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BIOACCUMULATION AND PHYSIOLOGICAL RESPONSES OF *Festuca arundinacea* (Poaceae) TO ZN(II) EXCESS

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SUMMARY

There is evidence showing that *Festuca arundinacea* plants can absorb and accumulate high amounts of Zn(II), greater than those considered as phytotoxic, without affecting plant growth. In order determine the usefulness of this species as a phytoremediation plant, a pot experiment was carried out to measure the physiological strategies employed by *F. arundinacea* 'Malma' Schreb plants growing in environments with an excess of Zn(II). The plants were grown until reaching adequate biomass, when increasing concentrations of Zn(II) were applied. Three months later, growth parameters (total biomass and leaf area), physiological parameters (relative membrane conductivity, chlorophyll and carotene contents, malondialdehyde in roots, and soluble proteins) and Zn(II) content were determined. Total biomass, leaf area, chlorophyll, carotene and protein contents in the aerial part showed a decline with the increase of Zn(II) concentration, whereas the relative conductivity, the protein content and malondialdehyde in roots showed the opposite pattern. Our results suggest that this species can be used for the phytostabilization of polluted soils with moderate concentrations of Zn(II).

Key Words: *Festuca arundinacea*, zinc stress, physiological parameters, bioaccumulation, phytostabilization.

BIOACUMULACIÓN Y RESPUESTAS FISIOLÓGICAS DE *Festuca arundinacea* (Poaceae) AL EXCESO DE ZN(II)

RESUMEN

Existe evidencia que demuestra que las plantas de *Festuca arundinacea* pueden absorber y acumular altas cantidades de Zn(II), superiores a las consideradas fitotóxicas, sin afectar el crecimiento de las plantas. Para determinar la utilidad de esta especie como planta fitorremediadora, se realizó un experimento en macetas para determinar las estrategias fisiológicas empleadas por *F. arundinacea* 'Malma' Schreb, que crecen en ambientes con exceso de Zn(II) para determinar su utilidad como especie fitorremediadora. Las plantas crecieron hasta alcanzar una biomasa adecuada para la aplicación de tres concentraciones crecientes de Zn(II). Tres meses después se determinaron parámetros de crecimiento (biomasa total y área foliar), parámetros fisiológicos (conductividad relativa de la membrana, contenido de clorofila y carotenos, malondialdehído en raíces y proteínas solubles) y contenido de Zn(II). La biomasa total, área foliar, contenido de clorofila, caroteno y proteínas en la parte aérea mostraron una disminución con el aumento de la concentración de Zn(II), mientras que la conductividad relativa, contenido de proteínas y malondialdehído en las raíces mostraron un patrón opuesto. Nuestros resultados sugieren que esta especie puede ser utilizada para la fitoestabilización de suelos contaminados con concentraciones moderadas de Zn(II).

Palabras clave: F*estuca arundinacea,* estrés por zinc, parámetros fisiológicos, bioacumulación, fitoestabilización.

INTRODUCTION

In recent years, contamination by heavy metals has become one of the most critical environmental concerns affecting our planet. Heavy met als are non-biodegradable and, because of their persistence, they are considered primary pollutants. These compounds have been introduced into the ecosystem through several anthropic activities such as mining, industry and waste disposal (Kumar *et al.*, 2017). Some heavy metals may be bioaccumulated due to their lipophilic nature, which allows them to quickly enter the food chain, while others can alter metabolic pathways (fatty acid synthesis and elongation, carbon fixation, photorespiration, chlorophyll biosynthesis, etc.), causing severe problems to the ecosystem and eventually affecting human health (Zhong *et al.*, 2017).

Zinc [Zn(II)] is an essential microelement for normal plant growth. It forms part of photosynthetic processes, protein synthesis and regulation, and the maintenance of the root membrane integrity, among other functions (Cakmak, 2015). However, in high concentrations, it becomes phytotoxic, causing damage to the root system, reducing plant growth for inhibition of CO_2 fixation and carbohydrate transport in the phloem and altering of cellular membrane permeability (Sagardoy *et al*., 2009). Phytotoxicity caused by Zn(II) is very common even more than that caused by Cu, Ni, Co, Cd or others. Zn(II) can reach phytotoxic concentrations in many soils and water resources, like urban streams and rivers, due to anthropic contamination from various sources (fertilizers, pesticides, manure, sewage sludge, foundries, incinerators, mines, galvanized products) (Papaioannou *et al.*, 2019).

Festuca arundinacea Schreb. is a native grass of Europe and North Africa, widely used as forage in extensive livestock production systems located in mild climates, given its tolerance to various environments (Zhang *et al.*, 2019). Several works have evaluated its use for phytoremediation of soils contaminated with heavy metals and/or organic compounds, evidencing their ability to grow and accumulate various metals in their biomass (e.g., Albornoz *et al*., 2016; Desjardins *et al.*, 2016; Khashij *et al*., 2018). However, few phytoremediation reports describe the physiological and biochemical mechanisms involved during this stressful situation. It is necessary to know not only the ability to accumulate a specific metal, but also the mechanisms that allow stress relief (Kavuličová *et al*., 2012).

There are some studies on the effects of high concentrations of heavy metals in *F. arundinacea* and the harmful potential of Zn(II) in the soil. Therefore, this study aimed to evaluate, the impact of Zn(II) excess on the growth and metabolism of *F. arundinacea* 'Malma' through the determination of physiological parameters and Zn(II) bioaccumulation. Results obtained were compared with parameters measured and published in the scientific literature to provide information for future phytoremediation research. We hypothesize that *F. arundinacea* plants have different sensitivities to the availability of Zn(II) observing, in the highest concentrations, a variety of physiological responses that seek homeostasis.

MATERIALS AND METHODS

Growth conditions

Ten *F. arundinacea* seeds were superficially disinfected with NaClO (10%) for 5 min, flushed with sterilized water and sown in 500 cm^3 pots, containing a soil-sand mixture (1:1 v/v). Then, thinning was manually performed, leaving five plants per pot.

When plants presented two expanded leaves, 100 mL of Zn(II) sulfate solutions ($ZnSO_4.7H_2O$) were applied weekly per pot until reaching the concentrations to be tested. Zn(II) concentrations were chosen by carrying out a literature search (Khashij *et al.*, 2018; Albornoz *et al.*, 2016, Cakmak, 2015) and were estimated through concentrations per application, the approximate weight of the substrate in the pot and the number of applications (Table 1). In all cases, seven pots per treatment were used.

Plants were grown under controlled greenhouse conditions from September 4th to December 20th, in the city of La Plata (SL 34°55'17.2''-LW 57°57'16.3''). After 90 days from the first application, plants were harvested to perform the different determinations.

Table 1. Zn(II) concentrations for the different treatments.

Zn(II) solutions (µM)	Zn(II) per pot (mg kg^{-1})
0	0
500	234
1000	332
1500	430

Measurements performed

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 shoot from roots. Before dry weight measurements, leaf area was measured with a LICOR Li-3000 (USA) leaf area meter. Leaves were placed on a transparent belt where they were automatically conveyed across a scanning bed. A press roller flattened any curled edges to feed the leaves properly between the transparent belts before they entered a scanner that measured the area of individual sheets and quickly calculated the accumulated area for a group of leaves. After the measurement, leaves were ejected from the scanning bed.

Relative conductivity (RC) of leaf and root cellular membranes

RC of leaf and root cellular membranes is a technique based on the permeability increase of the cellular membrane and the diffusion of electrolytes outside the cells when a stressful condition damages the tissue. Four disks of 1 cm diameter of fresh leaf material and equivalent root weight (300 mg) were taken from the different treatments. Measurements were performed according to Lutts *et al.* (1996), determining the electrical conductivity (dS m^{-1}) with a Jenco conductivity meter, model 3173 (USA). The RC was calculated according to the following formula:

Equation 1 $$RC\ (\%) = (RC_i/RC_f) \times 100$$

where $\mathbf{RC_i}$ is the initial relative conductivity and $\mathbf{RC_f}$ is the final relative conductivity.

Malondialdehyde content (MDA)

For all treatments, MDA determination was carried out from 200 mg of fresh root material by reaction with thiobarbituric acid (TBA), according to Heath and Packer (1968) method, as a marker of lipid peroxidation of cellular membranes. The fresh tissue (FW) was ground and 5 mL of TBA 0.5% in 20% of trichloroacetic acid (TCA) was added. The mixture was heated at 95°C for 30 minutes, when it was rapidly cooled in a cold bath. Then, the mixture was centrifuged at 10000 g for 10 min, the supernatant was separated, and the absorbance was read at 532 and 600 nm in a Shimadzu UV 160 UV/V spectrophotometer (Japan). The MDA concentration was calculated according to the following formula using a molar extinction coefficient of 155 $mM^{-1}\ cm^{-1}$:

Equation 2 $$\text{MDA equivalents (nmolgFW}^{-1}) = [(A532-A600)\ /155000] \times 10^6$$

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 according to Wellburn (1994) with a Shimadzu UV 160-A spectrophotometer (Kyoto, Japan). The results were expressed in µg of chlorophyll mL^{-1} and µg of carotenoids mL^{-1}.

Soluble proteins content

For all treatments, the content of soluble proteins was determined from 100 mg of fresh leaves and root material, employing the method described by Bradford (1976). The protein content calculation was carried out using a standard curve prepared with different concentrations of bovine serum albumin (BSA) (SiFMa Chemical Co.).

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

The content of Zn(II) was determined following the method proposed by Bonfranceschi *et al.* (2009), by causing acid digestion of 500 mg of aerial part, 500 mg of root and 1 g of substrate. Then, the absorbance was read using an atomic absorption spectrophotometer (Shimadzu AA6650F Atomic Absorption Spectrophotometer, Japan) with a limit of detection and quantitation of 0.005 mg L^{-1} and 0.015 mg L^{-1} for Zn(II), respectively. 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.

- Bioavailability index [BAI; mg Zn(II) kg^{-1} in root/ mg Zn(II) kg^{-1} in the substrate] indicates if the metal is extracted and accumulated in the root.
- Accumulation index [AI; mg Zn(II) kg^{-1} in aerial part/mg Zn(II) kg^{-1} of substrate] indicates if the metal is extracted and accumulated in the aerial part.
- Translocation index [TI; mg Zn(II) kg^{-1} in aerial part/mg Zn(II) kg^{-1} in root], indicates if the metal is translocated to the aerial part.
- Bioaccumulation index [BI; mg Zn(II) kg^{-1} in aerial part/mg Zn(II) kg^{-1} in root], indicates if the metal is accumulated in the biomass.

Experimental design and data analysis

The experimental design was fully randomized. The data were subjected to analysis of variance and the means compared by the 5% LSD test (InfoStat version 2018).

RESULTS

Growth and physiological parameters

A negative effect on growth was found, expressed in a decrease in total biomass (Figure 1). This result varied approximately 25% between the control (0 µM) and the maximum concentration of Zn(II) (1500 µM). The dry weight of the root and the aerial part decreased by 19% and 27%, respectively, and they were significantly lower for 1500 µM of Zn(II) than for 500 and 0 µM of Zn(II) (Figure 1a).

A reduction of the leaf area towards the highest Zn(II) concentrations was observed, finding significant differences in the concentration of 1500 µM Zn(II) with a decrease of 55% compared with the control (Figure 1b).

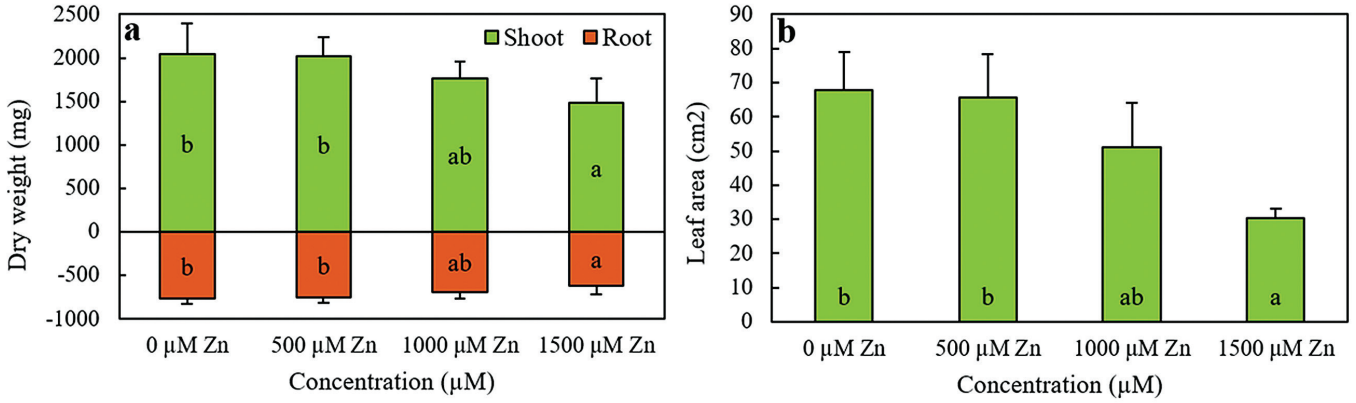

Figure 1. (a) Shoot and root dry weight (mg) of *F. arundinacea* plants obtained for the different concentrations of Zn(II). **(b)** Leaf area (cm^2) of *F. arundinacea* plants obtained for the different concentrations of Zn(II). Columns represent the mean (n=4), and vertical bars show the standard deviation. Different letters indicate significant differences ($p < 0.05$)

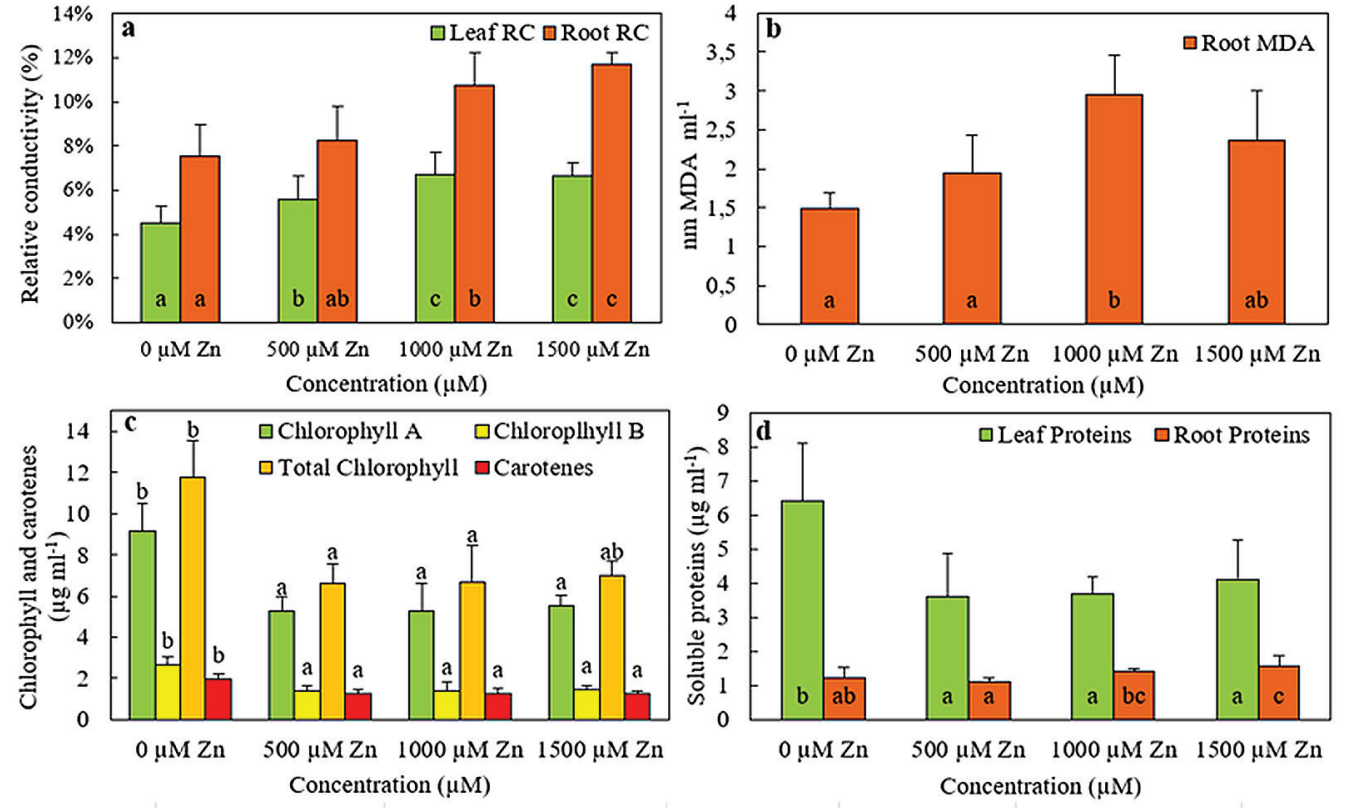

Figure 2. (a) Relative conductivity of roots and leaves cell membranes, **(b)** MDA content in the roots, **(c)** content of chlorophyll A, B, total and carotenes, and **(d)** content of soluble proteins in leaves and roots of *F. arundinacea* plants obtained for the different concentrations of Zn(II). Columns represent the mean (n=4), and vertical bars show the standard deviation. Different letters indicate significant differences ($p < 0.05$).

Although RC values were low, a gradual increase in the values for both roots and leaves was observed with the increase of metal concentration (Figure 2a). In all cases, higher values of RC were obtained in roots than in leaves, with differences of approximately 6% in the maximum concentration. In leaves, significant differences were observed among the control and all the metal concentrations, while in roots the significant differences were observed for 1000 µM and 1500 µM.

Zn(II) treatments increased root MDA levels (e.g., malondialdehyde content), finding significant differences when the metal concentration was 1000 µM (Figure 2b). There was a decrease in the MDA content in the 1500 µM concentration, but it was not statistically significant.

A significant decrease of chlorophyll and carotenes concentration was observed in all Zn(II) treatments in relation with the control (Figure 2c). This difference was approximately 50% and 60% for chlorophyll and carotenes content respectively. However, no significant differences were detected among Zn(II) treatments.

The application of Zn(II) determined a decrease by about 50% of the soluble protein content in leaves in relation to the control (Figure 2d). However, the protein content in the leaves did not differ significantly differences among the different Zn(II) concentrations (Figure 2d). In the roots, the protein content slightly increased towards the highest Zn(II) concentrations, being significantly greater for the highest concentration of 1500 µM.

Bioaccumulation of Zn(II)

The concentration of Zn(II) in the biomass of *F. arundinacea* plants significantly increased as the concentrations applied increased (Figure 3). For example, for a dose of 1500 µM, the amount of Zn(II) accumulated in the biomass was 482±19 mg Zn(II) kg^{-1} DW (±SD), being almost five times higher than the control. The values in the substrate were four times lower than those obtained in the biomass, denoting the absorption capacity of the plant. Concentrations of 500 and 1000 µM determined a higher accumulation of Zn(II) in the root than in the aerial part (shoot), with an increase of approximately 28% and 43%, respectively. On the other hand, the bioavailability (BAI), accumulation (AI), translocation (TI) and bioaccumulation (BI) indexes confirmed that mentioned above (Table 2). The values of BAI, AI and BI higher than 1 indicate that the plants were efficient in extracting the metal from the substrate, accumulating the largest amount of Zn(II) in the aerial part, as shown by TI values, which were lower than 1 (Table 2).

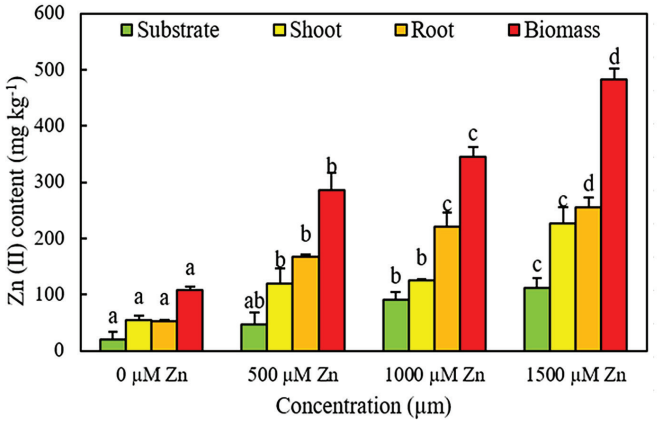

Figure 3. Zn(II) content in the substrate, shoot, root and total biomass of *F. arundinacea* plants for the different concentrations of Zn(II). Columns represent the mean (n=4), and vertical bars show the standard deviation. Different letters indicate significant differences ($p < 0.05$)

DISCUSSION

Growth and physiological parameters

The results obtained in this work indicate that the physiological and biochemical parameters of *F. arundinacea* were significantly different at high Zn(II) concentrations (Figures 1, 2 and 3). A decrease in biomass (aerial part and root) and leaf area (Figure 1), soluble proteins in the aerial part, the content of chlorophyll and carotenes (Figures 2c and 2d) was generally observed, whereas the values MDA in the root, the soluble proteins in root and the RC (aerial part and root) (Figures 2a, 2b

Table 2. Zn(II) accumulation indexes (mean ± SD) obtained for each Zn(II) concentration: BAI (bioavailability), AI (accumulation), TT (translocation) and BI (bioaccumulation).

Treatment	BAI (Root/Substrate)	AI (Shoot/Substrate)	TI (Shoot/Root)	BI (Biomass/Substrate)
0 µM	2.57 ± 0.48	2.58 ± 0.43	1.03 ± 0.34	5.16 ± 0.68
500 µM	4.46 ± 1.22	2.31 ± 0.71	0.65 ± 0.24	6.78 ± 0.74
1000 µM	3.29 ± 1.13	1.77 ± 0.79	0.53 ± 0.08	5.07 ± 1.1
1500 µM	2.28 ± 0.12	2.03 ± 0.26	0.89 ± 0.11	4.31 ± 0.32

and 2d) showed significant increments. Similar results were obtained for other species, such as *Lolium perenne* (Zamani *et al.*, 2015), *Brassica juncea* (Chaudhry *et al*., 2020), *Thlaspi caerulescens* (Bayçu *et al.*, 2017), *Hydrilla verticullata* (Xu *et al.*, 2013), *Triticum aestivum* (Glińska *et al*., 2016), *Camelia sinesis* (Mukhopadhyay *et al.*, 2013), *Zea mays* (Hosseini and Poorakbar, 2013), *Juncus acutus* (Mateos-Naranjo *et al*., 2014), *Pyllostachys edulis* (Peng *et al.*, 2015), *Lemma minor* (Radić *et al*., 2010), and *Solanum lycopersicum* (Cherif *et al*., 2011).

The decrease observed in the biomass and leaf area of *F. arundinacea* plants could result from modifications in the metabolic activity (Sidhu *et al*., 2020), inhibition of cellular division in the meristematic region, lengthening of root cells (Glińska *et al*., 2016), alteration in macronutrient absorption (Kaya *et al*., 2018) or the micronutrient distribution in different parts of the plant (Sidhu, 2016). Li *et al.* (2012) reported that the aerial part is more susceptible to high metal concentrations despite having lower contents than roots since they could act as a barrier to prevent an excess of accumulation in shoots. Roots are the first organs to come into contact with metals, and they are able to develop strong defense mechanisms against stress (Jan and Parray, 2016).

Zn(II) plays an essential role in maintaining membrane integrity, preserving the structural orientation of macromolecules and protecting the transportation systems of ions (Dang *et al*., 2010). In high concentrations, Zn(II) triggers reactions that promote oxidative stress and the breakdown of this integrity (Tsonev and Lidon, 2012). An increase in the relative conductivity (RC) of cellular membranes would indicate damage at membrane level; higher values than 30% indicate damage (Ruscitti *et al.*, 2017). In the present work, results show that RC significantly increased both in leaves and roots (Figure 2a). However, the values obtained were relatively low, which suggests the concentrations of Zn(II) employed here caused little damage.

In stress situations, the levels of reactive oxygen species (ROS) can significantly rise, causing damage to lipid membranes, proteins, pigments and nucleic acids (Sosa-Torres *et al.*, 2015). 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 (Ruscitti *et al*., 2017). In this work, MDA levels were low in comparison to the control (e.g., 0 µM of Zn(II); Figure 2b), suggesting that the antioxidant enzymes could have compensated for the damage caused by ROS (Jan and Parray, 2016).

The first and distinctive toxicity symptom of Zn(II) in plants is a reduction of the content of photosynthetic pigments (Marichali *et al*., 2014). Their levels are directly related to photosynthesis and plant growth, which explains why a decrease of the content of this pigment or damage done to chloroplasts results in lower CO_2 assimilation, causing a biomass decrease (Laidinen *et al.*, 2018). Carotenes are important components for the antioxidant system, acting as a defense against ROS (Laidinen *et al.*, 2018). The main sites generating ROS are chloroplasts, mitochondria and cellular membrane, which are interconnected to the electron transport system, so when oxidative stress occurs, these sites are the first to be affected (Gill and Tujeta, 2010). Zn(II) in phytotoxic concentrations may be equivalent to Mg, causing processes of substitution of the central ion of the tetrapyrrolic chlorophyll ring, inhibiting its function and decreasing its concentration (Bechaieb *et al.*, 2016). In tune, an effect observed in the present work was a decrease in chlorophyll and carotene contents (Figure 2c), which was associated with to the smaller biomass and leaf area in the highest Zn(II) concentrations and the increment of oxidative stress indicated by the increase of MDA and RC contents (Figures 1 and 2).

On the other hand, the level of soluble proteins in *F. arundinacea* was determined (Figure 2d). A significant decrease in the soluble protein content was observed in the aerial part concerning the control, whereas the opposite was found in roots (Figure 2d). The decrease in the level of soluble proteins is another symptom characteristic of the stress caused by metals (Radić *et al*., 2010). Zn(II) excess may induce the alteration of several mechanisms associated with protein formation and reduce the amino acid incorporation (Sidhu, 2016). Proteins not only act as metal chelators; they can also act in the movement towards the interior of the cell, for compartmentalization in vacuoles, as well as the exterior by an ion flow. Therefore, the increase of the protein content observed in the roots might be due to variations in the synthesis of resistance proteins highlighting the heavy metal pumps driven by ATP (CPx-ATPases), natural resistance-associated macrophage proteins (NRAMPs), facilitators of cation diffusion (CDF) and ZIP proteins (Bouain *et al*., 2014). Biosynthesis of various biomolecules is another way to tolerate or neutralize toxicity by Zn(II) 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 (Emamverdian *et al*., 2015).

Bioaccumulation of Zn(II)

In the present work, *F. arundinacea* accumulated values higher than the limits considered phytotoxic, reaching up to 482±19 mg kg^{-1} DW of Zn(II) (±SD) in the maximum tested concentration (Figure 3). In general, plants can contain Zn(II) in ranges from 30 to 100 mg kg^{-1} DW; concentrations higher than 300 mg kg^{-1} DW are considered phytotoxic (Bharagava *et al.*, 2020). Some species are capable of accumulating higher amounts of Zn(II) in their biomass (hyperaccumulators) exceeding 50-500 times the values considered phytotoxic, such as *Thlaspi caerulescens* and *Cardaminopsis halleri*, both Zn(II) hyperaccumulators (Jan and Parray, 2016). *F. arundinacea* presents an intermediate behavior between those plants that tolerate values higher than the phytotoxicity threshold and those plants that are considered hyperaccumulators. Genetic variability can also occur even within the same species, naturally generating new varieties that are highly resistant to metals (Sarma, 2011). Different works have reported data on the ability of *F. arundinacea* plants to accumulate Zn(II), showing variability in their responsiveness. For example, Cao *et al.* (2004) found that *F. arundinacea* plants accumulated by about 6000-9000 mg kg^{-1} DW of Zn(II) in the aerial part and root, respectively, when growing in a mine soil contaminated. Zamani *et al.* (2015) reported values of 432 and 1099 mg kg^{-1} of Zn(II) in leaf and root, respectively for *F. arundinacea* plants that were inoculated with endophyte *Neotyphodium* and grown in a substrate containing 1800 mg kg^{-1} of Zn(II). The existence of phenotypic variability and plasticity in the ability of *F. arundinacea* to accumulate Zn(II) depending on the environmental context is interesting for the selection of more efficient biotypes for phytoremediation.

Since Zn(II) is an inorganic pollutant that cannot be degraded, plants for phytoremediation must immobilize pollutants in their rhizosphere, accumulate them in the roots or translocate them to the aerial part (Saxena *et al*., 2020). The phytoextraction efficiency can be determined through indexes depending mainly on the bioaccumulation index (BI) and the translocation index (TI) (Antoniadis *et al.*, 2017). In this work, these indexes indicate that *F. arundinacea* plants accumulate Zn(II) in its total biomass, but with a low translocation to the aerial part in the first two concentrations, reaching values close to 1 in the maximum concentration (Table 2). Therefore, these data suggest that *F. arundinacea* plants could act as a phytostabilizer. Many studies have shown that *F. arundinacea* plants have roots with a high capacity for retaining metals, presenting a TI generally lower than 1 (Khashij *et al*., 2018). However, the amount of metal that plants can accumulate is highly variable. This depends, not only on the environmental or external plant conditions such as pH, organic matter content, associated microorganisms, quantity and bioavailability of the metal and competitive cations, but also on intrinsic plant characteristics (Singh *et al*., 2019). Even though in this work the concentrations found in *F. arundinacea* biomass were lower than those reported by the previously mentioned works, the same accumulation pattern was observed: a greater Zn(II) amount in root and less in leaves.

CONCLUSION

Festuca arundinacea can accumulate Zn(II), mainly in roots. Zn(II) excess causes a decrease of photosynthetic pigments content; however, festuca plants can bioaccumulate Zn(II) above the limit considered phytotoxic, due to the development of different physiological strategies, such as an increase of the antioxidant activity correlated with the MDA content, and protein synthesis in the root that would act as complexing agents, chelators and carriers of ions towards the interior of cells, for their compartmentalization in vacuoles, or towards the exterior as a method of elimination.

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