

Different strategies to deal with the same issue: the case study of four Brazilian native grasses exposed to an excess of Zn availability.

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Abstract

Introduction

The successive applications of fungicides containing zinc (Zn) in vineyards increase the levels of Zn contents in soils, contributing to the reduction of productivity in vineyards in tropical and subtropical regions. Thus, it is necessary to establish sustainable strategies to decrease the toxicity potential of Zn. The selection of tolerant native plants from the Pampa biome present in vineyards can reduce Zn toxicity in vines.

Objective

The study aimed to evaluate, considering physiological variables, the grass species native to the Pampa biome that are more tolerant to Zn excess.

Methods

The grass species of the Pampa biome used in the experiment were *Andropogon lateralis*, *Axonopus affinis*, *Paspalum plicatulum* and *Paspalum notatum*. Three Zn doses were added to the solution, corresponding to 2 μ M (original concentration of the nutrient solution), 150 and 300 μ M of Zn in the form of ZnCl_2 for the cultivation of the four grasses.

Results

The increase in Zn availability increased the photosynthetic rate in the four species. *Andropogon lateralis* increased shoot and root dry matter production with increasing Zn concentration in solution. *Andropogon lateralis* increased phosphorus (P) retention in the root system, contributing to the increase of Zn in the roots.

Conclusions

Axonopus affinis, *Paspalum notatum*, *Paspalum plicatulum* and *Andropogon lateralis* presented different values of growth rate, nutritional and photosynthetic parameters, and enzymatic activity, when cultivated in solution with Zn doses.

1. Introduction

Industrial, agricultural, urban and mining activities have very often contributed to the zinc (Zn) contamination of soils worldwide (Kan et al. 2021; Mandic-Rajcevic et al. 2021). This element is an essential micronutrient for plants, acting as a catalyst for the synthesis of phytohormones (e.g. auxins), stabilizing the structure of ribosomes and RNA, regulating cell division, photosynthesis reactions and enzyme activation (Lukhovitskaya et al. 2013; Andrejic et al. 2018). However, when present at excessive available levels, it affects the acquisition and transport of other essential ions as well as their homeostasis processes, chlorophyll biosynthesis and photosynthetic efficiency favoring, on the other hand, the production of reactive oxygen species (ROS) and the consequent lipid peroxidation (Phetchuay et al. 2019). When these aspects are considered in relation to the crops it is evident that the Zn toxicity can seriously compromise not only the productivity of the cultivated species (worsening also the qualitative traits) but even the possibility of completing the vegetative cycle of these plants. In this respect, considering the major challenge of the primary production system to feed a constantly growing population, disposing of the largest possible agricultural surface area for food production has an extremely high priority. In

this respect, the recovery for agricultural uses of all those soils presenting a too high availability of Zn appears extremely functional. To this purpose, it becomes strategic to set up practices for decreasing the metal availability preserving while maintaining soil fertility. The approach that best allows this compromise is phytoremediation based on the use of plants for a soft removal of the excess metal or of a generic pollutant (Sidhu et al. 2020). Regarding metals, there are two options for this approach: (1) phytoextraction: use of plants to extract metals from the contaminated soil and to accumulate it in the shoot which is then removed from the site (Remigio et al. 2020); (2), phytostabilization: precipitation, complexation or change in the valence of metals in the rhizosphere, thus, limiting their bioavailability for crops (Nedjimi 2021). In this latter option the metal acquisition by roots of a cover crop/a secondary plant species, limiting thus the metal availability in the rhizosphere for the crop, is also often included.

It is well known that the effectiveness of phytoremediation depends tightly on the plant species used (Saleem et al. 2020). In fact, those species able to survive in contaminated soil completing their vital cycle generally express tolerance mechanisms such as for example an efficient metal exclusion in the rhizosphere, a marked restraint of the metal allocation in shoots, an enhanced production of antioxidant enzymes, a metal compartmentation within the single cell and its complexation with ligands reducing the levels of the free active forms (DalCorso et al. 2019). It is interesting to note that several grass species have been used in the phytoremediation since they promote better soil protection mainly thanks to the large root system that they are able to develop and to the abundant production of shoot biomass (Saleem et al. 2020). It is well known that both these properties are crucial for a more effective removal of the metal from the soil. In South American countries, where the problem of copper (Cu) toxicity is especially severe due to the acidic nature of their soils, the Pampa with its about 450 native grasses (Rubert et al. 2019) represents a reserve of genetic resources of extreme interest. However, few studies have evaluated the potential of Pampa native grass species in Zn phytoremediation processes considering in particular the levels of metal acquisition and accumulation in plant tissues as well as the physiological and biochemical responses to the Zn excess. The study aimed to evaluate, physiological point of view, the native grass species of the Pampa biome most tolerant species to excess Zn.

2. Materials and methods

2.1. Selection of plants

The plants were collected in an area of the Pampa biome, in the Campanha Gaúcha region (30°46'36"S, 55°22'03"W), in Rio Grande do Sul State, South of Brazil. The soil was Typic Hapludalf and presented the following chemical characteristics: Zn (Mehlich-1): 23 mg kg⁻¹; Zn (USEPA): 20 mg kg⁻¹; Organic matter (Walkley-Black): 10 g kg⁻¹; pH (in H₂O): 6.4; Clay: 7 g kg⁻¹; P (Mehlich-1): 32,8 mg kg⁻¹; K (Mehlich-1): 56 mg kg⁻¹.

Sampling of the botanical composition was performed according to Botanal method (Tothill et al. 1992). The plants were identified to species level as *Andropogon lateralis*, *Axonopus affinis*, *Paspalum plicatulum* e *Paspalum notatum*. All these are common species in the native fields of the Pampa biome, located Southern Brazil state, Rio Grande do Sul. After plants collection, the tillers were separated, washed, and planted in plastic trays (15 liters capacity, inclined at a 20° angle to avoid solution accumulation), containing sand as substrate in a greenhouse. In the standardization process, through cuts, the seedlings remained with three roots and three leaves completely expanded per plant. The seedling preparation was repeated once a month, for seven months, in a greenhouse, allowing greater uniformity among individuals. The sand trays were irrigated daily with a complete nutrient

solution containing (in mg L⁻¹): 149.80 NO₃⁻; 24.80 H₂PO₄⁻; 39.27 SO₄²⁻; 41.31 Mg²⁺; 288.72 Ca²⁺; 234.60 K⁺; 0.03 Mo; 0.26 B; 0.06 Cu²⁺; 0.50 Mn²⁺; 0.22 Zn²⁺ e 4 Fe²⁺.

2.2. Plant cultivation and experimental design

The seedlings were removed from the pre-culture trays in sand, standardized with three roots and three fully expanded leaves, washed with distilled water and then placed in plastic containers. The specimens were cultivated in an aerated hydroponic cultivation system. Each container contained 5 L of nutrient solution, with the following nutrient concentration (mg L⁻¹): 85.31 N; 3.77 P; 11.54 S; 97.64 Ca; 23.68 Mg; 104.75 K; 181.08 Cl; 0.27 B; 0.05 Mo; 0.01 Ni; 0.13 Zn; 0.03 Cu; 0.11 Mn and 2.68 Fe. Five seedlings were added to each container. The seedlings were fixed by PVC sponges in styrofoam plates placed on the nutrient solution, constantly aerated with the aid of a compressor. (Chiaperini, CJ40 AP3V 360L, Brazil). After 14 days of acclimatization, the seedlings were submitted to three different concentrations of Zn, in form of zinc chloride (ZnCl₂).

The experimental design was completely randomized in a factorial 3 x 4 with four replications. Three Zn doses were added to the solution, corresponding to 2 µM (original concentration of the nutrient solution), 150 and 300 µM of Zn in the form of ZnCl₂ for the cultivation of the four grass species (*A. lateralis*, *A. affinis*, *P. plicatulum* e *P. notatum*). The nutrient solution was replaced every seven days and the pH was maintained at 5.5.

2.3. Physiological parameters and photosynthetic pigments

Gas exchange measurements linked to photosynthesis were evaluated at 40 days after transplanting the plants. The gas exchange of leaves was determined using a radiation of 1500 µmol m⁻² s⁻¹ and CO₂ concentration of 400 µmol mol⁻¹, using an infrared gas analyzer [Mod. Li-COR® 6400 XT]. Measurements were performed during the period from 8:00 am to 11:00 am in the penultimate fully expanded leaf of three tillers per experimental unit. The variables analyzed were net photosynthetic rate (A), intercellular CO₂ concentration (Ci), transpiration rate (E), stomatal conductance (Gs), water use efficiency (WUE), obtained by the relationship between the net photosynthetic rate and the amount of water transpired, and the instantaneous carboxylation efficiency of Rubisco (A/Ci), obtained by the relationship between the amount net photosynthetic rate and the intercellular concentration of CO₂.

At that moment, the same leaf was collected, immediately frozen in liquid N₂ and stored at -80°C. The determination of the concentration of chlorophylls and carotenoids followed the method of Hiscox and Israelstam (1979) and calculated according to Lichtenthaler (1987). For analyses, 0.05 g of frozen material was used. Fresh leaf samples were incubated at 65°C in dimethylsulfoxide (DMSO) until the pigments were completely removed from the tissue. For the determination of chlorophyll a, chlorophyll b and carotenoids, the absorbance of the solution was determined in a spectrophotometer (Bel Photonics, 1105, Brazil), at wavelengths of 663, 645 and 470 nm, respectively. Pigment concentration was expressed in mg g⁻¹ fresh matter (MF).

2.4. Oxidative stress enzyme activity

For the evaluation of superoxide dismutase (SOD) and guaiacol peroxidase (POD) activities, the crude enzymatic extract obtained from 0.5 g of frozen samples was homogenized in 3 mL of 0.05 M sodium phosphate buffer solution (pH 7.8), containing 1 mM EDTA and 0.5% Triton X-100. The homogenate was centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was used to enzymatic activity assay and protein content (Zhu et al., 2004; Bradford, 1976). The total soluble protein concentration of each sample was determined in a spectrophotometer at

595 nm as described by Bradford (1976). SOD activity was determined according to the spectrophotometric method described by Giannopolitis and Ries (1977), while POD activity was determined according to Zeraik et al. (2008).

Hydrogen peroxide (H_2O_2) was determined according to Loreto and Velikova (2001), and 0.3 g of roots and shoots were homogenized in 2.0 mL of 0.1% trichloroacetic acid (TCA) (w/v). Subsequently, the homogenate was centrifuged at 12,000 rpm for 15 min at 4°C and 0.5 ml of the supernatant was added to 0.5 ml of potassium phosphate buffer (10 mM) (pH 7.0) and 1 ml of KI (1M). Absorbance readings were performed in a spectrophotometer (Bel Photonics, 1105, Brazil) at 390 nm. Thiobarbituric acid reactive substances (TBARS) in shoots and roots were estimated using the methodology proposed by El-Moshaty et al. (1993). For this, cell damage was determined through the accumulation of malondialdehyde (MDA) as the end product of lipid peroxidation. Lipid peroxidation was expressed as nmol MDA mg^{-1} protein.

2.5. Root biometric parameters and leaf area measurements

The plants were harvested 40 days after transplantation; roots were transferred into a transparent tray containing distilled water and separated carefully from one other by hand. The root images of individual plants were obtained by Imagery Scan Screen (EPSON XL11000). The average diameter, volume, surface area, and total length of the roots of individual plants were measured and simultaneously analyzed using the WinRHIZO Pro image analysis system (Régent Instr. Inc.).

2.6. Plant growth and chemical elements

At 40 days after transplantation, the plants were collected to determine the dry matter production of the shoot and roots and the levels of P, K, Ca, Mg, Cu, Zn and Fe in the shoot. Root and shoot were separated and dried in a forced-air oven at 65° until reaching constant mass.

Samples of dried roots and aerial parts of the plants were ground in a Willey mill and passed through a 2 mm mesh sieve. The tissue was subjected to nitro-perchloric digestion (Embrapa 2017). The concentration of P, Mg, K, Ca, Zn, Cu Fe and Mn in was determined the digested extract in an atomic absorption spectrophotometer (Perkin Elmer, AAnalyst 200, United States). The P concentration was determined by colorimetry, according to Murphy and Riley (1962), in a spectrophotometer (SF325NM, Bel Engineering, Italy). The shoot translocation index (Eq. 1) and Zn bioaccumulation factor (Eq. 2) were calculated as below:

$$\text{Equation 1: TI} = [Zn]_{\text{aerial part}} / [Zn]_{\text{root}}$$

$$\text{Equation 2: BAF} = [Zn]_{\text{aerial part}} / [Zn]_{\text{solution}}$$

2.7. Statistical analysis

The data were submitted to analysis of variance (ANOVA), considering the variables observed for each plant species, and the treatment means were compared by the Scott-Knott test at 5% error probability, using the statistical program SISVAR software, version 4.0. In addition to the analysis of variance, an exploratory multivariate of principal components analysis (PCA) was performed, using the Canoco software version 4.5. The PCA was designed from the data on the concentration of elements in roots and shoots (P, K, Ca, Mg, Cu, Zn, Fe and Mn), plant growth parameters (root dry mass, shoot and total), photosynthetic parameters (chlorophyll a, b and total, carotenoids, net photosynthesis rate, stomatal conductance, intercellular CO_2 concentration, transpiration

rate, water use efficiency, instantaneous carboxylation efficiency of Rubisco), root morphology (average diameter, volume, specific surface area and length) and oxidative stress (POD, SOD, H₂O₂, TBARS).

3. Results

3.1. Growth and nutrient contents of the plant shoots

The highest Zn concentration (300 $\mu\text{Mol L}^{-1}$) reduced the dry mass of shoots of *P. notatum* and *P. plicatulum* on average of 19% with respect to the values measured in the treatment with 2 $\mu\text{Mol L}^{-1}$ (Fig. 1A). In *A. affinis* and *A. lateralis*, the increase in concentrations did not reduce the dry mass of the plants ($p < 0.05$); on the other hand, in *A. lateralis* there was an increase of 0.9 g of dry mass ($p < 0.05$) in 300 $\mu\text{Mol L}^{-1}$ in relation to that measured in plants fed with 2 $\mu\text{Mol L}^{-1}$ Zn. In the roots the excess of Zn restricted the dry mass accumulation in *A. affinis* (-24%) and *P. notatum* (-8%) (Fig. 1B). Roots of *A. lateralis* plants grown at the concentration of 300 $\mu\text{Mol L}^{-1}$ of Zn showed an increase of root dry mass of approximately 0.13 g with respect to those grown at the Zn concentration of 2 $\mu\text{Mol L}^{-1}$. For *P. plicatulum*, there was no effect of Zn concentrations on root dry mass ($p > 0.05$).

The root length of *P. notatum* and *P. plicatulum* increased by 21% and 27%, respectively, with increasing Zn concentrations, compared to plants grown in the 2 μM Zn solution (Fig. 1C). Root length of the specie *A. lateralis* was not affected by the increase of Zn availability ($p > 0.05$). Regarding the root surface, *A. lateralis* was the only one that presented the highest value when cultivated at a concentration of 300 μM of Zn (Fig. 1D). *A. lateralis* showed an increase in root diameter of 20% when cultivated in the treatment with 300 μM of Zn when compared to the plants cultivated in the solution with 2 μM of Zn (Fig. 1E). The species *P. plicatulum* and *A. lateralis* showed an increase in root volume with increasing concentrations of Zn. The volume of roots of *A. lateralis* tripled when the plants were grown in 300 μM of Zn, when compared to plants grown in the solution with 2 μM of Zn (Fig. 1F).

3.2. Zn concentration, translocation, and accumulation indexes in plant tissues

Zn concentrations in shoots and roots increased with Zn concentrations in the solution (Fig. 2A, 2B). The cultivation of the species *A. affinis* in the solution with 300 μM of Zn increased by 904% the concentrations of Zn in the plants shoot. The species *A. lateralis*, showed concentrations of Zn 9 and 16 times higher in the roots of plants cultivated in the solution with 150 and 300 μM of Zn in relation to the plants cultivated in the solution with 2 μM of Zn (Fig. 2B).

The plants *A. affinis*, *P. notatum* and *A. lateralis* showed higher translocation index in the presence of the lowest concentration of Zn in the solution, while *P. plicatulum* showed higher values at higher concentrations (Fig. 2C).

The Zn bioaccumulation index showed similar behavior in the four species studied, with emphasis on the highest values at the lowest concentration (2 μM of Zn), with an average increase above 1000% in relation to the average of the other concentrations (Fig. 2D).

The ratio between the amount of Zn-Shoot dry matter and dry matter in the shoot (Zn-SDM/SDM) and ratio between the amount of Zn-root dry matter and dry matter in the root (Zn-RDM/RDM) increased in all species as Zn concentrations increased (Fig. 2D). *A. lateralis* presented the highest ratio Zn-SDM/SDM, being on average 4.02

and 2.58 times higher when cultivated with 150 and 300 μM of Zn compared to the other species (Fig. 2E). In the Zn-MDR/MDR, the species *A. lateralis* obtained a superior ratio of 1405% and 967% at concentrations of 150 and 300 μM of Zn, respectively, compared to the concentration of 2 μM of Zn (Fig. 2F).

3.3. Nutrient concentrations in plant tissue

The concentrations of P, Ca, Mg and Mn in the shoot, in general, decreased with increasing concentrations of Zn in the solution (Table 1). The species *A. lateralis* presented the highest values of P in the plants shoot. The P concentrations in the roots of the species *A. lateralis* increased 1.75 times in relation to the treatment with a concentration of 2 μMol Zn.

A. lateralis presented mean concentrations of P in the root system of 6.25 times higher compared to other species. K concentrations in shoots and roots increased in *A. affinis* (39%shoot and 17%root) and *A. lateralis* (62%shoot and 18%root) with increasing Zn concentrations in the solution. For the species *A. affinis*, *P. plicatulum* and *A. lateralis* the concentrations of Cu increased in the shoot while in the roots there was a reduction only in the species *A. affinis*, with increasing concentrations of Zn in the solution. For *A. lateralis* the Fe concentrations significantly reduced (113.67 mg Zn_{shoot} and 149.32 mg Zn_{root}) at 300 μMol Zn in relation to the concentration of 2 μMol Zn.

Table 1
Concentrations of P, K, Ca, Mg, Cu, Fe and Mn shoots and roots of four native grass species in response to Zn concentrations in the nutrient solution.

Nutrients									
Nutrients	Zn Concentration (µM)	<i>A. affinis</i>		<i>P. notatum</i>		<i>P. plicatulum</i>		<i>A. lateralis</i>	
		Shoot	Roots	Shoot	Roots	Shoot	Roots	Shoot	Roots
g kg ⁻¹									
P	2	6.40 a ⁽¹⁾	4.09 a	4.54 a	2.06 c	4.83 a	4.02 b	6.90 a	11.32 c
	150	5.11 b	1.90 b	4.18 b	3.93 b	4.18 b	4.70 a	5.13 b	16.98 b
	300	4.81 b	1.43 b	4.58 a	5.38 a	4.50 c	1.73c	5.33 b	19.76 a
K	2	24.41 c	26.56 c	25.52 a	21.12 a	26.86 a	31.05 a	11.32 c	10.73 b
	150	33.37 a	28.46 b	23.51 b	18.09 b	26.78 a	24.10 b	16.98 b	11.57 b
	300	34.58 b	33.93 a	24.97 a	19.83 a	24.56 b	21.29 c	19.76 a	13.81 a
Ca	2	4.64 a	1.87 c	3.32 a	1.08 c	4.29 a	2.44 a	4.78 a	2.10 a
	150	4.20 b	2.73 a	3.10 a	1.69 a	4.06a	2.18 b	3.02 b	2.05 a
	300	4.23 b	2.23 b	3.41 a	1.46 b	3.99a	2.00 b	2.02 c	1.61 b
Mg	2	2.19 a	1.90 b	2.01 a	1.20 b	2.73 a	1.56 b	1.52 a	1.33 a
	150	1.97 b	2.86 a	1.60 b	1.60 a	1.88 b	1.60 b	1.17 b	1.53 a
	300	1.87 b	1.40 c	1.39 c	1.46 a	1.91 b	1.83 a	1.20 b	1.36 a
mg kg ⁻¹									
Cu	2	6.71 b	15.74 a	5.30 a	6.33 c	6.06 b	14.91 b	3.52 b	13.61 b
	150	7.24 b	12.04 c	4.58 a	8.49 b	6.23 b	16.75 a	3.27 b	15.80 a
	300	10.08 a	13.77 b	4.98 a	10.57 a	7.03 a	17.28 a	4.19 a	13.21 b
	2	175.79 b	437.89 a	121.44 b	451.81 c	156.59 a	711.37 a	246.3 a	1177.07 b
Fe	150	176.47 b	409.26 a	148.81 a	883.51 a	153.03 a	751.25 a	194.75 b	1390.00 a
	300	198.99 a	338.72 b	132.04 b	706.69 b	143.49 a	678.07 a	132.36 c	1027.75 c

		Nutrients							
Mn	2	48.08 a	51.23 a	37.58 a	19.27 a	52.47 a	17.06 b	64.04 a	51.02 a
	150	28.65 b	39.77 b	23.10 b	15.21 b	39.25 b	11.04 c	41.07 b	36.39 b
	300	30.91 b	22.91 c	20.66 b	14.59 b	31.96 c	22.03 a	41.10 b	21.02 c

Values were obtained from the means of three replications for each grasses species native to the Pampa Biome. (1) Different letters indicate significant differences ($p < 0.05$) between Zn treatments for the same species.

3.4. Gas exchanges

The net photosynthetic rate (A) increased in all species with increasing Zn concentrations in the solution (Fig. 3A). The species *A. lateralis* and *P. plicatum*, showed higher increased an average of 85% and 47% when cultivated in the highest Zn concentration, respectively, with respect to the plants fed with 2 μM Zn. The stomatal conductance of *A. affinis* and *A. lateralis* leaves was also increased with increasing Zn concentrations in solution (Fig. 3B). For the species *P. notatum* and *P. plicatum*, stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and transpiration rate (E) showed a reduction of 300 μM Zn (Fig. 3C). The water-use efficiency (WUE) in *A. affinis* and *A. lateralis* decreased with increasing Zn concentrations in the solution (Fig. 3E). The A/Ci values for *P. notatum*, *P. Plicatum* and *P. lateralis* increased with increasing Zn concentrations in solution (Fig. 3F).

3.5. Photosynthetic pigments

The highest values of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids were observed at the concentration of 300 μM Zn, compared to the control (Table 2). On the other hand, the Chl a/Chl b ratio was higher at 150 μM Zn. The lowest pigment values were found in *A. affinis* and *P. plicatum* (Table 2).

Table 2
Chlorophyll a, chlorophyll b, total chlorophyll, Chl a/Chl b, carotenoids concentrations in four native grass species in response to Zn concentrations in the nutrient solution.

	Chl a	Chl b	Chl total	Chl a/ Chl b	Carotenoid
	(mg g ⁻¹ Fw)	(mg g ⁻¹ Fw)	(mg g ⁻¹ Fw)		(mg g ⁻¹ Fw)
<i>Axonopus affinis</i>	1.275 c ⁽¹⁾	0.286 c	1.584 b	4.924 a	0.410 b
<i>Paspalum notatum</i>	1.472 a	0.415 a	1.888 a	3.704 b	0.516 a
<i>Paspalum plicatum</i>	1.393 b	0.344 b	1.753 a	4.043 b	0.437 b
<i>Andropogon lateralis</i>	1.514 a	0.383 a	1.822 a	4.054 b	0.497 a
2 μM	1.379 b	0.325 b	1.665 b	4.178 b	0.446 b
150 μM	1.375 b	0.328 b	1.735 b	4.517 a	0.448 b
300 μM	1.487 a	0.419 a	1.885 a	3.849 b	0.501 a

Values were obtained from the means of three replications for each native grass species to the Pampa Biome. (1) Different letters indicate significant differences ($p < 0.05$) between Zn treatments for the same species.

3.7. Antioxidant enzyme activity and oxidative stress indicators

The H_2O_2 concentrations of in the shoot of the *A. affinis*, *P. notatum* and *P. plicatum* were not altered with increasing Zn concentrations in the solution (Table 3). For these species there was a small increase in H_2O_2 concentrations in plant roots (on average $0.04 \mu\text{mol g}^{-1} \text{FW}$). However, the H_2O_2 concentrations in *A. lateralis* showed a 32% drop in the shoot with increasing Zn concentrations, while in the roots an increase of 27% was observed. The concentrations of MDA in the shoot of the species *A. affinis*, *P. notatum* and *P. plicatum* was not altered by increasing Zn concentrations. In shoots and roots of the *A. lateralis* species, the concentration of malondialdehyde (MDA) decreased with increasing Zn concentrations in the solution.

Guaiacol peroxidase (POD) activity of *A. lateralis* when cultivated in the Zn concentration of 300 μM presented an increase of 24.18 U/mg^{-1} in the POD activity in relation to the average of the other concentrations. For *A. affinis*, *P. notatum* and *A. lateralis* species, the superoxide dismutase (SOD) activity in the shoot increased with increasing Zn concentration in the solution.

Table 3

Hydrogen peroxide concentration (H_2O_2), malondialdehyde (MDA) concentration (TBARS), guaiacol peroxidase (POD) activity and superoxide dismutase (SOD) activity in four native grass species in response to Zn concentrations in the nutrient solution.

Native grasses	Dose de Zn	H_2O_2 Shoots	H_2O_2 Roots	TBARS Shoot	TBARS Roots	POD Shoots	POD Roots	SOD Shoots	SOD Roots
		($\mu\text{mol g}^{-1}$ FW)	($\mu\text{mol g}^{-1}$ FW)	(nM MDA/mg protein)	(nM MDA/mg protein)	(U/mg^{-1} protein)	(U/mg^{-1} protein)	(U/mg^{-1} protein)	(U/mg^{-1} protein)
	2	0,78 a ⁽¹⁾	0,10 a	15,44 a	34,50 a	14,86 a	247,11 a	5,13 b	3,71 a
<i>A. affinis</i>	150	0,68 a	0,11 a	13,62 a	19,85 b	15,04 a	167,57 b	5,4 b	4,11 a
	300	0,58 a	0,10 a	12,23 a	20,84 b	17,47 a	247,02 a	6,59 a	2,54 b
	2	0,23 a	0,09 b	14,03 a	31,59 a	63,06 b	241,71 a	10,93 b	11,88 b
<i>P. notatum</i>	150	0,21 a	0,12 a	12,54 a	26,63 b	77,25 a	237,77 a	11,39 b	15,12 a
	300	0,42 a	0,12 a	16,65 a	25,65 b	76,06 a	203,88 b	13,81 a	15,70 a
	2	0,25 a	0,06 b	20,69 a	23,98 a	43,45 a	99,84 a	14,33 a	4,83 b
<i>P. plicatum</i>	150	0,21 a	0,11 a	19,72 a	25,08 a	41,35 a	104,17 a	13,55 ab	6,23 a
	300	0,37 a	0,08 b	20,27 a	23,19 a	36,70 a	98,70 a	13,26 b	6,31 a
	2	4,07 a	0,15 b	45,97 a	22,45 a	15,96 a	112,56 b	5,86 b	17,59 a
<i>A. lateralis</i>	150	3,44 b	0,20 a	42,43 b	16,77 b	12,26 a	103,60 b	7,96 a	12,49 b
	300	2,76 c	0,19 a	37,85 c	19,92 a	9,31 a	132,26 a	7,61 a	10,89 c

Values were obtained from the means of three replications for each native grass species to the Pampa Biome. (1) Different letters indicate significant differences ($p < 0.05$) between Zn treatments for the same species.

3.8. Principal component analysis

Principal component analysis (PCA) was performed by extracting the first two components (PC1 and PC2), which combined explained 60% of the original data variability (Fig. 4). The main component 1 explained 37% of the variability and had the greatest influence on root and shoot dry mass, root and shoot K concentration, root Fe concentration, root and shoot H_2O_2 concentration, TBARS in shoots, in addition to all root biometric parameters. The main principal component 2 explained approximately 23% of the data variation, and the response variables

that most influenced were the concentration of Ca and Mn in the shoot, concentrations of chlorophyll b, carotenoids, net photosynthetic rate, and intercellular CO₂ concentration.

The *A. lateralis* when cultivated at Zn concentrations of 150 and 300 µM behaved similarly but differently of those treated with 2 µM Zn (blue ellipse). This phenomenon was not observed in the case of *P. notatum*, where the plant development was unaffected by the different levels of Zn availability (green ellipse). The species *A. affinis* and *P. plicatulum* (red ellipse) behaved similarly with a lower degree of distinction in the Zn concentrations of 2 and 150 µM with respect to the treatment with 300 µM Zn. This result may indicate possible similarities in the characteristics of these species at the same Zn concentrations in the solution.

4. Discussion

Results here presented show that from the physiological, nutritional, and biochemical point of view the 4 native grass species considered of the Pampa biome responded differently to the excess of Zn availability. Considering their capability to survive and complete the vital cycle even when exposed to very high levels of Zn availability (accumulating as well very high metal levels in their tissues), it can be speculated that these plant species possess traits that make them interesting candidates for phytoremediation programs. This hypothesis is further corroborated by the evidence that some of these plant species are naturally living and replicating in soils of southern Brazil characterized by an excessive Cu availability (e.g. *A. affinis*, *P. notatum* e *P. plicatulum*) (Silva et al. 2022; Schwalbert et al. 2022).

In all the four plant species, a close positive relationship between the availability of Zn in the growth medium and the levels of metal accumulated in the plant tissues was highlighted (Fig. 2). This feature is of particular interest when a recovery of Zn contaminated soil is considered. However, even if united by the marked tolerance to the elevated Zn concentrations, the four native plant species seem to adopt different resistance strategies or diverse combinations of them. In fact, while in *P. plicatulum* and *P. notatum* plant species, the higher was the Zn accumulation in the tissues, the more limited was their size, the development and the biomass accumulation of *A. affinis* plants were completely unaffected by the Zn availability, regardless of its concentration (i.e. 2 or 300 µMol L⁻¹) (Fig. 1A). In this context it is interesting to note that in *A. lateralis* plant species, there was not only an increase in the metal content in the tissues but also an increased accumulation of biomass even when the plants were fed with 300 µMol L⁻¹ Zn. Taken together, these results demonstrate that in addition to the metal availability level, exposure time to the metal, and the phenological stage of the plant development at the moment of the treatment, there is a significant genetic component at the basis of the tolerance to different levels of Zn availability in these four native grasses (Balafrej et al. 2020).

It is well known that Zn concentration levels in leaves above 150 mg Zn kg⁻¹ can normally induce the onset of toxicity symptoms in several plant species (Kabata-Pendias 2011) impacting, therefore, their development and reproduction capacity. It is worth to mention that in our work values ranging from 400 to 431 mg kg⁻¹ have been measured in shoots of the four plant species when fed at 150 and 300 µMol L⁻¹ (Fig. 1A). However, while in *P. plicatulum* and *P. notatum* plant species this metal accumulation process was accompanied by a marked inhibition of the plant development (about - 15% biomass accumulation) maybe due to the malfunction of different metabolic pathways as already described (Ghori et al. 2019), the plants of *A. lateralis* did not show any symptom of toxicity or stunted development. In this latter case, the activation of some specific physiological and biochemical processes linked to the tolerance to Zn excess (Sarwar et al. 2017) appears evident. The metal

compartmentation within the cell (Jogawat et al. 2021) as well as the restraint of the metal free form (Krippner and Schubert 2021) are well described strategies acting directly on the acquired metal. On the contrary, the enhancement of the antioxidant systems can be counted among the biochemical strategies implemented to counter the effects of Zn excess like the ROS formation (Kaur and Garg 2021). In this respect, it should be noted that, except for *P. plicatum* plant species, the levels of SOD activity were markedly increased in all the other three species when the plants were exposed to the highest level of Zn availability (Table 3).

From the nutritional and nutrients' interactions point of view, results here reported show that a high availability of Zn can hinder a balanced acquisition of Mn (Table 1). All the four plants species are affected by this problem, albeit at different levels. The similarities in the ionic radii of the two divalent cations can be, at least in part, at the base of this competitive phenomenon of Mn and Zn for the transmembrane transport (Tewari et al. 2008). Similarly, high levels of Zn also hinder the acquisition of Mg. In this case, the lower Mg content at the leaf level can certainly have consequences not only on the chlorophyll functionality, but also on the RUBISCO activation levels (Küpper and Andresen 2016). In this latter case, a competition phenomenon between Mg and Zn for the functional sites of these molecules/proteins cannot be excluded. On the contrary, a nutrient whose acquisition is strongly stimulated by high availability of Zn in the growth medium is P, reaching in particular in *A. lateralis* values 1.75 times higher than those measured when the plants were fed with $2 \mu\text{mol L}^{-1}$ Zn (Table 1). Considering the role played by P at the cell wall level in terms of pectin and hemicellulose contents and pectin methylesterases activity (Wei et al. 2021), the increase in the P content could be considered a contribution to the plant's ability to counteract the excess of Zn by limiting its toxic effects.

With respect to the shoot and its analyzed parameters, it is interesting to highlight that the net CO_2 assimilation rate (A) measured increased when the plants of the four native species were grown at the highest availability of Zn (Fig. 3A). Moreover, in this same growing conditions, *P. notatum*, *P. plicatum* and *A. lateralis* plants also showed an enhancement in the instantaneous carboxylation efficiency of Rubisco (A/Ci) which is also associated, limited to *P. notatum* and *P. plicatum* plant species, with an increased stomatal conductance, a higher internal CO_2 concentration and a more pronounced transpiration rate (Fig. 3). These phenomena are in agreement with the substantial integrity (even at the very high availability of Zn) of the photosynthetic system evaluated by analyzing the pigments' contents (Schwalbert et al. 2022). Moreover, the rather stable levels of H_2O_2 (with the only exception of *A. lateralis* plants) and malondialdehyde (MDA) concentration further corroborate the idea that not even the highest available Zn-dose is able to induce a significant level of stress in these plants. It is interesting to note that also at the root level the content of H_2O_2 and the levels of lipid peroxidation resulted not significantly affected by the Zn availability. Overall, it is clear that the plants of the four native plant species weren't suffering from significant oxidative stress due to Zn toxicity, at least from the type canonically described.

Considering the stress responses of each plant species analyzed individually, it is interesting to note that the plants of *A. affinis* are those not affected by the Zn excess in terms of biomass accumulation. Moreover, considering the Zn concentration at the root and shoot level (Fig. 2A and 2B) a mechanism of Zn exclusion at the rhizosphere (Taylor et al. 2008) appears to be adopted in this plant species. Furthermore, it is interesting to note that the values of the gas exchange and the oxidative stress parameters measured in *A. affinis* did not reflect a severe stress for these plant (Thakur et al. 2022), thus suggesting that there was still room of further plant's response expression to the high Zn availability, even if it was already at very high levels (i.e., $300 \mu\text{mol L}^{-1}$).

Differently, plants of *A. lateralis*, although the smallest, when exposed to high availability of Zn, stimulated the biomass accumulation, enhanced the Zn content at the root level, augmented the H₂O₂ and TBARS production in the roots. The plant showed the ability to maintain the metal in its roots, immobilizing so Zn thanks to the negative charges of the cell wall components or *via* a complexing process based on other elements such as P (Marta et al. 2019). It should be noted that limiting the metal content at the leaf level means preserving the photosynthetic system from the damage while maintaining the plant's ability to produce carbohydrates (Yang et al. 2011). Moreover, it is interesting to note that in these plants a very high content of Fe has been detected at the root level, particularly when the treatment with 300 µM Zn is considered. In this respect it should be mentioned that Wu et al. (2022) have shown a marked inhibition of Zn allocation at the shoot level when Zn competes with Fe for the binding sites at the root level.

With respect to *P. notatum* and *P. plicatulum* plants, data here reported show a clear toxic effect of Zn in the biomass accumulation process with a positive trend of inhibition linked to the levels of metal availability. However, the values of root volume and root length as well as those of several gas exchange parameters (A, WUE and A/Ci) were clearly increased by higher Zn availability. With respect to the root length, it is interesting to note that its increase has been proposed as a positive sign of Zn stress tolerance in plants (Michael and Krishnaswamy 2011). In addition, the recognized capacity of *P. plicatulum* roots to modify the rhizosphere pH (De Conti et al. 2020) can surely and markedly affect the metal availability fraction in the soil near the roots. Moreover, the enhanced POD and SOD activity detected in these plants is also counted among the tolerance mechanisms to counter Zn excess (Wei et al. 2022). Furthermore, the proneness to accumulate significant quantities of Zn at the shoot level makes *P. plicatulum* plants particularly promising for recovery plans of contaminated soils through phytoremediation programs (De Conti et al. 2020).

5. Conclusions

Results here presented show that *Axonopus affinis*, *Paspalum notatum*, *Paspalum plicaulum* and *Andropogon lateralis* plants behaved differently at increasing levels of Zn availability with respect to the growth rate, nutritional and photosynthetic parameters, enzymatic activity. *Axonopus affinis* has the ability to tolerate high Zn concentrations and to reduce the metal acquisition when supplied at intermediate levels. Very high Zn availability induced the enzymatic activities (SOD and POD) in *Paspalum notatum* that represent the main mechanism underlying the expression of the Zn tolerance in this plant species. Differently, a very pronounced allocation of the metal to the shoot level seems to be at the basis of the ability *Paspalum plicaulum* to survive with high levels of Zn availability in the growth medium. It is interesting to highlight that this feature makes this plant species particularly promising for phytoextraction to retrieve Zn contaminated soils. In *Andropogon lateralis* plant species, the particularly pronounced capacity to retain P at the root level and, consequently, high levels of Zn, seems to be the main strategy adopted to counteract the Zn excessive availability. The growth stimulation measured when these latter plants were exposed to very high Zn availability makes this plant species particularly interesting for phytoremediation plans. However, to this purpose a deeper understanding of the complex mechanism of Zn tolerance in this latter plant species appears crucial.

Declarations

Author contribution statement

Paulo A. A. Ferreira, Gabriela D. Milanese, Camila P. Tarouco and Liliane C. Machado: contributed to study design, methodology, accomplishment of the experiment, all laboratory analysis, interpretation of results and writing the article.

Natielo A. Santana, Mariana Coronas, Isley C. B. Silva: study design, interpretation of results and writing the article.

Gustavo Brunetto, Stefano Cesco, Tanja Mimmo and Fernando T. Nicoloso: interpretation of results and writing – review and editing the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. On behalf of all authors, the corresponding author states that there is no conflict of interest.

Submission declaration and verification

The authors would like to inform that the manuscript was not published previously, and its submission was approved by all the authors.

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Figures

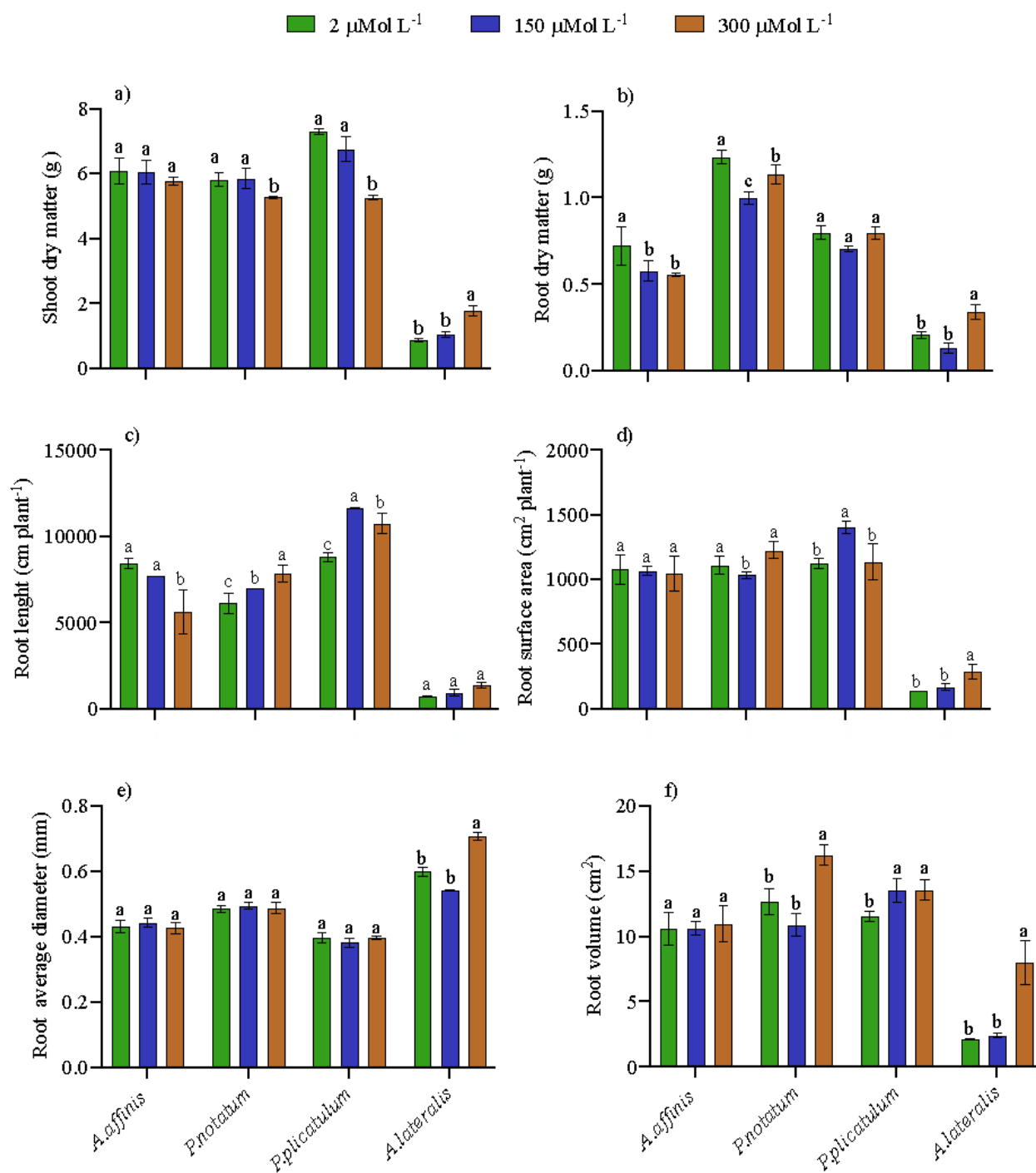


Figure 1

Shoot dry mass (a), root dry mass (b), root length (c), root surface area (d), root average diameter (e) and root volume (f) of four grass species native plants, in response to Zn concentrations in the nutrient solution. Different letters indicate significant differences ($p < 0.05$) between treatments with Zn for the same species.

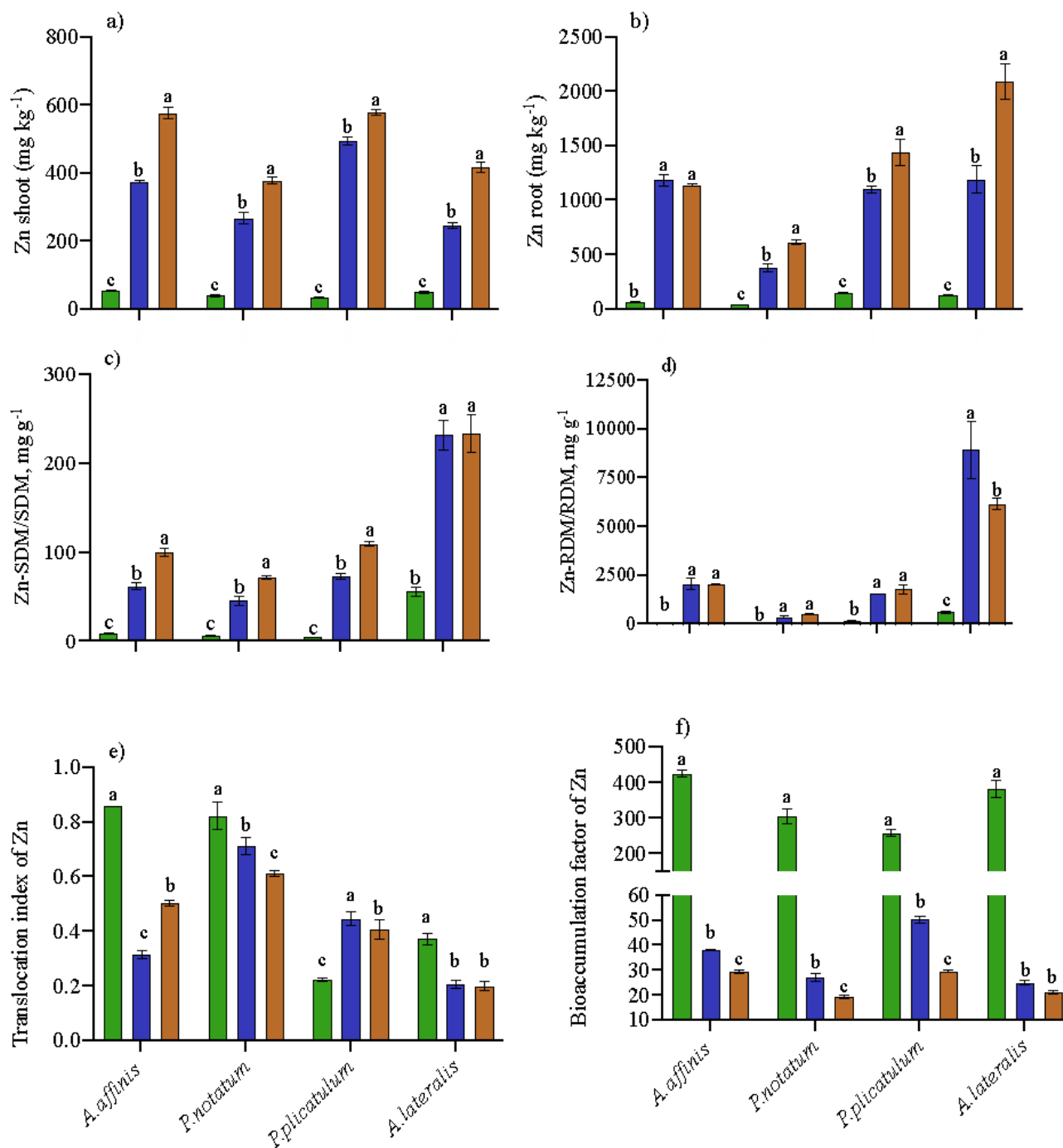


Figure 2

Zn concentration in shoots (a) and roots (b), ratio between Zn in the shoot and dry matter in the shoot (c), ratio between Zn in the root and dry matter in the root (d), Zn translocation for shoots (e) and Zn bioaccumulation factor in shoots (f), of four grass species native plants, in response to Zn concentrations in the nutrient solution. Different letters indicate significant differences ($p < 0.05$) between treatments with Zn for the same species.

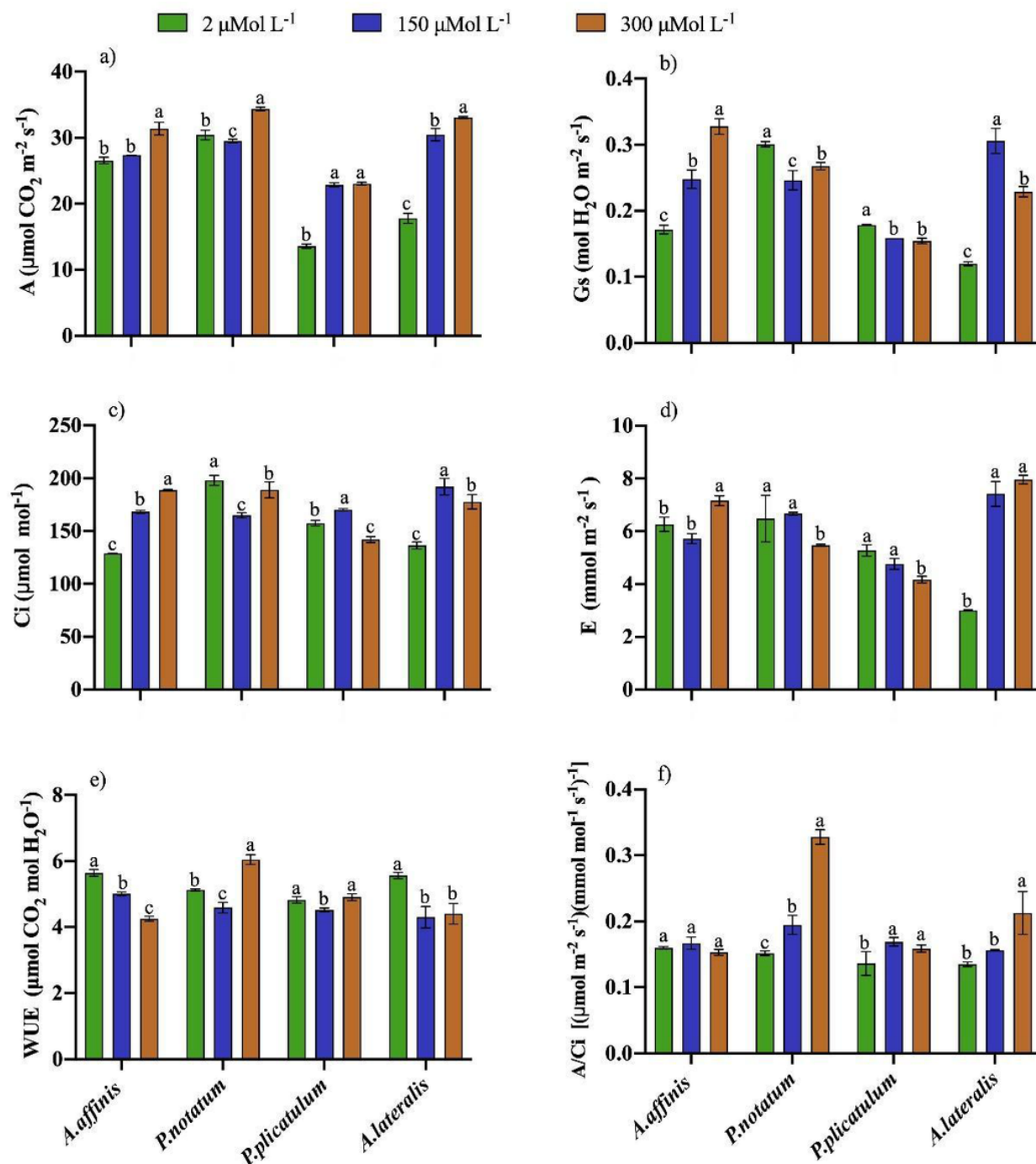


Figure 3

Net photosynthetic rate -A (a), stomatal conductance - Gs (b), intercellular CO₂ concentration - Ci (c), transpiration rate -E (d), water-use efficiency - WUE (e) and instantaneous efficiency of carboxylation of Rubisco – A/Ci (f) of four grass species native plants, in response to Zn concentrations in the nutrient solution. Different letters indicate significant differences ($p < 0.05$) between treatments with Zn for the same species.

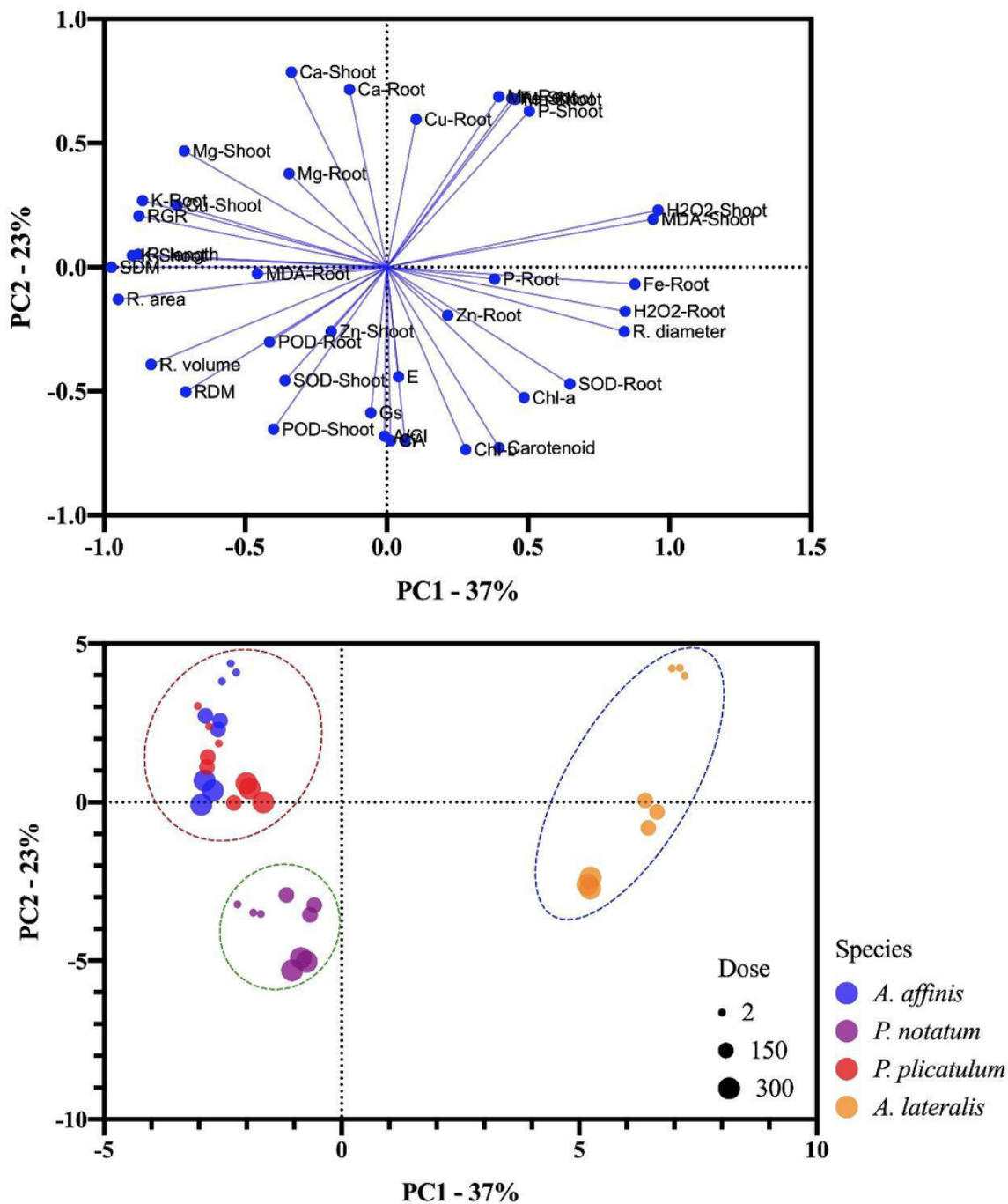


Figure 4

Relationship between principal component 1 (PC 1) and principal component 2 (PC 2) of photosynthetic parameters (A, E, Gs and Ci), concentration of chlorophylls a, b, and carotenoids, dry mass of shoot, root (SDM, RDM), concentration of P (P-Shoot and P-Root), K (K-Shoot and K-Root), Ca (Ca-Shoot and Ca-Root), Mg (Mg-Shoot and Mg-Root), Cu (Cu-Shoot and Cu-Root), Fe (Fe-Shoot and Fe-Root), Mn (Mn-Shoot and Mn-Root) and Zn (Zn-Shoot and Zn-Root) in roots and shoots, activity of superoxide dismutase enzymes in roots and shoots (SOD-Root and SOD-Shoot), activity of peroxidase enzymes in roots and shoots (POD-Root and POD-Shoot), concentration of hydrogen peroxide in roots and shoots (H_2O_2 -Root and H_2O_2 -Shoot) and lipid peroxidation in roots and shoots

(TBARS-Root and TBARS-Shoot), in four grasses of the Pampa biome cultivated in increasing Zn concentrations (2, 150 and 300 μM of Zn).