Changes in Tillering, Nutritional Status, and Biomass Yield of *Panicum Maximum* Used for Cadmium Phytoextraction

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Abstract

Although several grasses have been evaluated for cadmium (Cd) phytoextraction, there are no studies assessing how Cd is accumulated and distributed in the tissues of *Panicum maximum* grown in mildly polluted soils. The evaluation of tillering, nutritional status and biomass yield of this grass, mainly along successive shoot regrowths, is not well studied so far. Thus, *P. maximum* Jacq. cv. Massai was grown for two periods in an Oxisol presenting bioavailable Cd concentrations varying from 0.04 (control) to 10.91 mg kg^{-1} soil. Biomass yield of leaves and stems´ growth have decreased under the highest Cd exposure, but it did not occur in the regrowth period, indicating that Cd-induced toxicity is stronger in the early stages of development of *P. maximum*. The tillering was not compromised even the basal node presenting Cd concentrations higher than 100 mg kg^{-1} DW. We identified a restriction on Cd transport upwards from basal node, which was the main local of Cd accumulation. Apparently, P, K, Mg, S and Cu are involved in processes that restrict Cd translocation and confer high tolerance to Cd in *P. maximum*. The Cd-induced nutritional disorders did not negatively correlate with factors used to calculate phytoextraction efficiency. However, the nutritional adjustments of *P. maximum* to cope with Cd stress restricted the upward Cd transport, which decreased the phytoextraction efficiency from the available Cd concentration of 5.93 mg kg^{-1} soil.

Introduction

Several species of grass have been evaluated for cadmium (Cd) phytoextraction as they present desirable characteristics such as high biomass yield, fast growth, and successive shoot regrowth after shoot harvest (Rabêlo et al. 2021a). Between the grasses assessed, *Panicum maximum* has stood out for surviving when exposed to very high Cd concentrations (de Anicésio and Monteiro 2021), despite Cd is more transported to aboveground plant tissues at such conditions, compromising tillers emission, inducing nutritional disorders, and decreasing biomass yield, which results in lowered Cd phytoextraction efficiency (de Anicésio and Monteiro 2019). As tillering and nutrient homeostasis are essential for grass development, the use of *P. maximum* for Cd phytoextraction is supposed to be feasible only in mildly polluted soils (Rabêlo et al. 2021a), otherwise, the Cd-induced phytotoxicity could prevent further growth and biomass yield. However, to the best of our knowledge, there are no studies reporting how Cd is accumulated and distributed into the tissues of *P. maximum* in mildly polluted soils and how it affects the tillering, nutritional status and biomass yield of this grass, especially along successive shoot regrowth.

Each tiller in grasses is originated from the emission of a single apical meristem initiated in the axillary buds located at the basal node of the plant (Chrysler 1906). In other species of the family *Poaceae*, such as rice (*Oryza sativa*), there is evidence that Cd is highly accumulated in the basal node of such plant and compromises the tillering (Fujimaki et al., 2010). Nutritional disorders also can decrease the tillering of *P. maximum* by increasing the number of dormant buds (Garcez Neto et al. 2012). As Cd strongly induces nutritional disorders at high concentrations in such grass (Rabêlo et al. 2020a), the relationship between Cd accumulation, tillering and biomass yield of *P. maximum* used for Cd phytoextraction in mildly polluted soil should have better understood. Thereby, our aims with this study were to i) assess how Cd affects the growth and biomass yield of *P. maximum* used for Cd phytoextraction in mildly polluted soil along two successive shoot growths; ii) identify the main local of Cd accumulation (basal node?) in *P. maximum* used

for Cd phytoextraction; iii) check if a possible Cd accumulation in the basal node could limit successive shoot emissions due to reduced tillering; and iv) diagnose eventual nutritional disorders Cd-induced and correlate such event with the Cd phytoextraction efficiency of *P. maximum*.

Materials And Methods

Soil collection and physic-chemical characterization

The soil was collected from the upper layer (0.0-0.2 m depth) of an area under native pasture in Piracicaba, state of São Paulo, Brazil (S 22°43'04"; W 47°36'55"). The soil was classified as Typic Hapludox (USDA 1999). Soil characteristics were determined on air-dried soil sieved with a 2-mm (Table 1). The determination of pH (0.01 mol L^{-1} $CaCl_2$), Al (extraction with 1 mol L^{-1} KCl) and soil organic matter (oxidation with 0.2 mol L^{-1} $K_2Cr_2O_7$) followed the methods of the Brazilian Agricultural Research Corporation - EMBRAPA (EMBRAPA 1997). Potential acidity (H+Al) was estimated following the method of pH SMP (Raij et al. 2001). The available concentrations of P, K, Ca and Mg were extracted with ion exchange resin (Raij et al. 2001), S with 0.01 mol L^{-1} $Ca(H_2PO_4)_2$ (Tabatabai and Bremner 1970), B with hot water in a microwave oven (Bataglia and Raij 1990), and Cu, Fe, Mn and Zn with diethylene triamine pentaacetic acid - DTPA at pH 7.3 (Abreu et al. 2001). From these results the sum of bases, effective cation exchange capacity, total cation exchange capacity, base saturation, and saturation by aluminum were calculated (EMBRAPA 1997). The initial pseudo-total Cd concentration was determined following the method 3050B proposed by the United States Environmental Protection Agency - USEPA (USEPA 1996). The granulometric fractions sand, silt and clay were obtained by the hydrometer method (Gee and Bauder 2002).

Table 1
Descriptive analysis of chemical and physical properties of the Typic Hapludox used in this study

pH 0.01 mol L^{-1} $CaCl_2$	**4.6**	**Al ($mmol_c$ dm^{-3})**	**2**
		H+Al ($mmol_c$ dm^{-3})	25
P (mg dm^{-3})	4		
S (mg dm^{-3})	4.5	SB ($mmol_c$ dm^{-3})	15
K ($mmol_c$ dm^{-3})	0.5	CEC_t ($mmol_c$ dm^{-3})	40
Ca ($mmol_c$ dm^{-3})	10.5	CEC_e ($mmol_c$ dm^{-3})	17
Mg ($mmol_c$ dm^{-3})	4		
B (mg dm^{-3})	0.2	BS (%)	37
Cu (mg dm^{-3})	0.5	m (%)	11
Fe (mg dm^{-3})	39		
Mn (mg dm^{-3})	6.4	SOM (g kg^{-1})	14
Zn (mg dm^{-3})	2.1	Sand (g kg^{-1})	828
		Silt (g kg^{-1})	23
Cd (mg kg^{-1})	0.67	Clay (g kg^{-1})	149

SB - sum of bases, CEC_e - effective cation exchange capacity, CEC_t - total cation exchange capacity, BS% - base saturation, m% - aluminum saturation, SOM - soil organic matter

Plant Material And Experimental Set-up

Panicum maximum Jacq. cv. Massai was grown under greenhouse under natural conditions (31.5 ± 5 ºC and 63.7 ± 14% relative humidity during plant growth, and 26.0 ± 5 ºC and 66.0 ± 12% relative humidity during plant regrowth), in pots containing 5 kg of the Typic Hapludox (Table 1). Treatments were composed by a control (0.67 mg Cd kg^{-1} soil, Table 1) and three added Cd doses attempting to reach the final pseudo-total Cd concentrations of 7.2, 14.4 and 28.8 mg kg^{-1} soil. Such Cd concentrations were defined from the study of Farnezi et al. (2020), in which the authors estimated that *P. maximum* can grow up in soils presenting Cd concentrations close to 30 mg kg^{-1} soil. However, the final Cd concentrations reached were lower (Fig. 1). Pots were distributed in completely randomized design with four replicates per condition.

Soil Pollution, Growth Conditions And Plant Harvesting

The Oxisol was spiked with Cd by using $CdCl_2$, and then incubated for 30 days. The soil water content was maintained at constant level (70% of the maximum water holding capacity) throughout the study by adding deionized water. The basic fertilization was performed after the soil incubation by applying 100 mg N (NH_4NO_3), 150 mg P (KH_2PO_4), 100 mg K (KH_2PO_4) and 50 mg S ($MgSO_4.7H_2O$) per kg of soil, according to Werner et al. (1997). Seeds of *P. maximum* Jacq. cv. Massai were sown in the same day of the basic fertilization. A thinning leaving 10 seedlings per pot was carried out 21 days after sowing. Twenty-six days after sowing, the fertilization with 100 mg N (NH_4NO_3) and 100 mg K (KCl) per kg of soil was performed on the top (Werner et al. 1997). Aboveground plant parts were harvested (5 cm above the basal node to allow plant regrowth; Pautler et al. 2013) 54 days after sowing, adopting the beginning of the senescence of the mature leaves of plants in a more advanced physiological stage as the criterion for the harvest (Rabêlo et al. 2017).

To stimulate shoot regrowth, 100 mg N (NH_4NO_3) and 100 mg K (KCl) per kg of soil were top dressed one day after the first shoot harvest (Werner et al. 1997). Twenty-five days after, a second top-dressed fertilization was performed with 100 mg N (NH_4NO_3) and 100 mg K (KCl) per kg of soil (Werner et al. 1997). Nutrients were applied through a solution in the first and second growths. The second and final harvest was made 59 days after the first harvest, adopting the same criterion for the first harvest.

At the end of the study, the plant material was separated into roots, basal node, and shoot. The shoot collected at the end of the two growth periods was divided from the top to basal node into leaves I (the first fully expanded leaf), II, III and other leaves (leaves IV, V etc.), and stems. We counted the number of tillers one day before the first and second harvests. After the end of both growth periods the fresh weight of each plant tissue was recorded. Then, the plant tissues were placed in a forced ventilation oven at 60°C for 72 h to determine the dry weight and the concentrations of nutrients and Cd.

Determination of the concentration of nutrients and Cd in the plant tissues

The dried material was ground in a Wiley type mill (Model 4, Thomas Scientific, Swedesboro, NJ, USA). For determination of the concentrations of P, K, Ca, Mg, S, B, Cu, Fe, Mn, Zn and Cd, the plant material was digested in a microwave oven (Model ultraWAVE SRC - Single Reaction Chamber - Technology, Milestone, Sorisole, Italy) by using a mixture composed by nitric acid (HNO_3 20%) and hydrogen peroxide (H_2O_2 30%) (v/v), according to USEPA 3051A method (USEPA 2007). The contents were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 7000 SERIES, Thermo Fisher Scientific, Waltham, USA). Standard reference material (SRM 1515 - apple leaves) and blank reagent samples were used during the digestion to assure the accuracy and precision of the extraction and analytical method.

Calculation Of Nutrient Use Efficiency (Nue)

The NUE ($g^2\ mg^{-1}$ for macronutrients and $g^2\ ng^{-1}$ for micronutrients) was calculated for each nutrient: NUE = [(dried biomass of the plant tissue)^2 / nutrient accumulated in the plant tissue] (Siddiqi and Glass 1981), where nutrient accumulated in the plant tissue (mg/plant for macronutrients and µg/plant for micronutrients) was obtained by multiplying the nutrient concentration ($g\ kg^{-1}$ DW for macronutrients and $mg\ kg^{-1}$ DW for micronutrients) in each tissue by the dry weight of the respective tissue.

Calculation Of The Factors Related To Cd Phytoextraction Efficiency

Cadmium phytoextraction efficiency was calculated through the bioconcentration factor - BCF (BCF = Cd concentration in the aerial tissue / Cd concentration extracted by $CaCl_2$ in the Oxisol) and translocation factor - TF (TF = sum of Cd accumulated in the aerial tissues in the two growth periods / Cd accumulated in the roots) (adapted from Ali et al. 2013).

Statistical analysis

Normality and homoscedasticity were checked before proceeding with the analysis of variance. Then, the data were submitted to analysis of variance and post-hoc Tukey test ($p < 0.05$) to compare the means between Cd concentrations within each plant tissue for each growth period. Two-by-two comparisons were conducted using the t-test to compare the means between growth periods within each Cd concentration for each plant tissue. The statistical analyses were performed in the Statistical Analysis System v. 9.2 (SAS Institute 2008), whereas the graphs were plotted with SigmaPlot v. 10.0 (Systat Software Inc., Chicago, Illinois, USA). Results were expressed as mean ± standard error of the mean (SEM).

Results

The highest Cd concentrations decreased shoot biomass yield of P. maximum **in the growth but not in the regrowth period, indicating that Cd is more harmful in the early stages of development**

Only the leaf and stem biomass collected at the end of the growth period of *P. maximum* grown under the available Cd concentration of 10.91 $mg\ kg^{-1}$ soil was lower compared to control (Figs. 2A-B). The leaf and stem biomass of *P. maximum* at the end of the regrowth period was not affected by Cd concentrations in the Oxisol, as well as the biomass of basal node and roots (Figs. 2A-D). Plants of the control treatment presented lower leaf biomass in the regrowth compared to growth period of *P. maximum*, but when this grass was exposed to the available Cd concentration of 10.91 $mg\ kg^{-1}$ soil the opposite was observed (Fig. 2A). The stem biomass of *P. maximum* was higher in the regrowth compared to growth period, regardless of Cd concentration in the Oxisol (Fig. 2B), which indicates that other factor than the own Cd concentration affected stem biomass yield of this grass.

The number of tillers emitted by *P. maximum* was not affected by the available Cd concentrations in the Oxisol, in both growth periods. However, except for the control treatment, the number of tillers emitted during

regrowth period was higher compared to the growth period (Fig. 3A). Cadmium accumulated in the basal node presented a trend to reduce the number of tillers of *P. maximum* only in the growth period (Fig. 3B). Conversely from which was observed for the number of tillers, the leaf/stem ratio of *P. maximum* exposed to the higher available Cd concentration increased in relation to control (Fig. 3C) due to the more pronounced Cd-induced inhibition on the stem than leaf biomass yield under such conditions (Figs. 2A-B). The leaf/stem ratio in the regrowth period of *P. maximum* was not affected by the available Cd concentrations. Regardless of Cd concentration in the Oxisol, the leaf/stem ratio was lower in the regrowth compared to growth period (Fig. 3C), which reinforce the assumption that other factor than the own Cd concentration affected the grass regrowth.

Cadmium concentration, Cd BCF and Cd TF pointed out the existence of restrictive mechanisms on Cd translocation from lower plant parts to upper plant parts, mainly under highest Cd exposure

Cadmium concentration increased in all plant tissues of *P. maximum* due to Cd exposure (Fig. 4). However, the Cd concentrations measured in the leaves (Figs. 4A-D) were similar to those observed in the stems (Fig. 4E) and much lower in relation to basal node and roots (Figs. 4F-G) of *P. maximum* exposed to the highest available Cd concentrations in the Oxisol. The leaves I, II, III and other leaves presented Cd concentrations lower than 30 mg kg^{-1} DW in both growth periods (Figs. 4A-D), whereas Cd concentrations in the basal node and roots of *P. maximum* exposed to the available Cd concentration of 10.91 mg kg^{-1} soil were higher than 100 and 70 mg kg^{-1} DW, respectively (Figs. 4F-G). There was effect of the growth period on Cd concentration only for the stems of *P. maximum* grown with the available Cd concentration of 10.91 mg kg^{-1} soil, in which Cd concentration in the regrowth period was 86% higher compared to growth period (Fig. 4E).

In general, the highest Cd BCFs values were found in *P. maximum* exposed to the available Cd concentration of 2.86 mg kg^{-1} soil (Figs. 5A-E). The Cd BCFs values in the leaf II and stems of *P. maximum* exposed to the available Cd concentration of 2.86 mg kg^{-1} soil were higher in the regrowth compared to growth period (Figs. 5B and 5E). Cadmium BCFs remained higher than 1 when the grass was exposed to the available Cd concentrations of 2.86, 5.93 and 10.91 mg kg^{-1} soil, regardless of the aerial plant tissue. Similarly to which was observed for Cd BCFs, the highest Cd TF was found in *P. maximum* exposed to the available Cd concentration of 2.86 mg kg^{-1} soil, and Cd TF also remained > 1 when this grass was exposed to the available Cd concentrations of 2.86, 5.93 and 10.91 mg kg^{-1} soil in the Oxisol (Fig. 5F).

Both Cd exposure and growth period affected nutrients concentration and NUE of P. maximum, **but the decreased NUEs Cd-induced did not negatively correlate with Cd BCF**

In general, the concentrations of P, K, S and Cu increased, and the concentrations of B and Mn tended to decrease in the plant tissues of *P. maximum* exposed to the highest available Cd concentrations (Table 2). Phosphorus, Mg, S and Cu presented higher concentrations in the tissues collected at the end of the growth compared to regrowth period, whereas K, Fe and Mn were found in higher concentrations in the regrowth period. P, K, Ca, Mg and S tended to follow an increasing gradient of concentration in the sequence: roots <

basal node < stems < leaves. On the other hand, the higher concentrations of B, Cu and Fe were found in the roots, and Mn and Zn in the basal node of *P. maximum*.

Table 2
Effect of the available Cd concentrations, growth periods, plant tissues and their interactions on macronutrients and micronutrients concentrations of *Panicum maximum* established in non-polluted and Cd-polluted Oxisol

Available Cd concentrations (mg kg^{-1} soil)	**P**	**K**	**Ca**	**Mg**	**S**	**B**	**Cu**	**Fe**	**Mn**	**Zn**
	g kg^{-1} DW					**mg kg^{-1} DW**				
0.04 (control)	0.58 c	15.88 b	3.64 ab	2.11 ab	0.85 b	10.06 a	3.16 c	70.2	612.5 a	32.5
2.86	0.64 b	16.64 b	3.91 a	2.38 a	0.93 b	10.06 a	3.92 b	239.0	442.3 b	36.4
5.93	0.72 a	17.25 b	3.70 ab	2.37 a	0.97 b	8.22 b	4.07 b	75.2	541.9 ab	30.7
10.91	0.71 a	23.20 a	3.35 b	1.88 b	1.71 a	8.58 ab	5.94 a	69.4	474.6 b	36.0
Growth periods										
First (growth)	0.71 a	16.68 b	3.64	2.41 a	1.53 a	9.60	5.06 a	54.0 b	296.5 b	30.8
Second (regrowth)	0.62 b	19.80 a	3.66	1.96 b	0.69 b	8.87	3.49 b	173.0 a	739.2 a	36.8
Plant tissues										
Leaf I	0.71 ab	18.86 ab	3.31 c	1.71 d	1.10 ab	10.04 bc	4.78 cd	173.6 bc	433.0 bc	30.3 b
Leaf II	0.67 bc	18.19 ab	3.78 bc	1.87 cd	1.15 a	9.95 bc	4.28 cd	73.0 c	497.9 ab	27.9 b
Leaf III	0.61 cd	16.24 b	4.38 a	2.19 bc	1.22 a	10.70 b	3.76 cd	68.7 c	574.9 ab	26.0 b
Other leaves	0.75 a	18.19 ab	4.21 ab	2.56 ab	1.23 a	10.26 b	4.88 c	105.4 c	557.6 ab	39.4 b
Stems	0.56 de	19.72 a	2.57 d	2.60 a	0.87 c	5.21 d	3.66 d	146.6 c	525.8 ab	45.4 b
Basal node	0.50 e	6.43 c	1.75 e	1.18 e	0.73 c	7.15 cd	6.21 b	748.4 b	606.5 a	284.9 a
Roots	0.37 f	3.08 d	1.32 e	0.73 f	0.92 bc	18.16 a	9.08 a	3516.4 a	336.8 c	29.8 b
Statistical significance										

Different letters within the same column indicate differences between means (Tukey test, $p < 0.05$); significant effects for the main factors and for interaction between them are indicated with asterisks at the level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; ns = not significant

Available Cd concentrations (mg kg^{-1} soil)	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
	g kg^{-1} DW					mg kg^{-1} DW				
Cd concentration	***	***	**	***	***	**	***	ns	***	ns
Growth period	***	***	ns	***	***	ns	***	***	***	ns
Plant tissue	***	***	***	***	***	***	***	***	***	***
Cd concentration × Growth period	***	***	ns	ns	***	ns	***	ns	***	ns
Cd concentration × Plant tissue	ns	***	ns	*	ns	***	**	***	ns	***
Growth period × Plant tissue	***	***	**	**	***	ns	*	ns	***	ns
Cd concentration × Growth period × Plant tissue	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Different letters within the same column indicate differences between means (Tukey test, $p < 0.05$); significant effects for the main factors and for interaction between them are indicated with asterisks at the level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; ns = not significant

Cadmium exposure reduced the NUE of P, K, Ca, S, Cu, Fe, Mn and Zn compared to control (Table 3). However, there was no significant correlation between the NUE of each nutrient and the Cd BCFs (Supplementary Fig. 1). Only Mn-NUE was higher in the growth compared to regrowth period of *P. maximum*, whereas P, Ca, Mg, S, B, Cu and Zn presented higher NUEs at the end of the regrowth period. The highest NUEs for P, K, Ca, Mg, S, B, Cu, Mn and Zn were found in the roots (Table 3), followed by the lower plant parts and then the upper plant parts, differently from that observed for nutrients concentration (Table 2), when we analyzed the NUE of each nutrient within the plant tissues of *P. maximum*.

Table 3
Effect of the available Cd concentrations, growth periods, plant tissues and their interactions on macronutrients and micronutrients use efficiency (NUE) by *Panicum maximum* established in non-polluted and Cd-polluted Oxisol

Available Cd concentrations (mg kg^{-1} soil)	**P**	**K**	**Ca**	**Mg**	**S**	**B**	**Cu**	**Fe**	**Mn**	**Zn**
	g^2 mg^{-1}					**g^2 ng^{-1}**				
0.04 (control)	1.13 a	0.04 ab	0.17 ab	0.28	0.85 a	80	200 a	11 a	15 ab	19 ab
2.86	1.00 a	0.06 a	0.19 a	0.34	0.81 a	80	170 ab	11 a	19 a	20 a
5.93	0.82 b	0.03 ab	0.16 ab	0.22	0.73 a	80	140 b	8 ab	13 b	16 b
10.91	0.71 b	0.01 b	0.14 b	0.22	0.46 b	70	90 c	6 b	7 c	11 c
Growth periods										
First (growth)	0.62 b	0.02	0.12 b	0.18 b	0.34 b	50 b	100 b	9	17 a	15 b
Second (regrowth)	1.21 a	0.04	0.21 a	0.35 a	1.08 a	110 a	200 a	9	10 b	18 a
Plant tissues										
Leaf I	0.74 bc	0.03 b	0.16 b	0.33 b	0.60 cd	50 bc	120 bc	8 b	1 b	18 bcd
Leaf II	0.60 c	0.02 b	0.10 b	0.21 b	0.46 cd	40 bc	100 bc	6 bc	1 b	14 bcd
Leaf III	0.31 c	0.01 b	0.04 b	0.09 b	0.20 d	10 c	50 c	2 c	1 b	7 cd
Other leaves	0.95 bc	0.04 b	0.17 b	0.28 b	0.72 cd	60 bc	150 b	8 b	1 b	23 b
Stems	1.98 b	0.07 b	0.35 b	0.41 b	1.58 b	220 a	320 a	22 a	1 b	20 bc
Basal node	1.12 bc	0.11 b	0.34 b	0.53 b	0.90 c	80 b	100 bc	1 c	1 b	6 d
Roots	7.18 a	1.19 a	2.16 a	5.37 a	3.14 a	180 a	340 a	3 bc	12 a	89 a
Statistical significance										
Cd concentration	***	*	*	ns	***	ns	***	***	***	***

Different letters within the same column indicate differences between means (Tukey test, $p < 0.05$); significant effects for the main factors and for interaction between them are indicated with asterisks at the level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; ns = not significant

Available Cd concentrations (mg kg^{-1} soil)	P	K	Ca	Mg	S	B	Cu	Fe	Mn	Zn
	g^2 mg^{-1}					g^2 ng^{-1}				
Growth period	***	ns	***	***	***	***	***	***	***	**
Plant tissue	***	***	***	***	***	***	***	ns	***	***
Cd concentration × Growth period	***	ns	***	*	ns	*	ns	***	***	***
Cd concentration × Plant tissue	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
Growth period × Plant tissue	***	***	***	***	***	***	***	***	***	*
Cd concentration × Growth period × Plant tissue	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Different letters within the same column indicate differences between means (Tukey test, $p < 0.05$); significant effects for the main factors and for interaction between them are indicated with asterisks at the level of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$; ns = not significant

Discussion

Cadmium effect on biomass yield and tillering of P. maximum used for phytoextraction in mildly polluted Oxisol along of two successive shoot growths

The exposure of *P. maximum* to the highest Cd concentrations decreased both leaves and stems biomass yield in the growth period (Figs. 2A-B), but there was no reduction on the number of tillers per plant in this growth period (Fig. 3A). Thereby, the reduction observed on shoot biomass yield can be attributed to a Cd-induced reduced number of leaves and shortening of stems and leaves (Supplementary Fig. 2). As gibberellins regulate the stem elongation rate in grasses (Zhang et al. 2016), Cd may have repressed gibberellins synthesis in *P. maximum* by affecting the KNOTTED1- like homeodomain (KNOX) proteins. KNOX proteins either activate or repress gibberellins synthesis genes, modifying levels of active gibberellins in the meristems and boundary regions of grasses (Pautler et al. 2013), which is the tillers initiation region (Chrysler 1906). Cadmium-induced changes on shoot meristematic region are also pointed out as a factor to reduce the number and length of leaves in plants of the family *Poaceae*. The decreased leaf length Cd-induced in maize (*Zea mays*) grown in a mildly polluted soil (46.5 mg Cd kg^{-1} soil) was attributed to the lower number of meristematic cells, longer cell cycle duration and inhibition of cell elongation rate (Bertels et al. 2020).

During the regrowth, leaf and stem biomass yields of *P. maximum* exposed to the highest Cd concentrations did not differ from those plants of control treatment (Figs. 2A-B). These data indicate that *P. maximum* was able to cope with Cd-induced stress under prolonged exposure by adapting its mechanisms of tolerance against Cd-induced stress (for a comprehensive review we suggest Rabêlo et al. 2021a). Such assumption is supported by the fact the number of tillers increased during plant regrowth compared to growth period

(Fig. 3A), even the basal node (tiller initiation region) presenting high Cd concentrations (Fig. 4F). Moreover, there was not the trend of Cd accumulated in the basal node reduces the number of tillers during regrowth, differently from which was observed in the plant growth (Fig. 3B). It means that Cd probably is more harmful in the early stages of development of *P. maximum* grown in mildly polluted soils. Sunflower (*Helianthus annuus*) is also more susceptible to Cd-induced stress in the early stages of development because an uncontrolled Cd uptake that results in high Cd concentrations in its plant tissues (De Maria et al. 2013). However, as Cd concentrations in the leaf and stem tissues collected at the end of the growth and regrowth period were similar (Figs. 4A-E), other factors than Cd concentrations in the plant tissues limited the growth of *P. maximum* exposed to the highest Cd concentration in the first growth.

Interestingly, a decrease in Mg concentration in the leaves has been associated with plant protection against Cd-induced stress under prolonged exposure by improving the action of the antioxidant system (Chou et al. 2011; Hermans et al. 2011). Such mechanism possibly was employed by *P. maximum*, since lower Mg concentrations were observed in the leaves compared to stems, in the plants exposed to the highest Cd concentration compared to the other Cd concentrations, and in the regrowth compared to growth period (Table 2). This conferred higher tolerance to plants exposed to the highest Cd concentration in the regrowth period, but not in the growth period. Other nutritional adjustments occurred in *P. maximum* under Cd exposure (Table 2) and probably contributed for a higher Cd tolerance in the regrowth compared to growth period. Such nutritional adjustments are addressed in the next session.

As the number of tillers of *P. maximum* was higher in the regrowth compared to growth period (Fig. 3A), we can assume that plant density was higher in the regrowth period. Under such circumstance, plants show a clear increase in the stem fraction due to changes on carbohydrates allocation (Poorter et al. 2012). This explains why the stem biomass yield was higher and the leaf/stem ratio was lower in the regrowth than growth period (Figs. 2B and 3C). Furthermore, there was no reduction on basal node biomass under Cd exposure (Fig. 2B), which may have contributed for the higher tillering in the regrowth period, since the tillers grow up from the axillary buds located at the basal node of the plant (Chrysler 1906). As observed for basal node biomass, the root biomass of *P. maximum* did not decrease due to Cd exposure (Fig. 2D), even this structure presenting high Cd concentrations (Fig. 4G). Maybe such result is associated to the fact that *P. maximum* preferentially accumulates Cd bound to cell wall in the root apoplast (Rabêlo et al. 2021b). In this case, the deleterious effects of Cd are more noticeable on root length and root surface than root weight (Rabêlo et al. 2020b), since the thickening of the roots due to lignification and suberization (Lux et al. 2011) can compensate the root weight.

Distribution and accumulation of Cd within the plant tissues of P. maximum and its relationship with Cd phytoextraction efficiency and nutritional disorders

Cadmium concentration increased in all plant tissues of *P. maximum* as a consequence of Cd exposure, but our results suggest the existence of restrictive mechanisms on Cd translocation from lower to upper plant parts because Cd concentration followed a decreasing gradient in the sequence: basal node > stems > leaves (Fig. 4). During Cd translocation in the xylem, Cd^{2+} ions interact with the cell walls of xylem vessels and are partly adsorb on them (Sterckeman and Thomine 2020). Furthermore, Cd accumulated in the leaves

can be redistributed to other plant organs *via* phloem or even to roots where Cd could be excreted (Sterckeman and Thomine 2020). Thus, with exception of hyperaccumulators plants, a restriction on Cd translocation upwards is expected, mainly under higher Cd exposure. Cadmium translocation from roots to shoot was higher when *P. maximum* was grown in the Oxisol presenting the available Cd concentration of 2.86 mg kg^{-1} soil, but from this point there was a reduction on Cd TF (Fig. 5F). Our results are similar to those reported for lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), cauliflower (*Brassica oleracea*) and oat (*Avena sativa*), in which Cd concentrations were higher in the shoots than roots when plants were grown on low Cd-polluted soil, but Cd concentrations in the roots became higher than of the shoots when these plants were grown on more polluted soil (John 1973). Similarly to Cd TF, the higher Cd BCFs were observed when *P. maximum* was exposed to the available Cd concentration of 2.86 mg kg^{-1} soil, and from this point the Cd BCFs decreased (Figs. 5A-E). Although the two factors remained higher than 1 under Cd exposure, Cd TF and Cd BCFs decreased under the highest Cd concentrations in the Oxisol, indicating lowered phytoextraction efficiency in such conditions. The potential of phytoextraction tends to decrease when grass species are faced to more high Cd concentrations due to Cd-induced toxicity, such as nutritional disorders (Rabêlo et al., 2021a).

Nutritional disorders are common in grasses exposed to Cd (Rabêlo and Borgo 2016), which can decrease Cd phytoextraction efficiency (Rabêlo et al. 2020a), even the lower NUE of P, K, Ca, S, Cu, Fe, Mn and Zn observed in *P. maximum* under Cd exposure (Table 3) did not negatively correlating with Cd BCF (Supplementary Fig. 1). Changes on nutrients´ concentration and use are coupled to negative outcomes on the development of plants under Cd exposure, but there is evidence that nutritional adjustments are necessary for plants cope with Cd stress (for a review, see Carvalho et al. 2020). For instance, a reduction in leaves Mg concentration of plants exposed to Cd can improve the action of the antioxidant system (Chou et al. 2011; Hermans et al. 2011), making possible an increase on biomass yield (Carvalho et al. 2020), as observed in our study (Figs. 2A-B; Table 2). Another example is K, which is involved on biomass allocation due its role on carbohydrates loading into the phloem for long-distance transport. Under lower K concentration sucrose export into the phloem is reduced (Cakmak et al. 1994), but increased K^+ may promote sugar unloading in sink tissues and speed the conversion of sucrose to synthetic metabolites (Conti and Geiger 1982), which allow biomass yield. Thus, the increase in the stem biomass yield induced by the higher plant density in the regrown *P. maximum* (Figs. 2B and 3A), especially in those plants exposed to the highest Cd concentrations, possibly is associated with the increased K concentrations verified in the stems, in the regrown plants and in the plants exposed to the highest Cd concentration (Table 2).

The concentrations of P, S and Cu of *P. maximum* increased after Cd exposure (Table 2). Under high P concentrations, more P is accumulated in the root and may form insoluble phosphate precipitates with Cd in the cell wall and vacuoles, which prevents the transport of Cd to the protoplasm and xylem and inhibits the transport of Cd to the shoot (Guo et al., 2018). Indeed, Cd TF was reduced in *P. maximum* exposed to the available Cd concentrations of 5.93 and 10.91 mg kg^{-1} soil (Fig. 5F), where higher shoot P concentrations were found (Table 2). Plants under Cd exposure tends to uptake more S due its role on glutathione (GSH, γ-Glu-Cys-Gly) and phytochelatins [PCs, $(\gamma\text{-Glu-Cys})_n$-Gly, with n = 2-11] synthesis, which are peptides involved in plant tolerance against to Cd-induced stress (for a comprehensive review we suggest Gill and Tuteja

2011). Sulfur concentration was higher in the growth than regrowth period (Table 2), which makes sense, since Cd is more stored as chelates (*e.g.*, PC-Cd) in the vacuoles of plants in the early stages of development, whereas other detoxification mechanisms (*e.g.*, Cd bound to cell walls) are more employed under prolonged Cd exposure (Rabêlo et al. 2018, 2021a; Sterckeman and Thomine 2020). As an increase on antioxidant activity of *P. maximum* was speculated due to decreased Mg and increased S concentrations, an increase on Cu concentration due to Cd exposure, especially in the growth period (Table 2), makes sense since Cu is a cofactor of the enzyme superoxide dismutase (SOD, EC 1.15.1.1) (Gratão et al. 2005). Superoxide dismutases, such as the isoenzyme Cu/Zn-SOD, act as the first line of defense against reactive oxygen species by dismutating superoxide ($O_2^{\bullet -}$) in H_2O_2 (Gratão et al. 2005). In addition, the presence of Cu/Zn-SOD in the apoplast of spinach was positively correlated to sites of lignification (Ogawa et al. 1996). In this sense, is plausible to assume that the higher Cu concentration observed in the roots of *P. maximum* (Table 2) favored root lignification through the action of Cu/Zn-SOD in the root apoplast (main local of Cd storage in this species; Rabêlo et al. 2021b), which avoid a strong reduction on the root weight of plants exposed to the highest Cd concentrations in the Oxisol due to a root thickening (Fig. 2D).

The concentrations of B and Mn tended to decrease in the plant tissues of *P. maximum* exposed to the highest Cd concentrations, differently from which was observed for P, K, S and Cu (Table 2). In tomato (*Solanum lycopersicum*), Cd toxicity was related to B and Mn excess in leaves, in addition to the own Cd accumulation, since the symptoms of Cd toxicity in leaf tissues resembled those triggered by B and Mn excess (Carvalho et al. 2018). It is possible that *P. maximum* had decreased both B and Mn concentrations in its tissues as a strategy of adaptation to Cd-induced stress, as no visual symptoms similar to those triggered by B and Mn excess were observed in our study (Supplementary Fig. 2).

The most part of nutrients´ concentrations (Table 2), if not all, indicate that *P. maximum* cv. Massai poses strategies to cope with Cd-induced stress through nutritional adjustment (Carvalho et al., 2020). Even so, the NUE of P, K, Ca, S, Cu, Fe, Mn and Zn by *P. maximum* decreased under Cd exposure (Table 3). Lower NUEs in plants grown in polluted soils are expected due to phytotoxicity or internal adjustments which affect plant growth in such conditions (Baligar et al. 2001). Although there were no significant negative correlations between the NUEs and Cd BCFs (Supplementary Fig. 1), the data of NUE (Table 3) together with Cd TF and Cd BCFs (Fig. 5) support the statement of Rabêlo et al. (2021a), who described that the potential of phytoextraction of grass species faced to more high Cd concentrations tends to decrease due to Cd-induced toxicity.

Conclusions

Cadmium toxicity was stronger in the early stages of development of *P. maximum*, not by reduce the tillering but to induce a stem shortening. Tillering was not compromised by the high Cd accumulation in the basal node of *P. maximum*, suggesting that the deleterious effects of Cd are more related with processes involved on stem elongation than tiller initiation from the axillary buds located in the basal node of *P. maximum*.

P. maximum presented a clear restriction on Cd transport upwards from basal node, which was the main local of Cd accumulation in this grass. Such restriction was more evident in the plants exposed to the

highest Cd concentrations, and it was related to nutritional adjustments to cope with Cd-induced stress. Apparently, P, K, Mg, S and Cu are involved in processes that restrict Cd movement upwards and confer higher tolerance to Cd toxicity in *P. maximum*, but further studies are necessary to unravel the role of each one of these nutrients on Cd tolerance in this grass. The nutritional disorders Cd-induced did not negatively correlate with Cd BCF, but the data of NUE, Cd TF and Cd BCF suggest that phytoextraction efficiency of *P. maximum* decreases from the available Cd concentration of 5.93 mg kg^{-1} soil.

Declarations

Author contributions FHSR performed the experiment and drafted the original manuscript. FHS helped to collect the experiment, process the plant material and analyze the data. JL helped in the data analysis and manuscript editing. LRFA revised the manuscript and supervised the study. All authors contributed to the critical review of the manuscript and approved its final version.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material Not applicable.

Code availability Not applicable.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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quick development mutant of winter wheat (*Triticum aestivum* L.). Plant Physiol Biochem 107:228–236. https://doi.org/10.1016/j.plaphy.2016.06.008

Figures

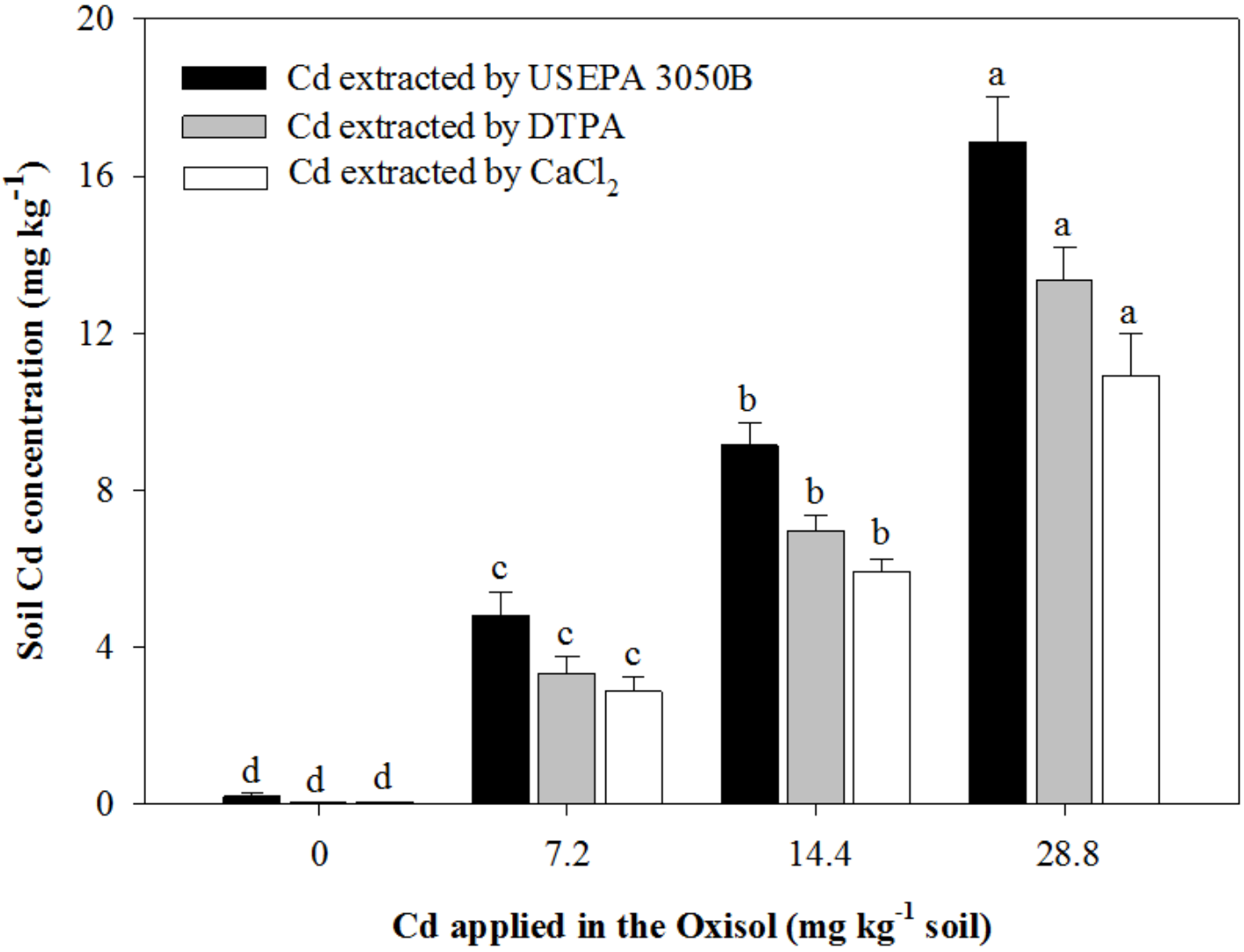

Figure 1

Cadmium concentration determined by USEPA 3050B, DTPA and 0.01 mol L^{-1} $CaCl_2$ methods in the Oxisol cultivated with *Panicum maximum* in function of the initial Cd doses applied. Distinct letters on the bars indicate difference between Cd concentrations for each Cd extractor (n = 4, Tukey test, $p < 0.05$). For the determination of Cd concentration extracted by DTPA [0.005 mol L^{-1} DTPA + 0.01 mol L^{-1} $CaCl_2$ + 0.1 mol L^{-1} triethanolamine (TEA), at pH 7.3] (Abreu et al. 2001), 5 g of soil were dispersed in 20 mL of DTPA solution, shaken for 2 h, and analyzed using an induced coupled plasma mass spectroscopy (ICP-MS, iCAP 7000 SERIES, Thermo Fisher Scientific, Waltham, USA). The concentrations of Cd extracted with 0.01 mol L^{-1}

$CaCl_2$ (Houba et al. 2000) were determined placing 4 g of soil in 50 mL centrifuge tubes and 40 mL of the extraction solution added. The samples were agitated for 2 h at 25 °C, centrifuged at 1800 ×g for 10 min, and filtered through filter paper (0.45 µm). Then, the extracts were analyzed by ICP-MS

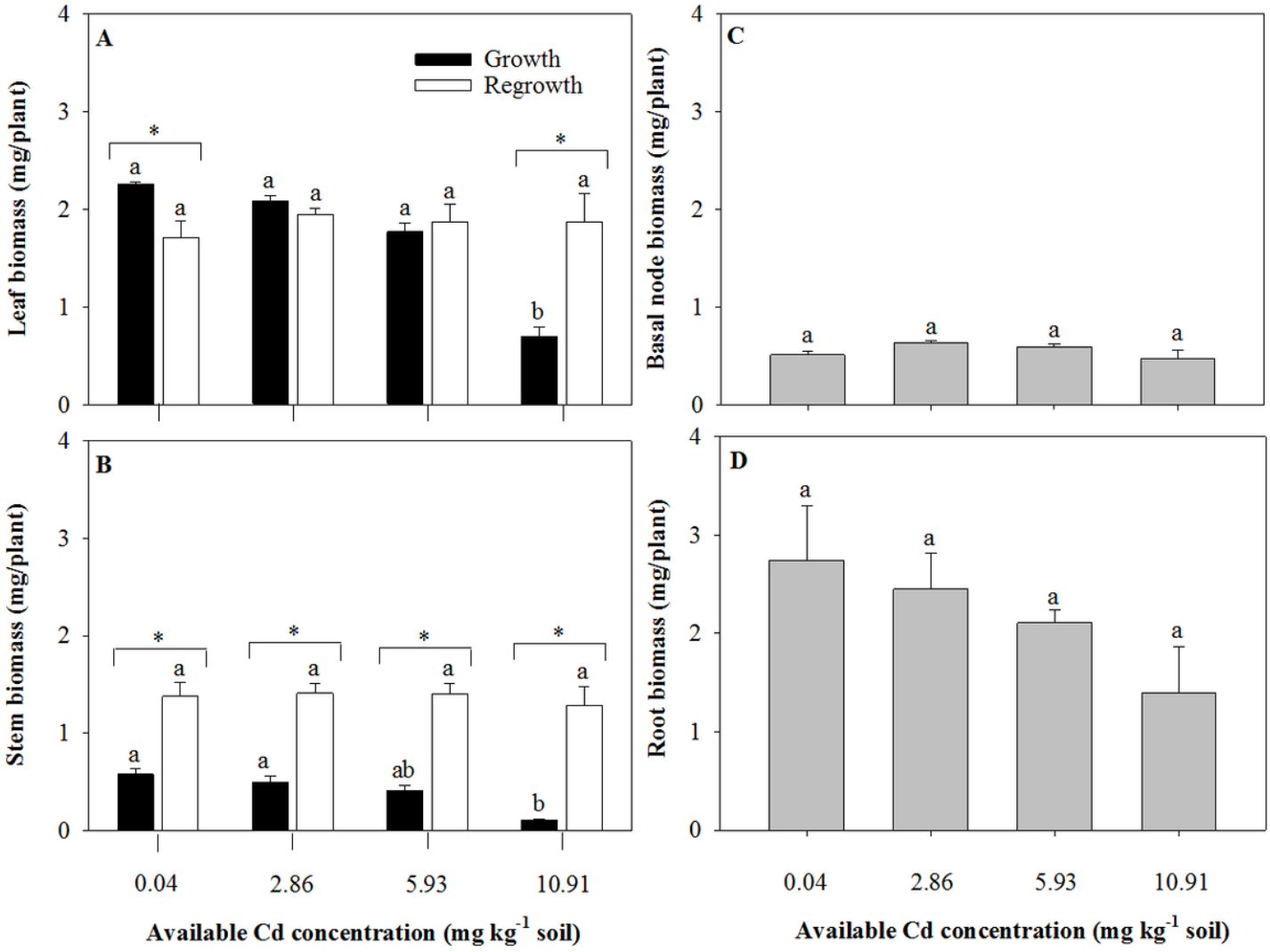

Figure 2

Biomass yield of leaves (**A**), stems (**B**), basal nodes (**C**) and roots (**D**) of *Panicum maximum* established in non-polluted and Cd-polluted Oxisol. Distinct letters on the bars indicate difference between Cd concentrations for each growth period of *P. maximum* (n = 4, Tukey test, $p < 0.05$). Asterisks represent differences at $p < 0.05$ between growth periods within each Cd concentration (ANOVA, t-test)

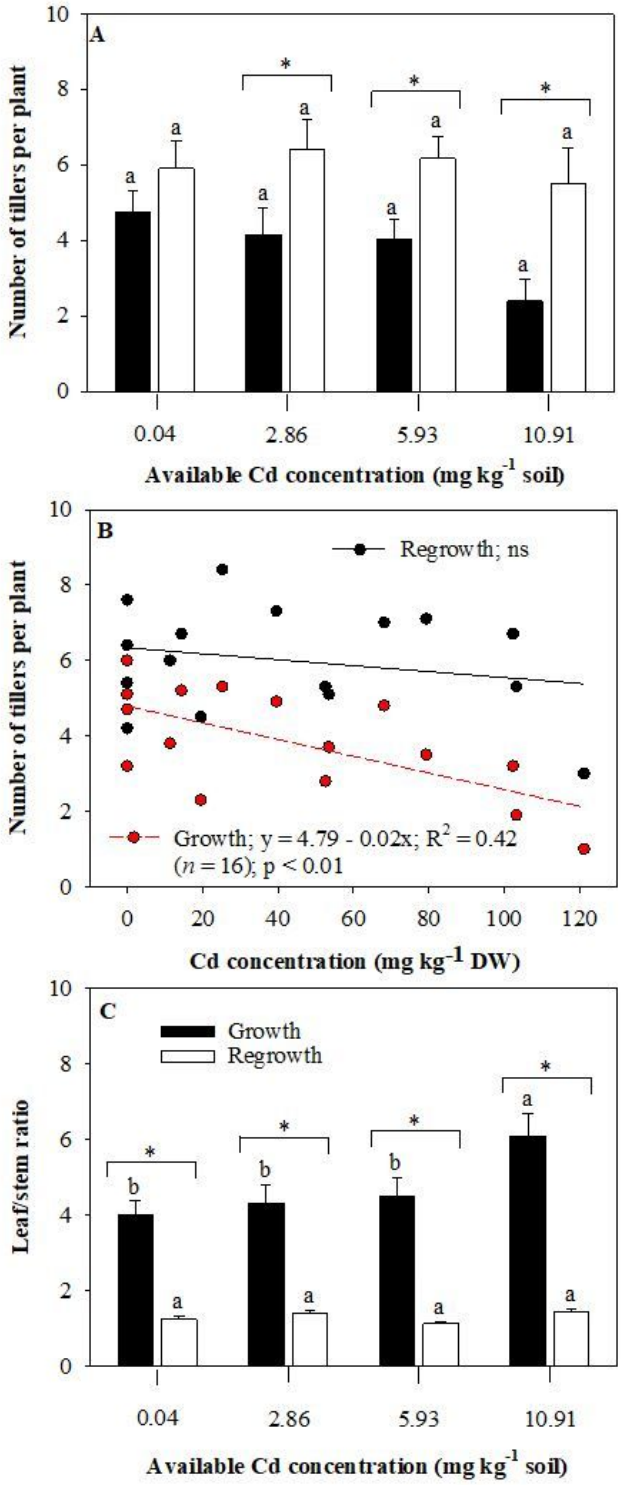

Figure 3

Number of tillers per plant (**A**), correlation between number of tillers per plant and Cd concentration in the basal node (**B**) and leaf/stem ratio (**C**) during the growth and regrowth of *Panicum maximum* established in non-polluted and Cd-polluted Oxisol. Distinct letters on the bars indicate difference between Cd concentrations for each growth period of *P. maximum* (n = 4, Tukey test, $p < 0.05$). Asterisks represent differences at $p < 0.05$ between growth periods within each Cd concentration (ANOVA, t-test). ns = not significant

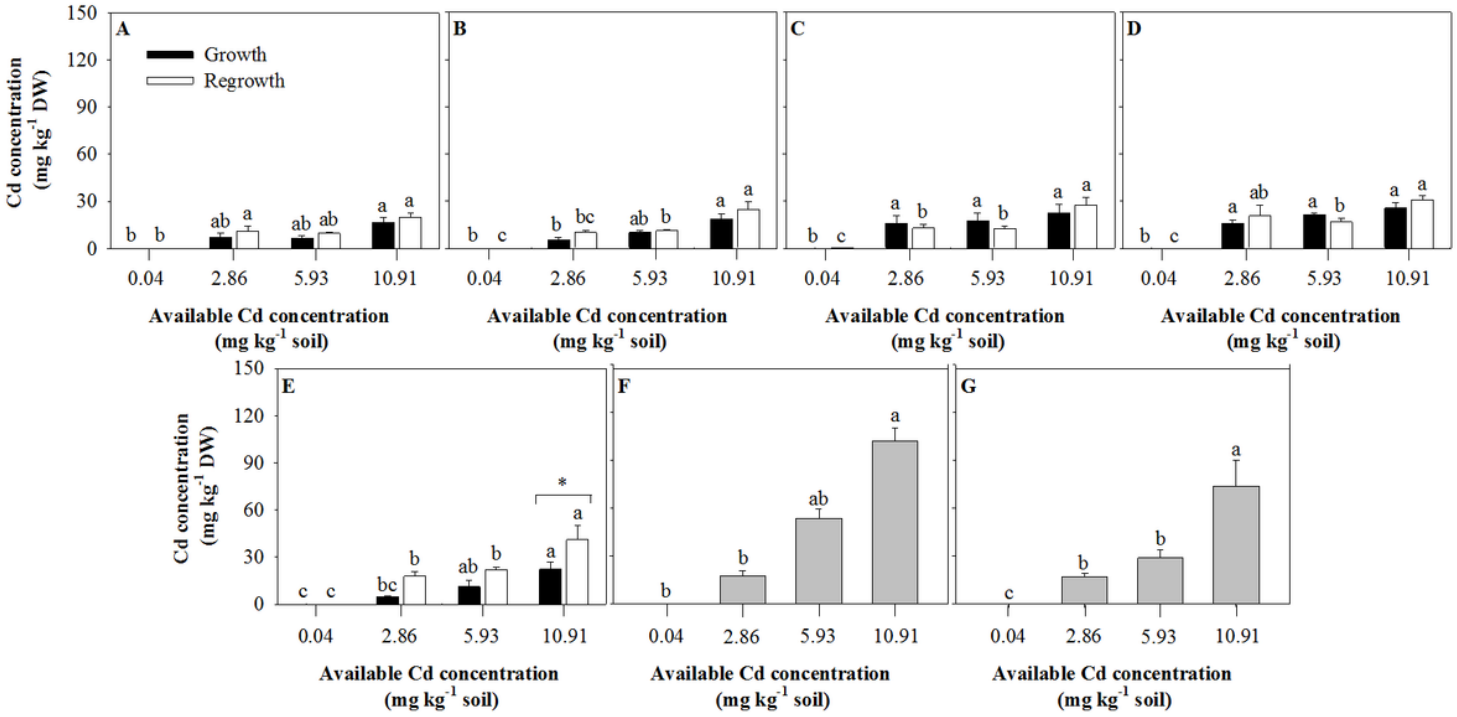

Figure 4

Cadmium concentration in the leaf I (**A**), leaf II (**B**), leaf III (**C**), other leaves (**D**), stems (**E**), basal node (**F**) and roots (**G**) of *Panicum maximum* established in non-polluted and Cd-polluted Oxisol. Distinct letters on the bars indicate difference between Cd concentrations for each growth period of *P. maximum* (*n* = 4, Tukey test, $p < 0.05$). Asterisks represent differences at $p < 0.05$ between growth periods within each Cd concentration (ANOVA, t-test)

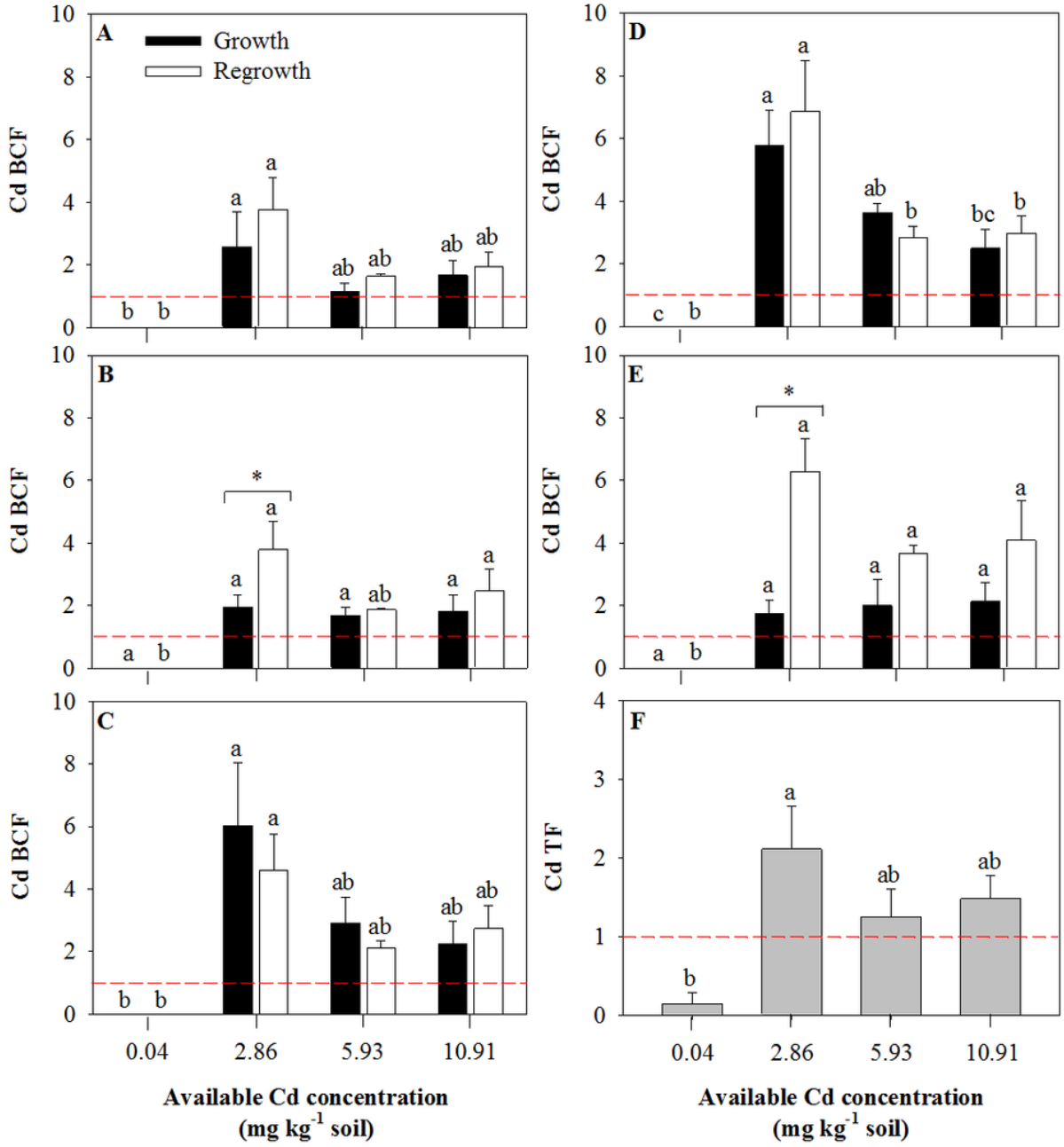

Figure 5

Bioconcentration factor - BCF in the leaf I (**A**), leaf II (**B**), leaf III (**C**), other leaves (**D**) and stems (**E**), and translocation factor - TF (**F**) of *Panicum maximum* established in non-polluted and Cd-polluted Oxisol. Distinct letters on the bars (**A**, **B**, **C**, **D** and **E**) indicate difference between Cd concentrations for each growth period of *P. maximum* (n = 4, Tukey test, $p < 0.05$). Asterisks represent differences at $p < 0.05$ between growth periods within each Cd concentration (ANOVA, t-test)

Supplementary Files

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