Diagnosis and Recommendation Integrated System and Nutritional Balance Index reveals nutritional disorders Cd-induced in *Panicum maximum* assayed for Cd phytoextraction

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ABSTRACT

The identification of nutritional disorders in plants induced by cadmium (Cd), based only on nutrient concentration, can fail and conceal links with biochemical and physiological events. In this study, the Diagnosis and Recommendation Integrated System (DRIS) and Nutritional Balance Index (NBI) were evaluated as auxiliary tools for diagnosing nutritional disorders in two *Panicum maximum* genotypes (Tanzania and Massai) which have contrasting behaviors for Cd translocation from roots to shoot. The correlation between nutritional disorders and Cd translocation in these plants was also checked. N, P, K, Ca, Mg, S, Cu, Fe, Mn and Zn concentrations in shoots of both grasses were extracted from studies previously published to form the database and develop DRIS, in which *P. maximum* cv. Tanzania was exposed to 0, 1 and 2 mmol L^{-1} of Cd, for eleven days, while *P. maximum* cv. Massai was exposed to 0, 0.1 and 0.5 mmol L^{-1} of Cd, for nine days. DRIS and NBI were obtained by calculations from nutrient concentrations in the shoot. Only P, S and Zn concentrations in the shoot of *P. maximum* cv. Tanzania and P, K, S, Cu and Fe in the shoot of *P. maximum* cv. Massai were Cd-disturbed from the point of view of nutrient concentration. However, DRIS revealed that the concentrations of Fe and Mn in the shoot of *P. maximum* cv. Tanzania exposed to Cd were considerably higher compared to other nutrients, enabling us to better understand certain biochemical and physiological Cd-induced events which occurred. Moreover, DRIS revealed that the Cd-induced nutritional disorders in the shoot of *P. maximum* cv. Massai were Cd-level dependent, and NBI confirmed that nutritional Cd-induced disorders in *P. maximum* increased when Cd translocation from roots to shoot was higher. In conclusion, DRIS and NBI revealed

Cd-induced nutritional disorders that had previously been obscure. Therefore, their use as auxiliary tools for diagnosing Cd-induced nutritional disorders is recommended.

KEYWORDS: DRIS; Forage grasses; Nutritional diagnosis; Nutrient use efficiency; Phytoremediation

Introduction

Cadmium (Cd) is a heavy metal that even in low concentrations can interfere with a series of physiological processes in plants, such as water uptake (Perfus-Barbeoch et al. 2002), root development (Rabêlo et al. 2018a), photosynthesis (Rabêlo et al. 2018b), and uptake, transport and assimilation of nutrients (Rabêlo et al. 2018a; Qin et al. 2020). Certain metabolic disorders (e.g., secondary metabolism and photosynthesis) can increase as a result of Cd disturbance in nutrient concentration and homeostasis depending on the species, variety and growth stage (Matraszek et al. 2016; He et al. 2017; Rabêlo et al. 2018b, 2018c). Küpper et al. (2007) stated that the effects of Cd on the photosynthesis of Cd hyperaccumulator *Thlaspi caerulescens* (also known as *Noccaea caerulescens*) strongly resemble those of Fe deficiency. Cd predominantly influences the transcriptional control of genes involved in the long-distance allocation of Fe in plants which results in Fe deficiency (Lešková et al., 2017).

Metabolic Cd-induced disorders suppress plant growth and can even lead to plant death. This fact is particularly worrying in plants assayed to clean up Cd-polluted environments, since phytoextraction efficiency is strongly dependent on biomass production (Vangronsveld et al. 2009). According to Li et al. (2019), the evaluation of characteristics related to plant growth that can be modified by heavy metal exposure is one of the hotspots of current research. Thus, it is essential to have a better comprehension of the Cd-induced metabolic disorders associated

with nutrient acquisition and homeostasis in plants assayed for Cd phytoextraction in order to optimize this low-cost and low-ecological footprint technology (Vangronsveld et al. 2009; He et al. 2017).

The small number of Cd hyperaccumulator plants and the difficulty in managing these plants has been a bottleneck that restricts their use for Cd phytoremediation (Li et al. 2019). For this reason, several non-hyperaccumulator plant species such as numerous forage grasses have been assayed for Cd phytoextraction potential (Rabêlo et al. 2018d). Among the grasses assayed, two genotypes of *Panicum maximum* (Tanzania and Massai) stood out as survivors of high Cd concentrations (Rabêlo et al. 2019, 2020a, 2020b; Sousa Leite and Monteiro 2019). Although *P. maximum* cv. Massai has higher Cd translocation from roots to shoot than *P. maximum* cv. Tanzania, high Cd concentrations in leaves can impair nutrient homeostasis and suppress plant growth (He et al. 2017; Qin et al. 2020) by reducing nutrient use efficiency - NUE (Borges et al. 2019).

In certain cases, it is not easy to identify Cd-induced nutritional disorders that suppress plant growth. Rabêlo et al. (2018a) reported that the growth of *P. maximum* cv. Massai was suppressed under Cd-induced stress, but the grasses exposed to 0.1 mmol L^{-1} of Cd had similar concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn and Zn in the leaf blades to those plants unexposed to Cd. In situations such as this, there may be a "concentration effect" of nutrients due to the suppression of biomass production which leads to a misinterpretation that there are no nutritional disorders (Jarrell and Beverly 1981). The use of NUE was also not efficient in identifying differences in Cd-induced nutritional disorders in contrasting tomato (*Solanum lycopersicum*) genotypes for Cd tolerance, since Cd-sensitive and Cd-tolerant genotypes had similar NUE values for P, K, Ca and S.

The Diagnosis and Recommendation Integrated System (DRIS) can be a helpful tool in identifying the Cd-induced nutritional orders (Matraszek et al. 2016), especially in genotypes

contrasting in Cd uptake. Elucidating the damage caused by heavy metals and the mechanisms involved in mitigating that damage in plants assayed for phytoremediation is a vital process in plant breeding, which is an important tool for increasing the phytoremediation potential of heavy metals (Li et al. 2019). DRIS uses the nutritional balancing concept (relationship between nutrients) for the interpretation of tissue analysis, which enables more precise detection of nutritional deficiencies and/or excesses induced by different stresses (Beaufils 1973). Matraszek et al. (2016) used DRIS to estimate Cd-induced nutritional disorders in lettuce (*Lactuca sativa*) and observed that DRIS enabled them to estimate the nutritional status of Cd-stressed plants with a high degree of accuracy. The Nutritional Balance Index (NBI) can be calculated from results obtained by DRIS (Hernandes et al. 2014). NBI can be an auxiliary DRIS tool that is capable of numerically measuring plant nutritional imbalances, in which the further values are from zero the higher the nutritional imbalance and the lower the biomass production (Mourão Filho 2004).

The use of DRIS has been previously established and validated for forage grasses (Bailey et al. 1997a, 1997b; Silveira et al. 2005a, 2005b). Thus, the goals of this study were i) to determine to what extent Cd disturbs N, P, K, Ca, Mg, S, Cu, Fe, Mn and Zn concentrations and the NUE in two genotypes of *P. maximum* (Tanzania and Massai) which have contrasting Cd translocation from roots to shoot; ii) to evaluate DRIS and NBI as auxiliary tools for diagnosing nutritional disorders in plants assayed for Cd phytoextraction; and iii) to check if there is correlation between nutritional disorders and Cd translocation from roots to shoot in *P. maximum* genotypes. The hypothesis was that a higher Cd translocation increases Cd-induced nutritional disorders more markedly by impairing nutrient homeostasis, which decreases the NUE and the biomass production by the grasses.

Materials and methods

Data used in the study

Certain previously published data were re-analyzed in which the authors evaluated the effect of sulfur (S) on mitigating Cd-induced stress in *P. maximum* Jacq. cv. Tanzania (Rabêlo et al. 2017a, 2017b) and *P. maximum* Jacq. cv. Massai (Rabêlo et al. 2018a, 2018b, 2018c, 2019). In these studies, both genotypes grown in a nutrient solution with 1.9 mmol L^{-1} of S had higher biomass production and Cd-tolerance compared to the other S levels assayed (0.1 and 3.7 mmol L^{-1}). Thus, in the current study, biomass production data and nutrient concentrations from plants that were grown with 1.9 mmol L^{-1} of S were used to evaluate nutritional Cd-induced disorders. Cd concentrations used in the studies mentioned above were different, and the reanalysis of data obtained under different conditions (including the level of Cd exposure and days under Cd exposure) is an important tool in the identification of trends in plants used for the phytoremediation of heavy metals (Li et al. 2019).

Plant growth, treatments, and experimental design

P. maximum Jacq. cv. Tanzania was germinated in trays containing sand and deionized water, in greenhouses (22°43″S; 47°38″W). Eleven days after sowing, 15 plants were transplanted to plastic pots (3.6 L of capacity) containing ground quartz as substrate and 1 L of a modified Hoagland and Arnon nutrient solution (1950) composed by 1 mmol L^{-1} KH_2PO_4, 4.5 mmol L^{-1} NH_4NO_3, 6 mmol L^{-1} KNO_3, 1 mmol L^{-1} KCl, 1.9 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} $CaCl_2$, 46 µmol L^{-1} H_3BO_3, 9 µmol L^{-1} $MnCl_2 \cdot 4H_2O$, 0.73 µmol L^{-1} $ZnCl_2$, 0.30 µmol L^{-1} $CuCl_2$, 0.11 µmol L^{-1} $H_2MoO_4 \cdot 4H_2O$ and 100 µmol L^{-1} Fe-EDTA and diluted at

25% of the ionic strength for seven days. The solution was added from the 8th to the 28th day after plant transplanting.

During this period, the plants were thinned periodically until six plants remained per pot. From the 29th day onwards after plant transplanting, Cd was added (0, 1 and 2 mmol L^{-1} applied as $CdCl_2$) to the nutrient solution, for 11 days to evaluate the effect of this metal on biomass production and mineral composition of leaf blades and roots. The solutions were replaced every seven days. During the first week after plant transplanting, the nutrient solutions were circulated through the substrate four times daily and remained in the pots during both the day and the night.

After the first week, nutrient solutions were circulated through the substrate three times daily and remained in the pots only during the day to allow root respiration. In the original study, plants were cultivated in two growth periods to study the effect of Cd on plant establishment (40 days of plant growth) and its residual effect on plant regrowth (18 days of plant growth). However, in the current study, only data from the rerooting of plants were used. Pots were prepared in a randomized block design with six replicates per treatment and six plants per replicate. For more information, see Rabêlo et al. (2017a, 2017b).

In a greenhouse, seeds of *P. maximum* Jacq. cv. Massai were germinated in a tray containing expanded vermiculite, which was irrigated with deionized water during the first 14 days, while the nutrient solution was added in the subsequent nine days to provide 0.1 mmol L^{-1} of S (diluted to 25% ionic strength). Twenty-three days after sowing, five plants were transplanted into each pot containing the undiluted nutrient solution (100% of the ionic strength) composed of 1 mmol L^{-1} KH_2PO_4, 4.5 mmol L^{-1} NH_4NO_3, 6 mmol L^{-1} KNO_3, 1.05 mmol L^{-1} KCl, 1.9 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} $CaCl_2$, 25 µmol L^{-1} H_3BO_3, 2 µmol L^{-1} $MnSO_4 \cdot H_2O$, 2 µmol L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.5 µmol L^{-1} $CuSO_4 \cdot 5H_2O$, 0.5 µmol L^{-1} $H_2MoO_4 \cdot 4H_2O$ (85% MoO_3) and 100 µmol L^{-1} Fe-EDTA for 21 days. After this

period Cd was added (0, 0.1 and 0.5 mmol L^{-1}; applied as $CdCl_2$) to the nutrient solution, for nine days, to evaluate the effect of this metal on biomass production and mineral composition of the leaf blades and roots. The solutions were replaced every seven days and remained continuously aerated. The pots used in the study were positioned in a randomized block design, with four replicates per treatment and five plants per replicate, over a plant growth period of 53 days. Readers are referred to Rabêlo et al. (2018a, 2018b, 2018c, 2019) for more details.

Plant material collection and determination of nutrients concentration

All leaf blades and roots from one plant per pot were collected at the end of the growth period of both genotypes to evaluate the effect of Cd on the mineral composition of leaf blades and roots. Samples were washed with deionized water to remove surface contamination, oven-dried at 60 °C for 72 h and ground in a Wiley type mill. The N concentration in the plant material from both grasses was determined using Kjeldahl's semi-micro method after the digestion, distillation and titration of sulphuric acid (Malavolta et al. 1997).

The concentrations of Ca, Mg, Cu, Fe, Mn and Zn in the tissues of *P. maximum* cv. Tanzania were determined by atomic absorption spectrophotometry (Analyst 400, Perkin Elmer, Waltham, USA) after nitric-perchloric digestion (HNO_3 65% and $HClO_4$ 70%), whilst K concentration was determined by flame photometry, P by colorimetry and S by turbidimetry (Sarruge and Haag 1974).

The extract to determine the concentrations of P, K, Ca, Mg, S, Cu, Fe, Mn, Zn and Cd in the tissues of *P. maximum* cv. Massai was obtained after nitric-perchloric digestion (HNO_3 65% and $HClO_4$ 70%). Blank reagent samples were used during digestion to verify lack of contamination. The concentrations of the elements in the tissues of *P. maximum* cv. Massai were determined by inductively coupled plasma optical emission spectrometry (ICP-OES,

iCAP 7000 SERIES, Thermo Fisher Scientific, Waltham, USA), as well as Cd concentration in the tissues of *P. maximum* cv. Tanzania. Blank reagent samples being used in this step too.

Calculation of Cd translocation factor (TF)

The Cd TF indicates the capacity of a plant to translocate Cd from roots to shoot and was calculated as described in equation 1 as follows (Ali et al. 2013):

$$\text{Cd TF} = Cd_{shoot}/Cd_{roots} \quad (1)$$

where Cd_{shoot} and Cd_{roots} were, respectively, the Cd concentrations determined in the shoot (specifically in the leaf blades) and the roots (mg kg^{-1} DW) at the end of the study.

Calculation of nutrient use efficiency (NUE)

The NUE (g^2 mg^{-1} for macronutrients and g^2 μg^{-1} for micronutrients) was calculated for each nutrient as described in equation 2 as follows (Siddiqi and Glass 1981):

$$\text{NUE} = \left[\frac{(\text{total dry biomass of the plant})^2}{\text{total nutrient content in the plant}}\right] \quad (2)$$

where total dry biomass of the plant was the dry weight of roots + shoot (g) determined at the end of the study. Total nutrient content in the plant (mg/plant for macronutrients or µg/plant for micronutrients) was obtained by multiplying the nutrient concentration (g kg^{-1} DW for macronutrients or mg kg^{-1} DW for micronutrients) in each tissue by the dry weight (g) of the respective tissue, and then adding in the nutrient content of the roots and shoot.

Procedures to calculate DRIS and NBI

The information used to form the database and develop DRIS were the concentrations of N, P, K, Ca, Mg, S (g kg^{-1} DW), Cu, Fe, Mn and Zn (mg kg^{-1} DW) in the leaf blade samples, and the shoot biomass production (leaf blades + stems and sheaths) of *P. maximum* cv. Tanzania and *P. maximum* cv. Massai which were determined after weighing the oven-dried plant material at 60 °C for 72 h. For the establishment of DRIS norms, the database was divided into two sub-populations: one with high shoot biomass production (or reference population) and another with shoot biomass production lower than 80% compared to the reference population. *P. maximum* cv. Tanzania and *P. maximum* cv. Massai unexposed to Cd were considered as the reference population, whereas plants presenting shoot biomass production lower than 3.3 g (this value corresponds to 80% of the shoot biomass production of the reference population for both genotypes) were referred to as the population of low-shoot biomass production (in this case, all plants exposed to Cd). This value was chosen somewhat arbitrarily such that maximum discrimination could be drawn between the populations of high- and low-shoot biomass while maintaining a reasonable number of observations in the population of high shoot biomass, as suggested by Walworth et al. (1986). Incidentally, the database size might not be directly related to standard quality, since too high generic DRIS norms (very sizeable database) can negatively affect the diagnosis efficiency. Notwithstanding the quantity of data, quality observation should be the goal when selecting the database (Mourão Filho 2004).

The mean, standard deviation and coefficient of variation of each nutrient as well as all the dual ratios between the nutrient concentrations were calculated for the reference population (plants unexposed to Cd, *x*) and population presenting shoot biomass production lower than 80% (plants exposed to Cd, *y*) for each genotype. In the calculations of the DRIS method, only

one type of expression is used to relate each pair of nutrients (N/P or P/N, for example) (Beaufils 1973). Thus, selected nutrient expressions for which the variance ratios (V*y*/V*x*) were large were chosen, which allow us to better differentiate between healthy and unhealthy plants (Singh et al. 2012). Once a set of norms had been developed, diagnoses were made on individual plant samples through the pair of nutrients selected in the previous step that were used to calculate unitless indexes for each plant through equations 3, 4 and 5 proposed by Beaufils (1973), using N as an example:

$$\text{N index}=\frac{\left[f\left(\frac{N}{P}\right)+f\left(\frac{N}{K}\right)+f\left(\frac{N}{Ca}\right)+f\left(\frac{N}{Mg}\right)\ldots+f\left(\frac{N}{Zn}\right)\right]}{9} \quad (3)$$

where, for instance, N/P is larger or equal to n/p:

$$f\left(\frac{N}{P}\right)=\frac{\left(\frac{N}{P}\right)-1}{\frac{n}{p}}\times\frac{1000}{CV} \quad (4)$$

or, when N/P is smaller than n/p:

$$f\left(\frac{N}{P}\right)=\frac{\left(1-\frac{N}{P}\right)}{\frac{n}{p}}\times\frac{1000}{CV} \quad (5)$$

In these equations, N/P is the nutrient ratio in the leaf blades of the plant to be diagnosed; n/p the optimum value or norm for that given ratio; CV the coefficient of variation associated with the norm; and nine the number of functions in the nutrient index composition. Values for other functions, such as *f*(N/K) and *f*(N/Ca) were calculated in the same way using appropriate norms and CV (Singh et al. 2012).

A positive index indicates sufficiency or excess of the nutrient under consideration while a negative index indicates insufficiency or deficiency (Beaufils 1973). Therefore, nutrient requirements can be ordered relative to one another. Then, the NBI was calculated from the absolute DRIS index for each nutrient, following equation 6 described by Hernandes et al. (2014) as follows:

$$NBI = |IN| + |IP| + |IK| + |ICa| + |IMg| + |IS| + |ICu| + |IFe| + |IMn| + |IZn| \quad (6)$$

Statistical analysis

The data of shoot and root biomass production, nutrient concentrations, Cd TF, NUE and NBI were submitted to analysis of variance (F test), and then, for significant results, the Tukey test was applied for multiple comparisons. The average of Cd TF and NBI between *P. maximum* cv. Tanzania and *P. maximum* cv. Massai were also compared by considering a randomized block design. The residuals' assumptions, normality and homoscedasticity, were checked for each model. The level of significance was set at 5% for all tests.

To investigate the relationship between Cd concentrations and shoot biomass production as well as Cd TF and NBI, and leaf Cd concentrations and NBI, several linear and non-linear regression models have been fitted, having been chosen to represent such relationships as those which presented the biggest coefficient of determination (R^2). All the statistical analysis was performed using R software version 3.0.2 (R Core Team 2019). Graphs were designed by using SigmaPlot (version 11.0, Systat Software Inc., San Jose, CA, USA).

Results

Comparison of Cd translocation factor in P. maximum cv. Massai and Tanzania

Cd concentrations in the leaf blades and roots of *P. maximum* cv. Tanzania exposed to 1 mmol L^{-1} of Cd were of 24 and 982 mg kg^{-1} DW, respectively, and only 9% of Cd absorbed was translocated from the roots to shoot (Figure 1). When *P. maximum* cv. Tanzania was exposed to 2 mmol L^{-1} of Cd, the concentrations of the element in the leaf blades and roots increased respectively by 17 and 28% compared to plants exposed to 1 mmol L^{-1} of Cd, but the percentage of Cd accumulated in the shoot, compared to Cd absorbed, decreased to 8%. On the other hand, *P. maximum* cv. Massai was exposed to lower Cd levels than *P. maximum* cv. Tanzania, but Cd translocation from roots to shoot was much higher in this genotype (Figure 1). Even *P. maximum* cv. Massai presented lower or similar Cd concentrations in the roots than *P. maximum* cv. Tanzania, Cd concentrations in the leaf blades of the plants exposed to 0.1 and 0.5 mmol L^{-1} of Cd were 135 and 983 mg kg^{-1} DW, respectively, and the percentages of Cd accumulated in the shoot, compared to total Cd absorbed, were 62 and 87%, respectively (Figure 1).

Relationship between biomass production and Cd translocation in P. maximum genotypes

Shoot biomass production of *P. maximum* cv. Tanzania exposed to 1 and 2 mmol L^{-1} of Cd decreased, respectively, by 22 and 44% compared to control (Figure 2A), whilst shoot biomass production of *P. maximum* cv. Massai exposed to 0.1 and 0.5 mmol L^{-1} of Cd decreased by 67 and 70% compared to control treatment, respectively (Figure 2B). These results are associated with Cd translocation from roots to shoot observed for each genotype. *P. maximum* cv. Tanzania had very low Cd TF (≤ 0.02), regardless of the increase in Cd level in the nutrient solution (Figure 2C). On the other hand, Cd TF in *P. maximum* cv. Massai was much higher

compared to *P. maximum* cv. Tanzania, especially when 0.5 mmol L^{-1} of Cd was added to the nutrient solution (Figure 2C). The lower Cd TF observed in *P. maximum* cv. Tanzania resulted in lower Cd concentration in their leaf blades compared to *P. maximum* cv. Massai, as was discerned when comparing Cd concentrations of the two genotypes exposed to the higher Cd levels (Figures 2D, E). The lower Cd concentration in the leaf blades of *P. maximum* cv. Tanzania resulted in a slow and linear decrease in shoot biomass production (Figure 2D), while the higher Cd concentration in the leaf blades of *P. maximum* cv. Massai resulted in a fast and exponential decay in shoot biomass production (Figure 2E).

Effect of Cd on nutrient concentrations and NUE of P. maximum genotypes

As was the case with the shoot biomass, the root biomass production of both genotypes decreased when the plants were exposed to Cd (Figure 3). This suppression in Cd-induced biomass production can change both nutrient uptake and NUE by plants. Consequently, the concentrations of N, P, K, Ca, Mg, S, Cu, Fe, Mn and Zn in the roots were determined, and the NUE of each nutrient for both grasses were calculated (Figure 3). Only the Fe concentration in the roots of *P. maximum* cv. Tanzania was affected by Cd exposure, in which plants exposed to 1 and 2 mmol L^{-1} of Cd had Fe concentrations much higher than plants not exposed to Cd, which indicates the formation of root Fe plaques. On the other hand, the NUE for this genotype was more disturbed when the plants were exposed to 2 mmol L^{-1} of Cd, with decreases in the NUE of N, P, Ca, Mg, Cu, Fe, Mn and Zn of 45, 51, 37, 35, 53, 62, 47 and 34% compared to control, respectively.

Differently from that observed for *P. maximum* cv. Tanzania, nutrient concentrations in the roots of *P. maximum* cv. Massai were strongly affected by Cd exposure. The concentrations of N, P, Cu and Fe increased, while Ca, Mg, Mn and Zn concentrations decreased in the roots

of *P. maximum* cv. Massai exposed to Cd compared to control, regardless of Cd levels. However, the root concentrations of K and S decreased only when *P. maximum* cv. Massai was exposed to 0.5 mmol L^{-1} of Cd (Figure 3). Regardless of the higher N, P, Cu and Fe concentrations in the roots of *P. maximum* cv. Massai, the NUE decreased when this genotype was exposed to both Cd levels. The NUE of N, P, K, Ca, Mg, S, Cu, Fe, Mn and Zn in the *P. maximum* cv. Massai exposed to 0.1 mmol L^{-1} of Cd decreased respectively in 70, 79, 71, 57, 56, 68, 78, 86, 50 and 70% compared to control (Figure 3).

DRIS and NBI to diagnose nutritional disorders in P. maximum genotypes exposed to Cd

Based on the results of Cd concentrations (Figure 1), shoot biomass production, Cd TF (Figure 2), root nutrient concentration and NUE (Figure 3), a clear distinction was observed in the responses to Cd due to the contrasting capacities of Cd translocation from roots to shoot in the two *P. maximum* genotypes. Consequently, a screening was undertaken to separate the plants of the two genotypes into two sub-populations: one with high shoot biomass production (plants unexposed to Cd) and the other with shoot biomass production lower than 80%, compared to plants unexposed to Cd. Thus, it was possible to compare the Cd-induced nutritional disorders in both contrasting genotypes of *P. maximum*.

To carry out the DRIS analysis, the nutrient expressions were chosen in cases where the variance ratios (V*y*/V*x*) were sizeable (Table 2), which allowed for better differentiation between healthy and unhealthy plants (Singh et al. 2012). The same number of nutrient expressions for each of the nutrients (N, P, K, Ca, Mg, S, Cu, Fe, Mn and Zn) was selected in this study to meet an orthogonal requirement of the mathematical model (Beaufils 1973). The exposure of *P. maximum* cv. Tanzania to 1 and 2 mmol L^{-1} of Cd-induced deficiencies of certain nutrients in the plants, especially S and Zn, whilst other nutrients such as Mn, P and Fe (Table

3) were present in excess. The order of nutrient limitation observed for *P. maximum* cv. Tanzania was less Cd-level dependent than for *P. maximum* cv. Massai. As examples, Mn and Mg were in deficiency while N and Zn were in excess compared to the other nutrients when *P. maximum* was exposed to a Cd level of 0.1 mmol L^{-1}. Conversely, in *P. maximum* cv. Massai exposed to 0.5 mmol L^{-1} of Cd, N and Zn were deficient, and Mn and Mg in excess (Table 3).

NBI was calculated from the DRIS indexes (Table 3) to verify if there was correlation of NBI with Cd exposure or Cd concentration in the leaf blades or Cd translocation from roots to shoot in the *P. maximum* genotypes. The NBI of *P. maximum* cv. Tanzania exposed to 1 mmol L^{-1} of Cd increased 74% compared to control, but not in the case of the NBI of the plants exposed to 2 mmol L^{-1} of Cd (Figure 4A). On the other hand, the Cd-induced nutritional disorders were much higher in *P. maximum* cv. Massai than in *P. maximum* cv. Tanzania, in which the NBI of plants exposed to 0.1 and 0.5 mmol L^{-1} of Cd increased 590 and 524%, respectively, compared to control (Figure 4A). There was positive correlation between the NBI and Cd concentrations in the leaf blades of both *P. maximum* genotypes (Figure 4B) and between the NBI and Cd TF (Figure 4C), in which the R^2 for this second correlation was higher (0.32 *vs*. 0.50; Figures 4B, C). Correlation between NBI and total Cd accumulated (roots + shoot), and that between NBI and Cd concentration in the roots were plotted but no significant correlations were found.

Discussion

Effect of Cd translocation in nutrient concentration in P. maximum genotypes

Cd translocation from roots to shoot is a biochemical and physiological process crucial to the achievement of an effective phytoextraction since harvesting the root biomass is generally not

feasible (Ali et al. 2013). Thus, *P. maximum* cv. Massai presented greater potential for use in Cd phytoextraction than *P. maximum* cv. Tanzania because of its higher Cd translocation from roots to shoot (Figures 1 and 2C). However, several factors other than Cd translocation from roots to shoot affect Cd phytoextraction efficiency, such as shoot biomass production under Cd-induced stress (Vangronsveld et al. 2009).

The shoot biomass of *P. maximum* cv. Massai was more strongly suppressed compared to *P. maximum* cv. Tanzania (Figures 2A, B), which can be attributed to higher Cd concentrations in its leaf blades (Figures 1 and 2D, E). Alterations in the chloroplast structure, stomatal conductance, leaf transpiration and inactivation of enzymes involved in the CO_2 fixation are the main Cd-induced factors that can suppress biomass production in plants of the *Poaceae* family (Kaznina and Titov 2014). In fact, Rabêlo et al. (2018c) observed a low number of chloroplasts, accumulation of starch grains and rupture of the tonoplast with disorders in the internal structures of the chloroplasts, and reduced stomatal conductance in *P. maximum* cv. Massai exposed to 0.5 mmol L^{-1} of Cd.

The exposure to Cd also impaired photosynthesis in *P. maximum* cv. Tanzania, as related by Sousa Leite and Monteiro (2019). As mentioned before, photosynthetic disorders can arise as a result of Cd-induced nutrient imbalance (Küpper et al. 2007; He et al. 2017; Rabêlo et al. 2018c). Therefore, a better comprehension of the metabolic disorders associated with acquisition, long-distance transport and nutrient homeostasis in plants assayed for Cd phytoextraction is essential.

Despite no specific Cd uptake mechanisms having been identified yet in plants, this metal is taken up and transported either actively or passively using membrane transporters of essential bivalent cations such as Ca, Fe, Mn and Zn in root cells (Küpper and Kochian 2010; Qin et al. 2020). Thereby, the competition between Cd and nutrients for the same transporters can reduce the nutrient concentration, as was the case for Ca, Mg, Mn and Zn in the roots of *P. maximum*

cv. Massai exposed to both Cd levels. On the other hand, Fe concentrations in the roots of both *P. maximum* genotypes exposed to Cd were much higher compared to control (Figure 3). Rabêlo et al. (2018a) reported that Fe was concentrated mainly on the root surface of *P. maximum* cv. Massai, indicating the formation of root Fe plaques.

The formation of Fe plaques is the result of an increase in radial oxygen loss in the roots. The release of oxygen from the root aerenchyma first oxidizes Fe^{2+} to Fe^{3+}, and then the Fe^{3+} precipitates on the root surface forming the root Fe plaques (Sebastian and Prasad 2016). However, the exposure of *P. maximum* cv. Massai to Cd resulted in an absence of aerenchyma in its roots (Rabêlo et al. 2018a). Thus, it is necessary to investigate further what brings about the formation of root Cd-induced Fe plaques in *P. maximum*. According to Ye et al. (1997), Fe plaques can adsorb Cu, and increase the concentration of this micronutrient in the plant roots, as had been the case with *P. maximum* cv. Massai (Figure 3). In contrast to this study, Kopittke et al. (2010) stated that the exposure of *Brachiaria decumbens* and *Chloris gayana* to Cd had no apparent influence on nutrient concentrations in the roots and shoot tissues other than Mn. This contrast observed in nutritional changes is closely associated with the ability of the plant species or genotypes to adapt to Cd-induced stress (He et al. 2017).

Interestingly, as observed in Fe and Cu, there were increases in N and P concentrations in the roots of *P. maximum* cv. Massai exposed to both Cd levels compared to control (Figure 3). N and P are involved in the synthesis of reduced glutathione - GSH (γ-Glu-Cys-Gly) and phytochelatins - PCs [$(\gamma\text{-Glu-Cys})_n$-Gly, in which n = 2-11] which are important thiol compounds related to Cd detoxification (Seth et al. 2012). Nitrogen is present in amino acid glutamate, cysteine and glycine requested for GSH synthesis, and P is present in the ATP molecule that provides energy to catalyze the synthesis of GSH (Seth et al. 2012). Glutathione is the main non-enzymatic antioxidant in plants, while PCs are synthesized from GSH are the main Cd-chelators (Clemens and Peršoh 2009; Seth et al. 2012).

Rabêlo et al. (2018b) reported that GSH and PCs synthesis were strongly Cd-induced in the roots of *P. maximum* cv. Massai, and speculated that in the roots of *P. maximum* cv. Tanzania exposed to Cd there was an increase in PCs synthesis (Rabêlo et al. 2017a). Phytochelatins act on the chelation of the free Cd^{2+} in the cytosol and then on its transporting from the cytosol to vacuoles that are organelles less sensitive to Cd toxicity (Seth et al. 2012). In higher plants, with the exception of Cd-hyperaccumulators, PCs synthesis is the main mechanism of Cd detoxification (Clemens and Peršoh 2009). Indeed, other metabolites involved in Cd-tolerance such as amino acids, sugars and organic acids were strongly employed in Cd detoxification in *P. maximum* only when the synthesis of GSH and/or PCs was lower (Rabêlo et al. 2017b, 2018b). However, the use of a high proportion of the metabolic energy in adapting to Cd-induced damages lead to a lower NUE and biomass production (Borges et al. 2019), as was the case with both *P. maximum* genotypes (Figures 2A, B and 3). Xie et al. (2014) reported that the metabolic profile of *Cynodon dactylon* was modified to mitigate Cd-induced stress in two genotypes contrasting in Cd-tolerance after 14 days of Cd exposure, which resulted in lower biomass production, especially in the Cd-sensitive genotype. Yang et al. (1996) stated that lower biomass production in Cd-sensitive genotypes is also strongly associated with the inhibition of the long-distance transport of nutrients, which lead to nutrient deficiency in the leaves (referred to as shoot from this point).

In this study, the Cd-induced changes in shoot nutrient concentration were stronger in *P. maximum* cv. Massai which had higher Cd translocation from roots to shoot (Figures 1 and 2C) than *P. maximum* cv. Tanzania (Table 1). The exposure of *P. maximum* cv. Massai to Cd resulted in higher P, K, S, Cu and Fe concentrations in its shoot compared to control (Table 1). Jiang et al. (2004) reported that the concentrations of P, K, Cu and Fe in the shoot of Indian Mustard (*Brassica juncea*) exposed to Cd were higher than the control. In cases of slowed plant growth due to toxic element exposure, certain nutrients can suffer a “concentration effect” that

has an increasingly detrimental effect on plant growth as well as its own toxic element (Jarrell and Beverly 1981).

Another hypothesis for the higher Cu and Fe concentrations observed in the shoot of *P. maximum* cv. Massai exposed to Cd is associated with the formation of Cd-induced Fe plaques in its roots (Figure 3; Rabêlo et al. 2018a), since Fe plaques can serve as a source of Fe to the shoot (Sebastian and Prasad 2016), which was probably the case in Cu adsorbed in the Fe plaques. Differently from that observed for *P. maximum* cv. Massai, only the P concentration increased in the shoot of *P. maximum* cv. Tanzania exposed to Cd compared to control, while the concentrations of S and Zn decreased (Table 1).

In graminaceous plants such as *P. maximum*, there is heightened Cd accumulation in the nodes (Fujimaki et al. 2010) that control nutrient distribution in these plants (Yamaji and Ma 2014). Thus, high Cd accumulation in the nodes of *P. maximum* cv. Tanzania impaired the distribution of both S and Zn to the shoot, which resulted in lower concentrations of S and Zn. Furthermore, the lower S concentration in the shoot of *P. maximum* cv. Tanzania can be associated with reduced root to shoot S-translocation due to higher S demand for PCs synthesis in the roots that were the main organ for Cd accumulation in this genotype (Figure 1). Matraszek et al. (2016) also found lower S concentration in the shoot than roots in lettuce exposed to Cd. In reference to the lower Zn concentration, Yamaji and Ma (2014) detailed that in graminaceous plants such as rice (*Oryza sativa*), Zn is preferentially distributed through phloem from the nodes by *HMA2* transporters that also present influx transport activity for Cd. Thus, high Cd concentrations in the nodes can disturb Zn distribution to the shoot and result in lower Zn concentrations.

DRIS and NBI as auxiliary tools for estimating nutritional disorders in P. maximum genotypes assayed for Cd phytoextraction

In plants that present slowed growth and biomass production due to exposure to toxic elements, certain nutrients can suffer a "concentration effect" and present similar concentrations to those of plants unexposed to the toxic elements (Jarrell and Beverly, 1981), and this often leads to a misinterpretation that there are no nutritional disorders, as observed by Rabêlo et al. (2018a) in *P. maximum* cv. Massai exposed to 0.1 mmol L^{-1} of Cd. DRIS is an alternative that can avoid misinterpretations of the nutritional status of plants exposed to Cd (Matraszek et al. 2016) and is a powerful tool created to cope with the difficulties inherent in foliar diagnostic procedures (Beaufils 1973; Walworth et al. 1986; Bailey et al. 1997a, 1997b; Silveira et al. 2005a, 2005b; Mourão Filho 2004; Singh et al. 2012).

DRIS uses the nutrient ratios instead of absolute and/or individual nutrient concentrations to interpret tissue analysis and allow for nutrient classification from the most deficient to the most excessive (Mourão Filho 2004). Values closer to zero indicate proper nutritional balance. Moreover, when DRIS is used together with NBI it is possible to detect limitations in biomass production due to nutrient imbalance, even when the nutrients are in apparently proper concentrations (Mourão Filho 2004; Hernandes et al. 2014).

Cd exposure resulted in lower or deficient S and Zn concentrations and in higher or excessive P concentrations in the shoot of *P. maximum* cv. Tanzania compared to control (Table 3), as previously revealed by the nutrient concentrations (Table 1). However, DRIS also showed that Fe and Mn concentrations in the shoot of *P. maximum* cv. Tanzania exposed to both Cd levels were in excess compared to the other nutrients (Table 3). Superoxide dismutase (SOD, EC 1.15.1.1), that acts on dismutation of superoxide ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and H_2O, can be activated by different metals, such as Mn (Mn-SOD) (Raychaudhuri and Deng 2000). Thus, the higher SOD activity observed in the shoot of *P. maximum* cv.

521 Tanzania exposed to Cd (Rabêlo et al. 2017b) could be associated with an excess of Mn (Table
522 3).

523 The products generated by the reaction catalyzed by SOD are H_2O_2 and H_2O
524 (Raychaudhuri and Deng 2000), and it is known that Fe^{2+} can interact with H_2O_2 to form
525 hydroxyl radicals ($^{\bullet}OH$) (Connolly and Guerinot 2002). Thus, Fe^{2+} excess can lead to
526 overproduction of $^{\bullet}OH$, which in turn can increase oxidative stress, lipid peroxidation and
527 photosynthesis impairment in plants of the *Poaceae* family (Pinto et al. 2016). Curiously, there
528 was an increase in Cd-induced oxidative stress in the shoot of *P. maximum* cv. Tanzania not
529 associated with the generation of H_2O_2 (Rabêlo et al. 2017a), although this could be associated
530 with an overproduction of $^{\bullet}OH$ Fe-induced from H_2O_2 (Pinto et al. 2016). This assumption was
531 totally obscure, and was revealed only by using DRIS. Thus, the use of this tool makes it
532 possible to identify C-d induced nutritional disorders that are quite difficult to recognize when
533 analyzing at data pertaining to nutrient concentration only.

534 From the data of nutrient concentration (Table 1), it was observed that the concentrations
535 of P, K, S, Cu and Fe in the shoot of *P. maximum* cv. Massai increased due to Cd exposure and
536 there was no Cd-induced reduction in the concentration of any nutrient. However, DRIS
537 revealed that certain nutrients were in deficient concentrations compared to others (Table 3).
538 There was a strong deficiency of Ca, Mn and Mg when *P. maximum* cv. Massai was exposed
539 to 0.1 mmol L^{-1} of Cd, and there was a deficiency of Zn, N and Cu when this genotype was
540 exposed to 0.5 mmol L^{-1} of Cd (Table 3). Calcium deficiency compromises cell extension and
541 membrane stabilization of plants, which can result in less leaf area (Marschner 2012;
542 Mehrabanjoubani et al. 2015), as recorded in *P. maximum* cv. Massai exposed to Cd by Rabêlo
543 et al. (2018c). Evidently, Cd toxicity can compromise cell extension *per se* by inducing the
544 rupture of cell membranes (Loix et al. 2017), but nutrient imbalance induced by toxic elements
545 have an additional detrimental effect on plant growth (Jarrell and Beverly 1981). As an

example, Cd can enter into the guard cells in competition with Ca^{2+} leading to stomatal closure, lower CO_2 conductance and inhibition of photosynthesis (Perfus-Barbeoch et al. 2002), and all these effects emerged on exposure of *P. maximum* cv. Massai to Cd (Rabêlo et al. 2018c).

Cd can replace Mg^{2+} in both chlorophyll *a* and *b*, which will degrade these molecules (Gillet et al. 2006). Consequently, an apparent Mg deficiency could facilitate its replacement by Cd^{2+} leading to chlorophyll degradation, as was also the case with *P. maximum* cv. Massai (Rabêlo et al. 2018c). The marked deficiency of Mn in the shoot of *P. maximum* cv. Massai compared to Mg (Table 3) can compromise the decarboxylation of oxaloacetate in the bundle sheath chloroplasts of C_4 plants by phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32) that have a requirement for Mn-ATP as a substrate rather than Mg-ATP (Burnell 1986). Thereby, limited Mn availability could decrease the efficiency of CO_2 assimilation, as was the case with *P. maximum* cv. Massai exposed to Cd (Rabêlo et al. 2018c).

The identification of the Cd-induced nutritional disturbance is quite difficult without auxiliary tools (in this study, DRIS and NBI). Kopittke et al. (2010) observed chlorosis of the veins in the leaves of *B. decumbens* and attributed this result to Cd-induced Mn deficiency, but the plants exposed to Cd presented similar or higher Mn concentrations than those observed in healthy plants. The changes revealed by DRIS in the order of nutrient limitation in the shoot of *P. maximum* cv. Massai were strongly Cd-level dependent, different from that observed in *P. maximum* cv. Tanzania (Table 3), which can be associated with Cd translocation from roots to shoot and shoot Cd concentration recorded for each genotype (Figures 1 and 2C, D, E). Rabêlo et al. (2019) observed that the accumulations of N, P, K, Mg, Cu and Fe in the shoot of *P. maximum* cv. Massai were strongly affected by Cd TF and shoot Cd concentration, and *vice-versa*. This result suggests that the Cd-induced nutritional disorders in the shoot of *P. maximum* cv. Massai tends to increase when there is higher Cd uptake and Cd translocation, as pointed out by NBI (Figures 4A, B, C). Thereby, the use of *P. maximum* cv. Massai for Cd

phytoextraction is recommended more for environments that present from low to moderate Cd-pollution (Rabêlo et al. 2020a), in order to avoid extensive nutritional imbalance that could lead to plant death.

Nutritional imbalance is the result of changes in nutrient ratios and often decreases the NUE by plants (Bailey et al. 1997a), as was observed in this study (Fig. 3). Matraszek et al. (2016) also reported that Cd induced changes in nutrient ratios in lettuce resulted in lower biomass production. Similarly, Cd-induced suppression in shoot biomass production in both *P. maximum* genotypes (Figures 1A, B, D, E) in this study was related to nutritional disorders (Figure 4A). Therefore, *P. maximum* cv. Tanzania that presented lower Cd translocation from roots to shoot than *P. maximum* cv. Massai is less susceptible to Cd-induced nutritional disorders (Figure 4C).

The simultaneous use of DRIS and NBI made possible the detection of limitations on biomass production in *P. maximum* due to nutrient imbalance, even when the nutrients were in apparently proper concentrations, as has already been pointed out by other researchers (Walworth et al. 1986; Bailey et al. 1997a, 1997b; Mourão Filho 2004; Singh et al. 2012; Hernandes et al. 2014). Nevertheless, to the best of our knowledge, this is the first study to propose the use of DRIS and NBI as auxiliary tools to identify nutritional disorders in plants assayed for Cd phytoextraction. These tools could be useful even in the screening of plants less susceptible to nutritional disorders in programs of genetic manipulation targeted at phytoremediation of metals.

Conclusions

P. maximum cv. Massai presented higher Cd translocation from roots to shoot than *P. maximum* cv. Tanzania and was more susceptible to Cd-induced nutritional disorders in terms of nutrient

concentrations and NUE. In this respect, the higher Cd translocation from roots to shoot favored Cd-induced nutritional disorders in *P. maximum* genotypes assayed for Cd phytoextraction.

The use of DRIS revealed Cd-induced disturbances that had not been previously observed when evaluating only the nutrient concentrations. DRIS revealed that the concentrations of Fe and Mn in the shoot of *P. maximum* cv. Tanzania exposed to both Cd levels were in excess compared to the other nutrients, which made it possible to link these Cd-induced changes to biochemical and physiological events that occurred in this genotype. Thus, the use of DRIS combined with nutrient concentrations made possible the identification of obscure Cd-induced nutritional disorders.

DRIS also revealed that the nutritional Cd-induced disorders in the shoot of *P. maximum* cv. Massai were Cd-level dependent, supported by NBI confirmation that nutritional Cd-induced disorders in *P. maximum* were associated with Cd translocation from roots to shoot.

NBI also revealed that the suppression of shoot biomass production is related to Cd-induced nutritional disorders, which opens a new gap in the use of forage grasses for Cd phytoextraction that merit investigation, since high Cd translocation from roots to shoot is desirable for Cd phytoextraction. However, high Cd translocation increases Cd-induced nutritional disorders, which in turn decreases the biomass production affecting the efficiency of phytoextraction.

The use of DRIS and NBI as auxiliary tools to evaluate Cd-induced nutritional disorders is recommended, and further studies are essential to validate their use in the diagnosing of nutritional disorders induced by heavy metals in forage grasses used to clean-up polluted environments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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