)$$

$$\text{Totalchlorophyll}(a+b)\left(\mu g\ cm^{-2}\right) = 17.67 \times A_{646} + 7.12 \times A_{663.8} \quad (3)$$

$$\text{Carotenoids } \left(\mu g\ cm^{-2}\right) = \frac{(1000 \times A_{480} - 1.12\ \text{Ca} - 34.07\ \text{Cb})}{245} \quad (4)$$

where A is absorbance, Ca is chlorophyll a content, and Cb is chlorophyll b concent.

2.2.3 Soluble proteins content

The soluble protein content was measured from 100 mg of fresh leaves and root material, employing the Bradford method [9]. The protein content calculation was carried out using a standard curve prepared with different concentrations of bovine serum albumin (BSA) (SiFMa Chemical Co.).

2.2.4 Proline content

Proline determination was carried out taking 100 mg of fresh leaf and root material and homogenized with 2 ml of a 3% sulfosalicylic acid solution in water. The homogenate was centrifuged at 12,000g for 15 min, and 1 ml of the extract obtained was taken. Then 1 ml of the acidic ninhydrin reagent and 1 ml of glacial acetic acid were added to the extract in a 15 ml tube and put in a water bath at 100°C for an h. After this period, the reaction was stopped by rapidly cooling the tube. After, 2 mL of toluene was added to the above reaction mixture and vortexed for 15–20 s. The phases were allowed to separate and the aqueous phase containing the toluene-proline chromophore was taken. The absorbance at 520 nm was read using toluene as a blank. Proline content per unit of fresh weight was calculated according to:

$$\mu\text{mol proline} \cdot g^{-1}\ \text{FW} = \frac{\frac{\frac{\mu g\ \text{proline} \cdot ml^{-1}}{ml\ \text{toluene}}}{115.5\ \mu g \cdot \mu mol^{-1}}}{\frac{g\ FW}{5}} \quad (5)$$

where, FW is fresh weight.

2.2.5 Malondialdehyde content (MDA)

The amount of malondialdehyde (MDA) content in fresh tissues was determined by the reaction with thiobarbituric acid (TBA) described in the Heath and Packer method [10]. In total, 200 mg of fresh leaf tissue and 200 mg of fresh root tissue were ground with 1 ml of 0.1% trichloroacetic acid (TCA) and then centrifuged. The supernatant was reacted with 1 ml of the trichloroacetic acid (TCA), butylhydroxytoluene (BHT) and thiobarbituric acid (TBA) reagent (20% trichloroacetic acid (TCA), 0.37% thiobarbituric acid TBA and butylhydroxytoluene BHT 0.01 g), then the tubes were incubated for 30 min at 95°C. After this period, they were placed in an

ice bath to rapidly stop the reaction, and then they were centrifuged at 10,000g for 10 min. Finally, the supernatant was separated, and the absorbance at 532 and 600 nm was read on a Shimadzu UV 160 UV/V spectrophotometer. The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}:

$$\text{MDA equivalents } \left(\text{nmol ml}^{-1}\right) = \frac{A_{532} - A_{600}}{155,000} \tag{6}$$

where MDA is malondialdehyde content, A is the absorbance.

2.2.6 Relative conductivity (RC) of cell membranes

The determination of the relative conductivity (RC) of the cell membranes was made from 200 mg of fresh leaf material and 200 mg of fresh root material, from the different treatments, according to the Lutts method [11]. Immediately after sampling, the tissues were washed three times with redistilled water for 15 s, to remove the electrolytes adhering to the surface and those released by the wounds produced by the cut. Subsequently, each sample was immersed in a tube with 10 ml of double-distilled water where they remained for 4 h at room temperature. Following this, the electrical conductivity (dS m^{-1}) was determined using a Jenco model 3173 conductivity meter. Then, the tubes were capped and taken to an autoclave where they were kept for 20 min at a one-atmosphere pressure and 120°C, to affect the integrity of the membranes. Finally, the tubes were allowed to cool to room temperature, and the electrical conductivity of the medium was measured again. Based on the data obtained, the relative conductivity of cell membranes was estimated from the following formula:

$$RC\ (\%) = \left(\frac{L1}{L2}\right) \times 100 \tag{7}$$

where RC is the relative conductivity; $L1$ and $L2$ are the electrical conductivity readings before and after autoclaving, respectively.

2.2.7 Zn(II) and Cu(II) content in aerial part, root, and substrate

Plant tissues were digested in triplicate with concentrated perchloric and nitric acids in a 1:4 ratio (Merck, analytical grade), for the analyses of Cu(II) and Zn(II) (FAO & SIDA, 1983). Luoma method [12] was used to analyze the Cu(II) and Zn(II) labile fraction of sediments, being mineralized with hydrochloric acid (1 N, Merck analytical grade) by shaking for 24 h. Then, the absorbance was read using an atomic absorption spectrophotometer (Shimadzu AA6650F Atomic Absorption Spectrophotometer, Japan). The data obtained were employed for calculating the bioavailability, accumulation, translocations, and bioaccumulation indexes. All values were expressed on the dry weight of the respective sample [13].

$$\text{BAI} = \frac{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in roots}}{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in the substrate}} \tag{8}$$

$$\text{AI} = \frac{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in aerial part}}{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in the substrate}} \tag{9}$$

$$TI = \frac{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in aerial part}}{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in roots}} \quad (10)$$

$$BI = \frac{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in the biomass}}{\text{mg Zn(II)} \cdot \text{kg}^{-1}\text{in the substrate}} \quad (11)$$

where BAI is bioavailability index and indicates if the metal is extracted and accumulated in the root; AI is accumulation index and indicates if the metal is extracted and accumulated in the aerial part; TI is translocation index and indicates if the metal is translocated to the aerial part; BI is bioaccumulation index and indicates if the metal is accumulated in the biomass.

2.2.8 Statistical analysis

The experimental design was fully randomized with a control (without addition of heavy metals solutions), two metals, and three concentrations for each one. The number of repetitions per treatment was $n = 5$. The data were subjected to analysis of variance (ANOVA) and the means compared by the 5% least significant difference test (LSD test) and the Pearson correlations using the software InfoStat version 2019.

3. Results

3.1 Growth, physiological and biochemical parameters

A negative effect on growth was found, expressed in a decrease in total biomass, as in **Figure 1A** and **B**. This result varied approximately 82 and 59% between the control (0 ppm) and the maximum concentration of Zn(II) (3000 ppm) and Cu(II) (1500 ppm), respectively. The dry weight of the root and the aerial part decreased by 82% for Zn(II), whereas 62 and 56% for Cu(II), respectively. A significant reduction was observed from the lowest concentration of Zn(II) (1000 ppm) while for Cu(II), this decrease was observed from the middle concentration (1000 ppm). The reduction of biomass, both shoot and root, shows the same pattern, as the metal concentration increases, the decrease of biomass becomes greater (**Figure 1A** and **B**).

Figure 1A and **B** represent chlorophyll and carotenes concentration in Zn (II) and Cu(II) systems, respectively. A significant decreased of chlorophyll and carotenes concentration was observed in Cu(II) treatment (1500 ppm) compared with the control (**Figure 1B**). This difference was approximately 47 and 16% for chlorophyll and carotenes content, respectively. However, chlorphyll and carotens concentration in Zn(II) systems (**Figure 1A**) were not affected.

Figure 3 shows the relativity conductivity (RC) percentage in roots and leaves in Zn(II) (A) and Cu(II) (B) systems. A gradual increase of relativity conductivity (RC) in roots with increasing Zn(II) and Cu(II) concentrations was noted. On the other hand, the RC in leaves biomass was not affected by Zn(II) and Cu(II) concentrations (**Figure 3A** and **B**).

Figure 4A and **B** represent malondialdehyde (MDA) content in the roots and leaves of *Canna indica* plants in Zn(II) (A) and Cu(II) (B) systems, respectively. As observed in **Figure 4A** and **B**, malondialdehyde (MDA) content in leaves had significant differences at maximum concentrations of Zn(II) and Cu(II)

compared with the control. However, statistically significant increase of malondialdehyde (MDA) content was only detected in roots at 1500 ppm Cu(II) system (**Figure 4B**).

The soluble protein content in leaves and roots is shown in **Figure 5**. In general, it was determine there are not statistically significant differences of soluble protein content in roots for Zn(II) and Cu(II) systems, whereas the soluble protein content in leaves biomass decreased about 26% compared with the Cu(II) maximum concentration and the control (**Figure 5A** and **B**).

Figure 6 represents proline content in leaves and roots for Zn(II) and Cu(II) systems. The proline content in leaves increased with the increase of Zn(II) and Cu(II) concentrations, but statistically significant differences were determine only in the maximum concentrations for both metals compared with control system (**Figure 6A** and **B**).

3.2 Bioaccumulation and extraction of Zn(II) and Cu(II)

Figure 7A and **B** show the mean bioaccumulation values for Zn(II) and Cu(II) in shoot, roots, and total biomass of *Canna indica*, respectively. A higher bioaccumulation of Zn(II) and Cu(II) in the root than in the aerial part was observed. The results demonstrated that *C. indica* bioaccumulated 872.99 ± 694.68 mg Zn(II) kg^{-1} dry weight (DW) of total biomass (±SD), almost 77 times higher than the control (withouth heavy metal) (**Figure 7A**). The maximum concentration of Cu(II) in total biomass was 1432.15 ± 91.13 mg Cu(II) kg^{-1} DW (±SD) (**Figure 7B**).

On the other hand, the bioavailability (BAI), accumulation (AI), translocation (TI), and bioaccumulation (BI) indexes were calculated with the results mentioned above (**Table 1**). It was determined that BAI, AI, and BI indexes > 1 for Zn(II) and Cu (II) system. These results mean *C. indica* plant was efficient in extracting Zn(II) and Cu(II) from the substrate. However, *C. indica* plant did not translocate Zn(II) and Cu (II) to the aerial part as TI index was < 1 (**Table 1**).

3.3 Zn(II) and Cu(II) bioaccumulation correlated with physiological effects by Pearson stadistical method

Pearson coefficients (r) showed, for Zn(II), a significant negative correlation for shoot and root dry weight, whereas shoot malondialdehyde (MDA) and proline

Treatment	BAI (root/substrate)	AI (shoot/substrate)	TI (shoot/root)	BI (Biomass/substrate)
1000 ppm Zn(II)	5.409 ± 0.68	3.574 ± 0.32	0.663 ± 0.03	8.983 ± 1
2000 ppm Zn(II)	3.940 ± 0.12	1.922 ± 0.12	0.488 ± 0.04	5.862 ± 0.14
3000 ppm Zn(II)	14.283 ± 0.27	4.700 ± 0.27	0.329 ± 0.02	18.982 ± 0.31
500 ppm Cu(II)	3.966 ± 0.35	0.632 ± 0.02	0.160 ± 0.01	4.597 ± 0.37
1000 ppm Cu(II)	4.907 ± 0.74	0.706 ± 0.09	0.144 ± 0.01	5.613 ± 0.83
1500 ppm Cu(II)	2.540 ± 0.07	0.318 ± 0.02	0.125 ± 0.01	2.858 ± 0.09

Note: *(mean ± SD).*

Table 1.
BAI (bioavailability), AI (accumulation), TI (translocation), and BI (bioaccumulation) for Zn(II) and Cu(II) systems.

Variable 1	Variable 2	Zn (II) (*r*)	*p*-value	Cu(II) (*r*)	*p*-value
Shoot Pearson correlation coefficient (r)					
Metal concentration	Shoot dry weight	−0.74	0.001*	−0.67	0.0048*
Metal concentration	Chlorophyll	−0.05	0.8524	−0.61	0.0113*
Metal concentration	Carotenes	0.33	0.2525	−0.36	0.175
Metal concentration	Relative conductivity	−0.23	0.3991	0.11	0.6825
Metal concentration	MDA content	0.53	0.0339*	0.32	0.2257
Metal concentration	Soluble proteins content	0.33	0.2068	−0.58	0.0195*
Metal concentration	Proline content	0.6	0.0144*	0.66	0.0053*
Root Pearson correlation coefficient					
Metal concentration	Root dry weight	−0.8	0.0002*	−0.78	0.0003*
Metal concentration	Relative conductivity	0.63	0.0086*	0.93	<0.0001*
Metal concentration	MDA content	−0.28	0.3254	0.44	0.1188
Metal concentration	Soluble proteins content	−0.1	0.6989	−0.22	0.4279
Metal concentration	Proline content	−0.03	0.9212	0.28	0.3267

Note: *Asterisks indicate significant differences (*p < *0.05), and (*r*) is Pearson correlation coefficient.*

Table 2.
Zn(II) and Cu(II) bioaccumulation correlated with physiological effects by Pearson stastical method.

content and root-relative conductivity showed the opposite. For Cu(II), negative significant correlations were found for shoot dry weight, chlorophyll, and protein content while positive correlations were found for shoot proline content and root-relative conductivity. Positive correlations show an increase of both variables, whereas a negative correlation indicates a decrease in the second variable when the first variable increases (**Table 2**).

4. Discussion

4.1 Growth, physiological and biochemical parameters

Zinc is an essential trace element for normal plant growth. There are important enzymes that contain zinc, such as the enzyme alcohol dehydrogenase, carbonic anhydrase, ribonucleic acid (RNA) polymerase, and superoxide dismutase, a key enzyme in protection against oxidative stress. Zinc activates different enzymes responsible for the synthesis of certain proteins. It is involved in the formation of chlorophyll and some carbohydrates. It is essential in the formation of auxins, which help regulate stem development and elongation, in addition to being the precursor of tryptophan [14]. Copper also plays a key function in normal plant growth. For example, it participates in CO_2 assimilation and adenosine triphosphate (ATP) production [15]. It is the main constituent of diverse proteins such as plastocyanin of the photosynthetic system and cytochrome oxidase of the electron transport chain [16]. It plays a significant function in cell wall metabolism, signaling to the transcription protein trafficking apparatus, oxidative phosphorylation, iron armament, and biogenesis of

molybdenum cofactor [17]. Both are essential micronutrients necessary for the correct growth and development of plants; however, in high concentrations, they turn out to be phytotoxic, generating various negative metabolism modifications.

The results of our experiment indicate that some physiological and biochemical parameters of *C. indica* were significantly different at high Zn(II) and Cu(II) concentrations (**Figures 1-6**). The biomass decreased (both aerial part and root) for both metals (**Figure 1**), but only Cu(II) treatments showed a decline in the content of chlorophyll and carotenes (**Figure 2**). Root-relative conductivity (RC) increased with the Zn(II) and Cu(II) increasing concentrations (**Figure 3**), and the same occurred for the malondialdehyde (MDA) content in shoots with both metals, whereas, in roots, only Cu(II) treatments showed an increase (**Figure 4**). The soluble proteins content increased in the roots of the plants treated with Zn(II) but decreased in shoots of Cu (II)-treated plants. (**Figure 5**). For proline shoot content, a decline was shown in the lowest concentrations of both metals but increased at the highest concentrations while, in roots, increased only in the lowest concentration of Zn(II) but then decreased again to the levels of control treatment, showing no significant difference (**Figure 6**).

The decrease observed in the biomass of *C. indica* is highly reported in this and other species for zinc [18–20] and copper [21, 22] toxicity as one of the most obvious symptoms of plants growing in these conditions.

The biomass reduction related to Zn(II) toxicity is a consequence of mitosis inhibition that causes growth alterations product of the inhibition of deoxyribonucleic acid (DNA) synthesis [23]. Also could be the result of the alteration in macronutrient absorption [24] or the micronutrient distribution in different parts of the plant [25] such as lower uptake of Fe^{+2} and Fe^{+3}; modification of the metabolic activity [26], inhibition of cellular division in the meristematic region, lengthening of root cells [27], reduction of cell viability, and death in the root tips [28].

Additionally, copper excess generates reactive oxygen species, which causes oxidative stress [29] that disrupts numerous metabolic pathways and modifies essential macromolecules [30]. Also, high copper concentrations cause negative modifications to DNA, photosynthesis, cell membrane integrity, enzyme activity, and respiration leading to general growth reduction [31]. Excess of copper in the roots can trigger alterations in the root system design that causes growth reduction, bronzing, necrosis, and nutritional inequities [32, 33].

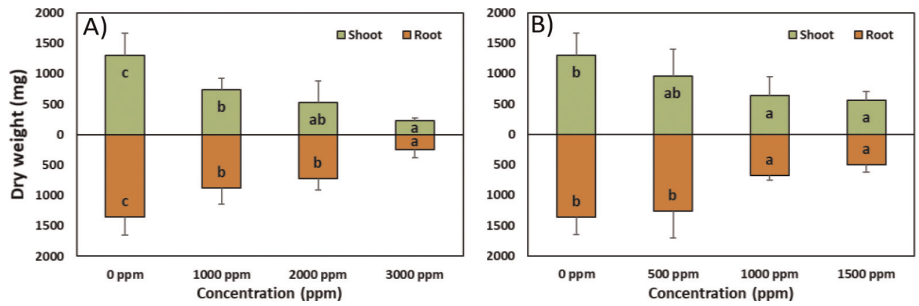


Figure 1.
Shoot and root dry weight (mg) of Canna indica *plants in Zn(II) (A) and Cu(II) (B) systems. Columns represent the mean* (n = 5)*, and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b-c) represent statistically significant differences* ($p < 0.05$)*, e.g., "a" is statistically different from "b" and "c", but not from "ab".*

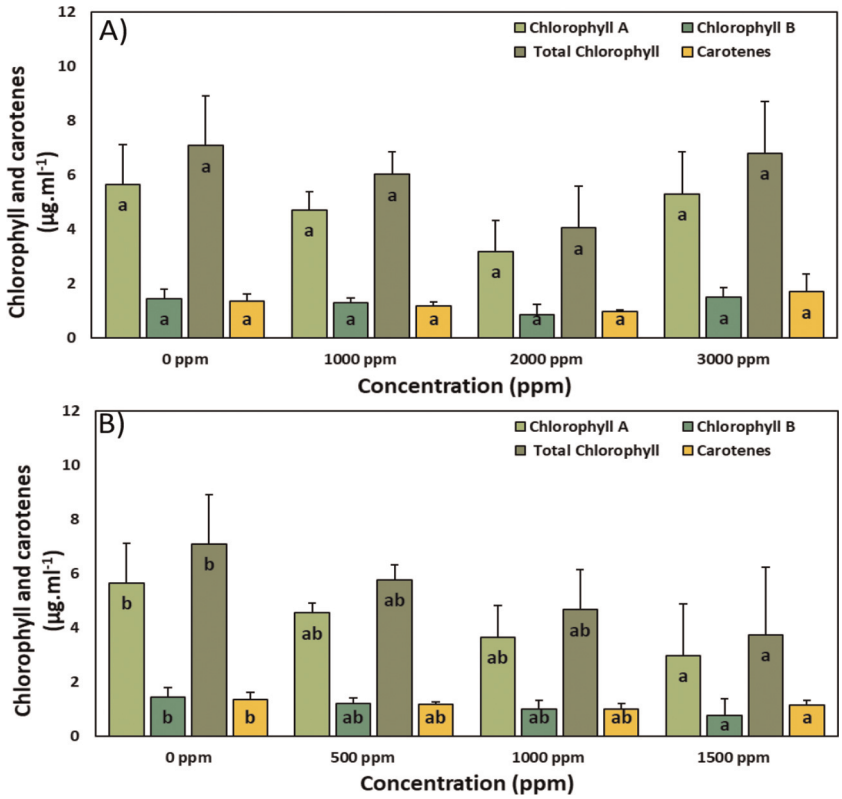


Figure 2.
Chlorophyll A, B, total and carotenes content of Canna indica *plant in Zn(II) (A) and Cu(II) (B) systems. Columns represent the mean* (n = 5)*, and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b) represent statistically significant differences* ($p < 0.05$)*, e.g., "a" is statistically different from "b", but not from "ab".*

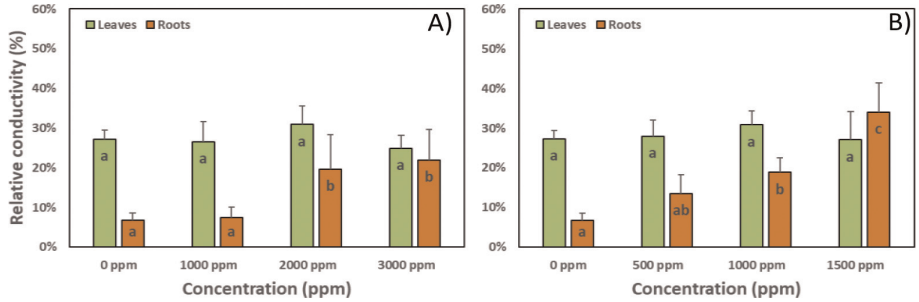


Figure 3.
Relative conductivity (RC) percentage (%) in roots and leaves biomass of Canna indica *plants in Zn(II) (A) and Cu(II) (B) systems. Columns represent the mean (n = 5), and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b-c) represent statistically significant differences* ($p < 0.05$)*, e.g., "a" is statistically different from "b" and "c", but not from "ab".*

Zinc helps to maintain membrane integrity, preserving the structural orientation of macromolecules and protecting the transportation systems [18], but in high concentrations, triggers reactions that promote oxidative stress and the breakdown of membrane integrity [24]. Similar behavior happens with copper excess, causing the

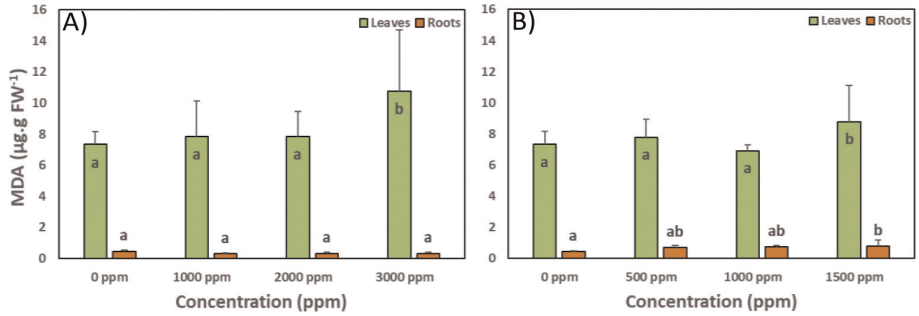


Figure 4.
Malondialdehyde (MDA) content in the roots and leaves of Canna indica *plant in Zn(II) (A) and Cu(II) (B) systems. Columns represent the mean (n=5), and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b) represent statistically significant differences* ($p < 0.05$)*, e.g., "a" is statistically different from "b", but not from "ab".*

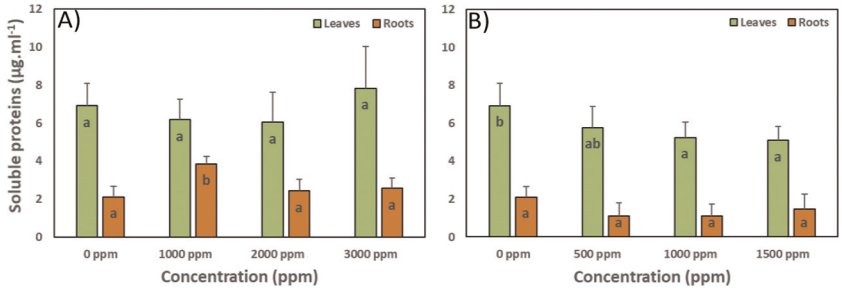


Figure 5.
Soluble protein content in the roots and leaves of Canna indica *plant in Zn(II) (A) and Cu(II) (B) systems. Columns represent the mean* ($n = 5$)*, and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b) represent statistically significant differences* ($p < 0.05$)*, e.g., "a" is statistically different from "b", but not from "ab".*

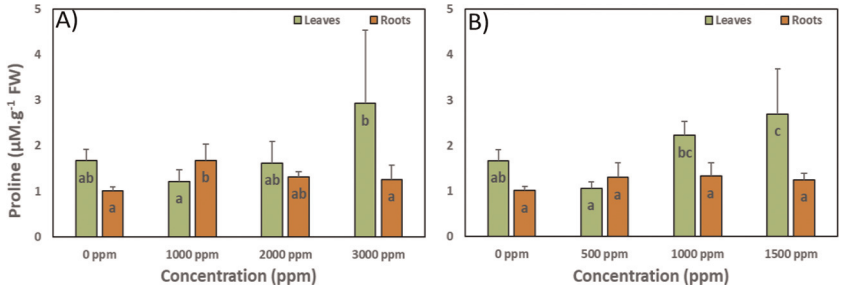


Figure 6.
Proline content in the roots and leaves of Canna indica *plants in Zn(II) (A) and Cu(II) (B) systems. Columns represent the mean* ($n = 5$)*, and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b-c) represent statistically significant differences* ($p < 0.05$)*, e.g., "b" is statistically different from "a" and "c", but not from "ab" and "bc".*

disruption of cell wall integrity and deposition of electron-dense material in the cytoplasmic membranes [34]. An increase in the relative conductivity (RC) of cellular membranes would indicate damage at the membrane level; higher values than 30%

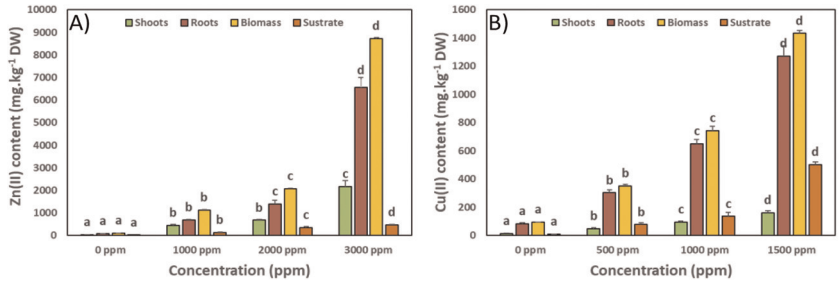


Figure 7.
(A) Zn(II) and (B) Cu(II) bioaccumulation in shoot, root, and total biomass of Canna indica *plants and heavy metal accumulation in substrate. Columns represent the mean* (n = 4)*, and vertical bars show the standard deviation (S.D.). Means followed by different letters (a-b-c-d) represent statistically significant differences* ($p < 0.05$)*, e.g., "a" is statistically different from "b", "c" and "d".*

indicate damage [35]. In this work, results show that RC significantly increased only in roots for both metals. However, the values obtained were relatively low, showing damage only in the highest concentrations. The degree of peroxidation of lipids and the degree of membrane damage are related and can be analyzed from the malondialdehyde (MDA) concentration and RC [36]. Increased levels of reactive oxygen species (ROS) caused by heavy metal stress could develop in damage to lipid membranes, proteins, pigments, and nucleic acids [37]. The malondialdehyde is a product of the lipid peroxidation of polyunsaturated fatty acids in cell membranes caused by oxidative stress and the production of ROS [35]. In this work, shoot MDA levels increased in the maximum concentration, in comparison to the control, for both metals, while in roots only copper treatments showed an increase in the maximum concentration. Also, this suggests that the antioxidant enzymes present in the roots of zinc treatments could have compensated the damage caused by ROS [38]. Similar results were found in different species such as *Salix fragilis* and *Salix aurita*, which showed an increase in the electrolytic leakage (similar parameter associated to relative conductivity) related to heavy metal concentrations [39], or *Canna orchioides*, which also showed an increase in the relative conductivity and MDA accumulation associated to this type of stress [40]. Metal-induced stress induces reactive oxygen species (ROS) generation, which can lead to lipid peroxidation, protein impairment, enzyme inactivation, and DNA damage [23]. Membrane disruption and lipid peroxidation are generally contemplated as dependable biomarkers of oxidative status in plants [24].

Another distinctive heavy metal toxicity symptom in plants is a reduction of the content of photosynthetic pigments [41]. They are directly related to photosynthesis and plant growth so, a decrease of the content of these pigments or damage done to chloroplasts results in lower CO_2 assimilation and a biomass decrease [42]. Carotenoids participate in antioxidant defense systems and impart a significant role in ROS sequestration [43], preventing the peroxidation of lipid membranes. [42]. Chloroplasts, mitochondria, and cellular membranes are some of the main sites that generate ROS. They are interconnected to the electron transport system, so when oxidative stress occurs, these sites are the first to be affected [44]. The decline in chlorophyll content in plants exposed to heavy metals stress is related to the inhibition of important enzymes, such as 6-aminolevulinic acid dehydratase (ALA-dehydratase) and protochlorophyllide reductase associated with chlorophyll biosynthesis, and the reduction of Mg^{+2} and Fe^{+2} supply. Zinc in phytotoxic concentrations may be equivalent to magnesium, causing processes of substitution of the central ion of the

tetrapyrrolic chlorophyll ring, inhibiting its function and decreasing its concentration [45]. Similar effects are caused by excessive copper concentrations. Photosynthetic pigments decrease might be the result of displacement of magnesium required for chlorophyll biosynthesis or ultra-structural alteration of chloroplast under metal toxicity [46]. Also, this reduction might be due to the inhibited activities of various enzymes associated with chlorophyll biosynthesis [47]. A similar effect was observed in the present work but only with statistical significance in copper-treated *C. indica* plants where a decrease in chlorophyll and carotene contents was observed with the increment of this metal. This can be associated with the smaller biomass and the increment of oxidative stress indicated by the increase of MDA contents found in the highest concentrations of copper. Similar diminution in chlorophyll and carotenes caused by copper excess was found in different species such as *Citrus aurantium* [48], *Phragmites australis* [49], *Lemna minor* [50], and *Camellia sinensis* [51].

Shoot-soluble protein content of *C. indica* plants decreased with the increase of copper concentrations concerning the control, whereas the opposite was found in the roots of the lowest zinc treatment. Similar results were found in *L. minor* [52] and *Hordeum vulgare* [53] treated with high concentrations of heavy metals. The decrease in the level of soluble proteins is another symptom characteristic of the stress caused by metals [54]. Proteins not only can act as metal chelators; they can also act in the movement toward the interior of the cell, for compartmentalization in vacuoles, as well as the exterior by an ion flow [55]. Therefore, the increase of the protein content observed in the zinc-treated *C. indica* roots might be due to a nutritional boost caused by the lowest zinc concentration. Also, biosynthesis of various biomolecules is another way to tolerate zinc excess; this process includes the induction of metallochaperones, proteins of low molecular weight, or chelators such as nicotianamine, putrescine, spermine, mugineic acid, organic acids, glutathione, phytochelatins, and specific metallothioneins, such as proline and histidine [56]. A similar increment was found in different poplar clones [57] and was associated with antioxidant enzymes synthesis during oxidative stress induced by heavy metals. On the contrary, in this work, shoot-soluble protein content decreased in copper-treated *C. indica* plants. A similar reduction was found in *Brassica napus* growing on copper excess [58]. This decrease may be due to ROS generation. ROS are likely to target proteins that contain sulfur-containing amino acids and thiol groups [59]. Proteins can also be damaged in oxidative conditions by their reactions with lipid peroxidation products [60], and it can result in the deleterious effect of the normal protein form by disrupting the pathways and protein synthesis [61].

Proline is an amino acid that helps in activating many physiological and molecular responses in stress conditions. Its accumulation is a widespread response to heavy metal stress [62]. Shoot proline content con *C. indica* in this work showed a tendency to increase with the increment of both metal concentrations, whereas for roots only an increment in the first concentration of zinc treatment was observed. Proline accumulation increases the tolerance to heavy metals through several mechanisms, such as osmoregulation, stabilization of protein synthesis, and enzyme protection against denaturation [63]. It is suggested that proline accumulation is triggered by ROS, which allows their direct detoxification without the intervention of antioxidant enzymes [64]. Oxidative stress can lead to lipid peroxidation that produces a disruption at the cellular level, especially plasma membrane and leaking potassium from the plant cell; exogenous proline applications suppress the heavy metal induces [65]. Several authors found an increment in proline content in different species growing in excessive zinc [66–68] and copper [69–71] concentrations.

4.2 Bioaccumulation of Zn(II) and Cu(II)

Heavy metals are inorganic pollutants that cannot be degraded, so the principal strategy for plants should be to immobilize them in their rhizosphere, accumulate them in the roots, or translocate them to the aerial part [72]. They enter the root either by crossing the plasma membrane of the root endodermal cells, by entering the root apoplast through the space between cells, or with the aid of membrane transporter proteins. These transporters are present in membranes of different organelles such as tonoplasts, endoplasmic reticulums, mitochondria, or chloroplasts [73]. Inside the plant, they can be chelated by glutathione (GSH), phytochelatins (PCs), or metallothioneins (MTs), chelators that have thiol (—SH) groups, which gives them a high affinity for metal cations [74]. Also, this process may work synergistically with secondary stress-defensive antioxidative systems to combat metal-induced oxidative stress [75]. Metals in roots can be stored in vacuoles, cell walls or exported to the shoot via the xylem. Vacuoles are considered the main storage site for metals in plant cells, being a part of the tolerance mechanism [76].

In general, plants can contain, in their total biomass, Zn(II) in ranges from 30 to 100 mg kg^{-1} dry weight (DW); concentrations higher than 300 mg kg^{-1} DW are considered phytotoxic [77], but for other authors, this limit is set at 100 mg kg^{-1} DW [78]. For Cu(II), normal total biomass content ranges from 2 to 50 mg kg^{-1} DW, depending on the plant species. However, 5–20 mg kg^{-1} DW seems to be optimal, as toxicity symptoms appear above and deficiency symptoms below this critical range [79]. In the present work, *C. indica* accumulated values higher than the limits considered phytotoxic, reaching up to 8723.99 ± 694.68 mg kg^{-1} DW for Zn(II) (±SD) and 1432.15 ± 91.13 mg kg^{-1} DW for Cu(II) (±SD) in the total biomass in the maximum tested concentrations. Numerous authors showed the capacity of Zn(II) and Cu(II) accumulation of *C. indica* growing on different substrates [80–82].

Indexes are calculated to determine the phytoextraction efficiency, mainly being the bioaccumulation index (BI) and the translocation index (TI) [83]. An effective phytoextraction process requires the translocation of metals to easily harvestable parts. Plants with BI values less than 1 are unsuitable for phytoextraction. In this work, *C. indica* indexes suggest that this plant could act as a phytostabilizer because it showed low translocation to the aerial part but a high accumulation of both metals in the roots. Under this type of stress, the root suffers the first exposure, limiting transmission of heavy metals to other tissues [84]. Many studies found the same for the *Canna* genus for different heavy metals [85–87].

4.3 Correlation between physiological and biochemical parameters and Zn(II) and Cu(II) bioaccumulation: indicators for different applications

Some associations between physiological and biochemical parameters and the exposition of metals can be estimated by Pearson's correlation coefficient (r). In this work, *C. indica* plants showed a significant negative correlation for shoot ($r = -0.74$) and root dry weight ($r = -0.8$) in Zn(II) treatments and shoot dry weight ($r = -0.67$), chlorophyll ($r = -0.61$) and protein ($r = -0.58$) content in Cu(II) treatments showing that when the concentration of this metals increases, these parameters are affected negatively. The opposite occurred for shoot MDA ($r = 0.53$) and proline ($r = 0.6$) content and root-relative conductivity ($r = 0.63$) in Zn(II) treatments and shoot proline content ($r = 0.66$) and roots-relative conductivity ($r = 0.93$) in Cu(II) treatments. Proline accumulation in shoots, relative conductivity increment in roots, and

the diminution of dry weight could be useful indicators of the strategies of this plant to overcome heavy metal stress and could be used to monitor the phytoremediation process.

The analysis of the correlation between metal accumulation and physiological parameters could be useful in different areas, such as variety selection, genetic improvement, environmental monitoring, or index construction as an indirect indicator of the phytoremediation process [88]. Various studies have demonstrated the correlation between metal accumulation and the antioxidant system. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), show an increased production to protect the plant from the damage caused by reactive oxygen species (ROS) under metals exposure [89]. Also, malondialdehyde (MDA) could act as an indicator of lipid peroxidation and is usually related to assessing oxidative damage [28]. Lipid peroxidation and oxidative damage cause alterations in metabolic processes [90] such as photosynthesis or protein productions leading to a decrease of photosynthetic pigments, less CO_2 assimilation, and diminution of biomass [91]. On the other hand, the accumulation of metabolites is another mechanism that plants use for stress tolerance. Proline is an amino acid that is involved in different stress mechanisms; it performs functions such as osmoregulation, stabilization of protein, and enzyme synthesis or even can chelate metal ions to help in the vacuolar sequestration [92]. These correlations are another way to demonstrate the tolerance mechanisms, and it helps to create comparations between species from the same genus or different cultivars to select the best for specific phytoremediation techniques becoming these, indicators of phytoremediation efficiency parallel to heavy metal accumulation [93].

Another use of these correlations is the construction of biomarkers. These represent the biological response to environmental disturbances or contamination, and they allow the detection of pollution at different contamination levels corresponding to concentrations difficult to achieve or when yield is not easy to form an integrative sample. There are three types of biomarkers: biomarkers of exposure: such as DNA breaks, stress proteins, and phytochelatins; biomarkers of effects such as morphological and physiological parameters; and biomarkers of susceptibility such as genetic mutations [94]. The use of such tools is currently increasing in the field of biomonitoring and bioremediation. Some biomarkers that have already been reported in plants are the following: oxidative stress by the production of reactive oxygen species [95], the reduction of macromorphological parameters such as plant height, stem diameter, and the number of leaves and negative modifications in chloroplasts with implications in photosynthesis [96]. These have been useful biomarkers for showing the adverse effects of metal exposition on the development, growth, and physiology of different plants exposed to this type of stress [97, 98].

5. Conclusion

Physiological and biochemical parameters are essential to understand the processes involved in the detoxification strategies employed by the plants during heavy metal stress. Some of them could be used as indirect indicators of the status of the phytoremediation process. In this work, *C. indica* plants could accumulate Zn(II) and Cu(II), mainly in roots. This affected some physiological and biochemical parameters due to the development of different physiological strategies, such as an increase of the antioxidant activity or the accumulation of proline, but these were not significant to

produce high negative modifications in the physiological apparatus. Pearson analysis showed some negative correlations such as dry weight and chlorophyll, but also some positive correlations such as MDA, proline concentration, and relative conductivity, which could be useful to understand the strategies employed by *C. indica* plants to overcome heavy metal stress.

The plant could grow without great problems, accumulating high concentrations of both metals so it could be used in phytoremediation programs as a phytostabilization species, and parameters such as proline content, relative conductivity, and dry weight could be used to monitor the phytoremediation process.

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