University of São Paulo "Luiz de Queiroz" College of Agriculture

Nitrogen forms and the mitigation of cadmium toxicity in tanzania guinea grass

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Dissertation presented for the degree of Master in Science. Area: Soils and Plant Nutrition

Piracicaba 2018 Tiago de Sousa Leite Agronomist

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1. Adubação nitrogenada 2. Amônio 3. Atividade fotossintética 4. Enzimas antioxidantes 5. Fitoextração 6. Metabolismo do nitrogênio 7. Nitrato 8. *Panicum maximum* I. Título DEDICATION

To my beloved parents, Antonia and Moacir (*in memoriam*), and my dearest girlfriend, Mara.

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# ABSTRACT

# Nitrogen forms and the mitigation of cadmium toxicity in tanzania guinea grass

Adequate nutrition plays a key role in the phytoextraction of soil contaminants and may influence the response of plants to heavy metal toxicity. Nevertheless, there is no information on the influence of nitrogen (N), particularly in the forms of nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>), on tanzania guinea grass (Panicum maximum cv. Tanzania) under cadmium (Cd) stress. Thus, in the present work, it was aimed to study the effect of these ions on the mitigation of Cd toxicity in this grass. Plants were grown in a greenhouse, using plastic pots containing nutrient solution and ground quartz as substrate. The experiment was laid out in a randomized complete block design, in a  $3 \times 3$  factorial arrangement, with six replications. The factors tested were  $NO_3^-/NH_4^+$  ratios (100/0, 70/30 and 50/50) and Cd rates (0.0, 0.5 and 1.0 mmol L-1). Morphological, physiological and biochemical changes in the plants were studied throughout two growth periods. In addition to causing oxidative stress and visual toxicity symptoms, Cd exposure resulted in reduced chlorophyll content and photosynthetic activity, negatively affecting shoot and root growth. Moreover, it altered N metabolism and induced the accumulation of NO3<sup>-</sup> and NH4<sup>+</sup> mainly in shoots, increasing plant total N concentration. The exclusive use of NO<sub>3</sub><sup>-</sup> mitigated toxicity symptoms by favoring Cd accumulation in roots, maintaining normal N metabolism and increasing guaiacol peroxidase activity. On the other hand, although the simultaneous supply of NO<sub>3</sub><sup>-</sup> and NH4<sup>+</sup> increased the uptake, transport and accumulation of this metal by the grass, it also increased the photosynthetic capacity along with the synthesis of proline and protein. The use of these N forms at a 50/50 ratio increased the tolerance of the plants to Cd by inducing high superoxide dismutase and glutathione reductase activities in shoots and roots, respectively, maintaining cellular homeostasis and reducing oxidative stress. Cd uptake and accumulation in tanzania guinea grass are strongly related to the form of N available, and the negative effects of this metal on photosynthesis and on the balance between oxidants and antioxidants are attenuated by the partial replacement of  $NO_3^-$  by  $NH_4^+$  in the nutrient solution.

Keywords: Ammonium; Antioxidant enzymes; Nitrate; Nitrogen fertilization; Nitrogen metabolism; *Panicum maximum*; Photosynthetic activity; Phytoextraction

## RESUMO

# Formas do nitrogênio e a mitigação da toxidez por cádmio no capim tanzânia

A nutrição adequada desempenha papel fundamental na fitoextração de contaminantes do solo e pode influenciar a resposta das plantas à toxidez por metais pesados. No entanto, não há informações sobre a influência do nitrogênio (N), particularmente nas formas de nitrato (NO<sub>3</sub><sup>-</sup>) e amônio (NH<sub>4</sub><sup>+</sup>), no capim tanzânia (Panicum maximum cv. Tanzânia) sob estresse por cádmio (Cd). Assim, no presente trabalho, objetivou-se estudar o efeito desses íons na mitigação da toxidez por Cd nesse capim. As plantas foram cultivadas em casa de vegetação, utilizando vasos plásticos contendo solução nutritiva e quartzo moído como substrato. O experimento foi arranjado em delineamento de blocos completos ao acaso, em esquema fatorial 3 × 3, com seis repetições. Os fatores testados foram proporções de  $NO_{3}^{-}/NH_{4}^{+}$  (100/0, 70/30 e 50/50) e doses de Cd (0,0, 0,5 e 1,0 mmol L<sup>-1</sup>). Foram estudadas alterações morfológicas, fisiológicas e bioquímicas nas plantas ao longo de dois períodos de crescimento. Além de causar estresse oxidativo e sintomas visuais de toxidez, a exposição ao Cd resultou em baixos teor de clorofila e atividade fotossintética, afetando negativamente o crescimento da parte aérea e das raízes. Além disso, ela alterou o metabolismo do N e induziu o acúmulo de NO<sub>3</sub><sup>-</sup> e NH<sub>4</sub><sup>+</sup> principalmente na parte aérea, aumentando a concentração de N total na planta. O uso exclusivo do NO3<sup>-</sup> mitigou sintomas de toxidez favorecendo o acúmulo de Cd nas raízes, mantendo o metabolismo normal do N e aumentando a atividade da guaiacol peroxidase. Por outro lado, embora o suprimento simultâneo de NO3<sup>-</sup> e NH4<sup>+</sup> tenha aumentado a absorção, o transporte e o acúmulo desse metal pelo capim, também aumentou a capacidade fotossintética juntamente com a síntese de prolina e proteína. O uso dessas formas de N na proporção de 50/50 aumentou a tolerância das plantas ao Cd induzindo altas atividades da superóxido dismutase e da glutationa redutase na parte aérea e nas raízes, respectivamente, mantendo a homeostase celular e reduzindo o estresse oxidativo. A absorção e o acúmulo de Cd no capim tanzânia estão fortemente relacionados à forma de N disponível, e os efeitos negativos desse metal na fotossíntese e no balanco entre oxidantes e antioxidantes são atenuados pela substituição parcial de NO3<sup>-</sup> por NH4<sup>+</sup> na solução nutritiva.

Palavras-chave: Adubação nitrogenada; Amônio; Atividade fotossintética; Enzimas antioxidantes; Fitoextração; Metabolismo do nitrogênio; Nitrato; *Panicum maximum* 

# **1. INTRODUCTION**

The area devoted to pasture cultivation in Brazil has increased over the last few decades, given that it constitutes the basis of ruminant production in the country [1, 2]. In the genus *Panicum*, the *P. maximum* species has been widely cultivated, in both tropical and subtropical regions, due mainly to its tolerance and adaptability to diverse edaphoclimatic conditions [3]. Tanzania guinea grass (*P. maximum* cv. Tanzania) is one of the most important cultivars, because of its agronomic and nutritional value. In addition to a rapid growth and high biomass production, this forage grass uses its extensive root system to regrow over successive cycles. These are some characteristics that indicate a high potential for phytoextraction of soil contaminants [1, 4].

Cadmium (Cd) is a heavy metal widespread in the environment and highly toxic to animals and plants [5]. This element has no known biological function and enters the root via transmembrane carriers responsible for the uptake of other divalent cations, such as Ca, Mg, Fe, Cu and Zn [6]. One of the factors determining the suitability of plants for phytoextraction purposes is the ability to accumulate a given contaminant in their aboveground biomass. Silva et al. [3] found that tanzania guinea grass effectively transports Cd from roots to shoots, increasing the concentration of this metal in leaves and culms. Consequently, it may induce physiological and biochemical dysfunctions and affect the overall plant growth. However, the response of plants to soil contaminants depends on several factors, including their nutrient status [7].

Apart from carbon, oxygen and hydrogen, nitrogen (N) is the most abundant element in plants. The adequate supply of this nutrient is indispensable for plant growth and development, since it is found in amino acids, proteins, nucleic acids, nucleotides and other organic compounds. In addition, N is required for the formation of new cells and tissues and is also essential for carbon assimilation [8]. Nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>) are the main inorganic forms of N absorbed by plants, which benefit from the simultaneous supply of these ions [9]. Nevertheless, although excess NH<sub>4</sub><sup>+</sup> is rapidly stored in the vacuoles, in large quantities it can cause toxicity by dissipating transmembrane proton gradients and affecting photosynthesis and respiration [8].

Santos et al. [9] studied the growth and productive responses of *P. maximum* cv. Aruana to six  $NO_3^-/NH_4^+$  ratios (100/0, 85/15, 70/30, 55/45, 40/60 and 25/75) and observed that the N use efficiency was improved with the use of ratios ranging from 70/30 to 55/45. There were increased shoot (numbers of tillers and leaves and leaf area) and root (length and surface area) growth in plants supplied with combinations of  $NO_3^-$  and  $NH_4^+$ , proving the influence of N

forms on the grass yield. The authors found that when N is supplied, absorbed and assimilated as  $NH_4^+$ , less energy is spent in the  $NO_3^-$  assimilation pathway. The energy saved could then be used in other metabolic processes, enhancing plant growth.

Several studies have addressed the effect of N on the phytoextraction of soil contaminants, and showed that this nutrient can influence their availability and uptake by plants [7, 10, 11]. In this context, the supply of  $NO_3^-$  and  $NH_4^+$  can help to mitigate the deleterious effects of Cd on perennial forage grasses. The use of strategies to stimulate the tolerance of tanzania guinea grass to heavy metals is of paramount importance for its agronomic management in contaminated sites [3, 12]. Adequate nutrition plays a key role in the phytoextraction of heavy metals [4, 13]. However, there is no information on the influence of N forms on this grass under Cd stress.

Therefore, morphological, physiological and biochemical responses of tanzania guinea grass grown with  $NO_3^-/NH_4^+$  ratios and exposed to Cd rates were evaluated. The hypothesis was that the supply of N in the form of adequate  $NO_3^-/NH_4^+$  ratios improves the plant nutrient status and enhances Cd phytoextraction. In addition, the simultaneous use of these N forms mitigates Cd toxicity, attenuating oxidative stress and improving the tolerance of the grass to this metal.

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# 2. NITROGEN FORM REGULATES CADMIUM UPTAKE AND ACCUMULATION IN TANZANIA GUINEA GRASS USED FOR PHYTOEXTRACTION

#### ABSTRACT

Plants benefit from the mutual uptake of nitrate (NO3-) and ammonium  $(NH_4^+)$ , and these ions are known to influence the bioaccumulation of heavy metals. Nevertheless, there are no studies on the effect of nitrogen (N) forms on forage grasses used for cadmium (Cd) phytoextraction. Therefore, the objective was to evaluate the development of tanzania guinea grass (Panicum maximum cv. Tanzania) under  $NO_3^{-}/NH_4^{+}$  ratios and Cd rates. A 3 × 3 factorial experiment was conducted under greenhouse conditions, in a randomized complete block design replicated three times. The factors consisted of three  $NO_3^-/NH_4^+$  ratios (100/0, 70/30 and 50/50) and three Cd rates (0.0, 0.5 and 1.0 mmol L<sup>-1</sup>). Changes in growth parameters, the activity of key enzymes of N metabolism, and plant nutrient status were studied. Although it increased plant total N concentration, Cd exposure reduced the chlorophyll content and negatively affected shoot and root growth. In addition, it altered N metabolism and induced the accumulation of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> mainly in shoots. The supply of 50/50 disturbed the glutamine synthetase activity and changed leaf and root morphology under Cd toxicity. However, while the exclusive use of NO<sub>3</sub><sup>-</sup> mitigated toxicity symptoms, by favoring Cd accumulation in roots and maintaining normal N metabolism in shoots and roots, plants grown with 50/50 showed increased uptake, transport and accumulation of this metal. Thus, Cd uptake and accumulation in tanzania guinea grass are strongly related to the form of N available, and the supply of 50/50 increases the phytoextraction of this metal by enhancing its uptake, rather than increasing plant growth.

Keywords: Ammonium; Cadmium toxicity; Nitrate; Nitrogen metabolism; Panicum maximum

### 2.1. Introduction

The deposition of heavy metals in the environment has attracted the interest of the scientific community in the search for alternatives that can minimize the impacts resulting from this process [1-3]. One of these alternatives is the cultivation of metal-accumulating plants, which promote the uptake and accumulation of soil contaminants in their biomass [4, 5]. The success of this technique relies on the use of species that are not only tolerant, but also able to show rapid growth, high biomass production and the capacity to concentrate the toxic element [6, 7].

Despite these requirements, the plants commonly used for phytoextraction purposes have low biomass production and need to be constantly replanted after harvesting, thus reducing the efficiency of the process [8]. In this sense, the use of perennial forage grasses in phytoremediation programs emerges as a promising alternative. Recent studies have demonstrated the potential of tanzania guinea grass (*Panicum maximum* cv. Tanzania) for remediation of barium- [9], copper- [10, 11] and cadmium-contaminated sites [7, 8]. This grass is easily established and has excellent yield potential, given its regrowth capacity, resistance to biotic and abiotic stresses and good response to fertilization [7, 8].

Nitrogen (N) is often a limiting factor for plant growth and yield [12], especially in tropical forage grasses [13]. Plants uptake N mainly in two forms, nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>) [14]. The uptake of NO<sub>3</sub><sup>-</sup> is an active process mediated by cotransporters in the roots, being its reduction to NH<sub>4</sub><sup>+</sup> dependent of photochemical energy [15]. Thus, the supply of N directly as NH<sub>4</sub><sup>+</sup>, with uptake and assimilation in this form, reduces energy expenditure by the plant, which can be used in other metabolic activities [11]. However, it is important to consider that such supply must be balanced with that of NO<sub>3</sub><sup>-</sup>, as a high availability of any of these ions may exceed the capacity of the plant to assimilate it [14]. This leads to their accumulation in the tissues and can ultimately cause toxicity.

The supply of N using  $NO_3^-/NH_4^+$  ratios results in increased plant growth, given that the simultaneous uptake and assimilation of these ions helps to maintain the balance between cations and anions in the plant [14]. For *P. maximum* cv. Aruana, the highest shoot growth and N use efficiency were observed in plants supplied with  $NO_3^-/NH_4^+$  ratios of 70/30 and 55/45 [13]. Moreover, the form of N applied to the soil is known to influence the availability of contaminants, as well as their uptake and accumulation in plants [16, 17]. The  $NO_3^-$  assimilation pathway is the main route for the synthesis of glutamate, which in turn is required for the synthesis of N compounds involved in biochemical mechanisms of adaptation to heavy metals [18].

Cadmium (Cd) is a heavy metal found naturally in the environment and considered one of the most hazardous to animals and plants [3], causing phytotoxicity even when present at low levels in the soil [19]. This metal can affect the balance between  $NO_3^-$  and  $NH_4^+$  in the plant by altering the activity of enzymes of N metabolism [18, 20, 21]. Nevertheless, despite the demonstrated influence of the supply of  $NO_3^-$  and  $NH_4^+$  on Cd toxicity [6, 22], no studies have addressed the effect of these ions on tanzania guinea grass used for Cd phytoextraction.

Therefore, the objective was to evaluate the development of tanzania guinea grass under  $NO_3^-/NH_4^+$  ratios and Cd rates. Changes in growth parameters, the activity of key enzymes of N metabolism, and plant nutrient status were studied, relating the availability and uptake of Cd and N forms to the observed effects. It was hypothesized that, by increasing plant growth, the simultaneous supply of  $NO_3^-$  and  $NH_4^+$  enhances Cd phytoextraction.

## 2.2. Material and Methods

## 2.2.1. Plant material and growth conditions

Tanzania guinea grass was grown under greenhouse conditions during the summer season. A  $3 \times 3$  factorial experiment was conducted in a randomized complete block design replicated six times. The factors consisted of three NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios (100/0, 70/30 and 50/50) and three Cd rates (0.0, 0.5 and 1.0 mmol L<sup>-1</sup>). Growth parameters were evaluated in three replications, while the other three were used for metabolic analyses.

Seeds were germinated in washed sand and the seedlings were irrigated exclusively with deionized water for two weeks, and then transplanted to 3.6 L plastic pots filled with ground quartz and nutrient solution (Table 1) [23]. During the first week after transplanting, plants were thinned to five per pot and provided with 1/5 strength nutrient solution, after which a full strength solution was used. The N concentration was set at 15 mmol L<sup>-1</sup> throughout the experimental period, while the Cd rates were applied at 28 days after transplanting.

$NO_3^-/NH_4^+$ ratio	100/0	100/0	100/0	70/30	70/30	70/30	50/50	50/50	50/50
Cd rate (mmol L <sup>-1</sup> )	0.0	0.5	1.0	0.0	0.5	1.0	0.0	0.5	1.0
Stock solution	Volume (mL L <sup>-1</sup> )								
$CdCl_2 (1 mol L^{-1})^a$	-	0.5	1	_	0.5	1	-	0.5	1
$KH_2PO_4 (1 \text{ mol } L^{-1})$	1	1	1	1	1	1	1	1	1
KNO <sub>3</sub> (1 mol L <sup>-1</sup> )	5	5	5	2	2	2	-	-	_
KCl (1 mol L <sup>-1</sup> )	2	2	2	5	5	5	7	7	7
$Ca(NO_3)_2 (1 \text{ mol } L^{-1})$	5	5	5	2	2	2	_	_	_
$CaCl_2 (1 mol L^{-1})$	_	_	_	3	3	3	5	5	5
$MgSO_4 (1 mol L^{-1})$	2	2	2	2	2	2	2	2	2
$NH_4NO_3 (1 \text{ mol } L^{-1})$	_	_	-	4.5	4.5	4.5	7.5	7.5	7.5
Micronutrients -Feb	1	1	1	1	1	1	1	1	1
Fe-EDTA (90 µmol L <sup>-1</sup> )	2	2	2	2	2	2	2	2	2
DCD (7 mmol L <sup>-1</sup> ) <sup>c</sup>	1	1	1	1	1	1	1	1	1

**Table 1.** Composition of the nutrient solutions provided to *Panicum maximum* cv. Tanzania in the experiment with three  $NO_3^-/NH_4^+$  ratios and three Cd rates.

<sup>a</sup> Applied at 28 days after transplanting. <sup>b</sup> Composition (µmol L<sup>-1</sup>): H<sub>3</sub>BO<sub>3</sub> = 46; MnCl<sub>2</sub> = 9; ZnCl<sub>2</sub> = 0.73; CuCl<sub>2</sub> =

0.3; Na<sub>2</sub>MoO<sub>4</sub> = 0.08. <sup>c</sup> Nitrification inhibitor (DCD - dicyandiamide).

The nutrient solution was aerated by circulating it through the substrate three to six times a day (depending on the growth stage) and replaced biweekly. Whenever needed, deionized water was added to the pots to compensate for evapotranspiration losses. The experiment was conducted for two growth periods, which were defined based on the development of the grass in response to Cd toxicity. The first one lasted for 33 days after transplanting, when shoots were harvested and plants provided with a new nutrient solution (without Cd). The second period comprised 27 days after the first harvest and plants were subjected only to the residual effect of Cd in the substrate.

#### 2.2.2. Visual diagnosis and relative chlorophyll index (RCI)

Throughout the experimental period, annotations and images were used to record any visible changes in the plants. These included symptoms of Cd toxicity, as well as signs of its mitigation by the supply of  $NO_3^-$  and  $NH_4^+$ .

Immediately before each harvest, the RCI was determined using a SPAD-502 Plus chlorophyll meter (Spectrum technologies, Plainfield, IL, USA). The RCI values were obtained by the average of 10 readings performed in the middle third of newly expanded leaves (diagnostic leaves) of each experimental unit [24].

# 2.2.3. Assessment of plant growth, morphology and yield

At the end of each growth period, tillers and leaves were counted and leaf area was measured using a LI-3100C area meter (LI-COR Biosciences, Lincoln, NE, USA). At the first harvest, shoots were cut at 4 cm above the substrate surface and allowed to regrow. However, at the second harvest, cutting was performed at the substrate level and roots were also collected. About 20% of the fresh roots were immersed in a gentian violet solution and scanned for calculation of root length and surface area, using the SIARCS software (version 3.0) [25]. Shoots and the remaining root system were packed in paper bags and placed in a forced-air drying oven at 65 °C for 72 h for dry weight determination and further laboratory analyses.

## 2.2.4. Enzymes of N metabolism

# 2.2.4.1. Nitrate reductase (NR, EC 1.7.1.1)

Twelve hours before sampling, plants were transferred to a growth chamber in order to ensure uniform light conditions. The NR activity was determined in vivo based on the reduction of  $NO_3^-$  to  $NO_2^-$  in 4 mL of 100 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.5), containing 250

mmol  $L^{-1}$  KNO<sub>3</sub> and provided with 0.2 g of finely cut diagnostic leaves [11]. After 2 h in a water bath at 35 °C, the extracts were homogenized with 58 mmol  $L^{-1}$  sulfanilamide, 770 µmol  $L^{-1}$  *N*-(1-Naphthyl)ethylenediamine dihydrochloride and 2 mol  $L^{-1}$  sodium acetate, and the NO<sub>2</sub><sup>-</sup> concentration was measured colorimetrically at 540 nm.

# 2.2.4.2. Glutamine synthetase (GS, EC 6.3.1.2)

The production of  $\gamma$ -glutamyl hydroxamate was used to estimate GS activity [26]. Shoot and root samples were wrapped in aluminum foil, frozen in liquid N<sub>2</sub> and stored in a freezer at -80 °C. Then, 0.5 g of the frozen material was macerated in 1.5 mL of extraction buffer (50 mmol L<sup>-1</sup> Tris-HCl, 2 mmol L<sup>-1</sup> mercaptoethanol and 1 mmol L<sup>-1</sup> EDTA; pH 7.5) and centrifuged at 10,000 rpm for 10 min at 4 °C. A 300 µL aliquot of the supernatant was homogenized with 500 µL of 200 mmol L<sup>-1</sup> Tris-HCl, 200 µL of 50 mmol L<sup>-1</sup> ATP, 500 µL of 500 mmol L<sup>-1</sup> glutamic acid, 100 µL of 1 mol L<sup>-1</sup> MgSO<sub>4</sub>, 300 µL of 100 mmol L<sup>-1</sup> hydroxylamine and 100 µL of 100 mmol L<sup>-1</sup> cysteine, and placed in a water bath at 30 °C for 30 min. The  $\gamma$ -glutamyl hydroxamate produced was determined colorimetrically at 540 nm after a 1:1 dilution with a solution of 200 mmol L<sup>-1</sup> FeCl<sub>3</sub>, 500 mmol L<sup>-1</sup> TCA and 330 mmol L<sup>-1</sup> HCl.

# 2.2.5. NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, total N and Cd concentrations in plant tissues

The concentrations of  $NO_3^-$ ,  $NH_4^+$ , total N and Cd were determined in the shoot and root dry matter. For  $NH_4^+$  and  $NO_3^-$ , a 1 g sample was shaken in 15 mL of 1 mol  $L^{-1}$  KCl, subjected to steam distillation with 0.2 g of calcined MgO and 0.2 g of Devarda's alloy, respectively, and titrated with 2.5 mmol  $L^{-1}$  H<sub>2</sub>SO<sub>4</sub> [27]. The total N in 0.1 g samples was determined by the Kjeldahl method, using sulfuric acid digestion followed by steam distillation with 25 mL of 10 mol  $L^{-1}$  NaOH and titration with 5 mmol  $L^{-1}$  H<sub>2</sub>SO<sub>4</sub> [28]. For Cd, a 0.5 g sample was subjected to nitric-perchloric digestion and the concentration in the extract was determined using inductively coupled plasma optical emission spectrometry (ICP/OES) [8]. The accumulation of this metal in the plant was calculated by multiplying the Cd concentration in shoots and roots by their respective dry weight.

# 2.2.6. Statistical analysis

The data were subjected to two-way analysis of variance and means were compared by Tukey's test at the 5% level, using the Assistat software (version 7.7) [29]. For significant interaction effects,  $NO_3^-/NH_4^+$  ratios were compared within each Cd rate, as well as Cd rates

compared within each  $NO_3^-/NH_4^+$  ratio. When no significant interaction was observed, each factor was analyzed independently.

#### 2.3. Results

## 2.3.1. Visual symptoms and RCI

Five days after Cd application, based on visual symptoms, the plants were subjectively classified as slightly, moderately or highly susceptible to Cd toxicity. Plants grown exclusively with  $NO_3^-$  and subjected to 0.5 mmol  $L^{-1}$  Cd did not differ from those grown in the absence of the metal (control), being only slightly susceptible to 1.0 mmol  $L^{-1}$  Cd. On the other hand, plants supplied with 70/30 showed to be severely affected by Cd exposure and were classified as moderately and highly susceptible to 0.5 and 1.0 mmol  $L^{-1}$  Cd, respectively. An intermediate response was observed with the supply of 50/50. Visual symptoms included wilting, leaf rolling, chlorosis and the formation of reddish-brown spots in diagnostic leaves (Fig. 1), whose intensities varied with the level of susceptibility. Throughout the second growth period, stunted growth was the main visual symptom observed in all plants subjected to Cd.



**Fig. 1.** Wilting and leaf rolling (a), followed by chlorosis and the formation of reddish-brown spots in diagnostic leaves (b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates.

The RCI of diagnostic leaves at the first harvest decreased proportionally to the availability of Cd in the nutrient solution (Fig. 2a). Plants subjected to 1.0 mmol  $L^{-1}$  Cd had a RCI 25.1% lower compared with those of the control. At the end of the second growth period, the RCI was significantly influenced only by the NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios (Fig. 2b). In this case, the highest RCI was observed in plants supplied with combinations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, although with 70/30 they did not differ from those grown with 100/0.



**Fig. 2.** RCI of diagnostic leaves (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05).

## 2.3.2. Plant growth, morphology and yield

At the first harvest, the number of tillers varied as a function of both  $NO_3^-/NH_4^+$  ratios and Cd rates, but the interaction  $NO_3^-/NH_4^+$  ratios × Cd rates was not significant. Regarding N forms, plants supplied with 70/30 produced over 25.6% more tillers than with any other ratio (Fig. 3a). As for Cd, application of this metal reduced tillering by up to 30.2%, as observed at the highest rate tested (Fig. 3b). Control plants had similar number of tillers at the second harvest, regardless of  $NO_3^-/NH_4^+$  ratios (Fig. 3c). In contrast, exposure to 0.5 mmol L<sup>-1</sup> Cd caused the number of tillers in plants supplied with 50/50 to be 57.9% higher than in those grown with 100/0. Nevertheless, only plants supplied exclusively with  $NO_3^-$  were able to maintain normal tillering at 1.0 mmol L<sup>-1</sup> Cd.

The response pattern of the number of leaves at the end of both growth periods was analogous to that found for the number of tillers. Thus,  $NO_3^-/NH_4^+$  ratios and Cd rates had independent effects at the first harvest, when the highest number of leaves was observed in plants supplied with 70/30 (Fig. 4a) and not subjected to Cd (Fig. 4b), respectively. Nonetheless, at the second harvest, the difference between plants supplied with 100/0 and 50/50 and subjected to 0.5 mmol L<sup>-1</sup> Cd was even greater, and the number of leaves in the latter was more than two-fold higher than in the former (Fig. 4c). Furthermore, there was no significant difference between plants exposed to 1.0 mmol L<sup>-1</sup> Cd, which negatively affected the number of leaves in all  $NO_3^-/NH_4^+$  ratios.



**Fig. 3.** Number of tillers (a-c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **c**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.



**Fig. 4.** Number of leaves (a-c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **c**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

The leaf area was compared with the numbers of tillers and leaves, and it was found to be proportional to these parameters at the first harvest (Fig. 5a and 5b). Nevertheless, at the end of the second growth period, the leaf area of control plants supplied with 100/0 and 70/30 was 47.4 and 70.4% higher, respectively, than that determined in 50/50 (Fig. 5c). Moreover, even though the highest number of leaves at the second harvest was observed in plants supplied with 50/50 and subjected to 0.5 mmol  $L^{-1}$  Cd, their leaf area was actually low compared with that of any of the controls. Regardless of NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios, leaf area was reduced to a minimum after the plants were exposed to 1.0 mmol  $L^{-1}$  Cd.



**Fig. 5.** Leaf area (a-c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **c**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

Root growth in terms of length and surface area decreased sharply after Cd exposure (Fig. 6). In relative terms, plants supplied with 100/0 were the most affected by the addition of 0.5 mmol L<sup>-1</sup> Cd to the nutrient solution. Their root length and surface area were 74.3 and 77.0% lower, respectively, compared with the control. For 50/50, these values were limited to 45.3 and 46.2%, respectively. On the other hand, only in plants supplied with 100/0 both parameters were not further affected by exposure to 1.0 mmol L<sup>-1</sup> Cd.



**Fig. 6.** Root length (a) and surface area (b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). Lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.



**Fig. 7.** Shoot (a-c) and root (d) dry weight of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **c** and **d**, lowerand uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

At the end of the first growth period, there were independent effects of  $NO_3^-/NH_4^+$  ratios and Cd rates on shoot dry weight. Shoot biomass production was increased when both  $NO_3^-$  and  $NH_4^+$  were available to plants, especially at a 70/30 ratio (Fig. 7a). Conversely, it was 36.5% lower in plants exposed to 1.0 mmol L<sup>-1</sup> Cd, in comparison with those grown without application of the metal (Fig. 7b). As for the second harvest, despite the overall high numbers of tillers and leaves recorded at 0.5 mmol L<sup>-1</sup> Cd, there was also a sharp decrease in shoot dry matter production (Fig. 7c). A similar pattern was found for root dry weight, although at 0.5 mmol L<sup>-1</sup> Cd, like for root length and surface area, the lowest values were observed in plants supplied with 100/0 (Fig. 7d).

## 2.3.3. NR and GS activities

For the NR activity, the interaction  $NO_3^-/NH_4^+$  ratios × Cd rates was not significant. The activity of this enzyme at the first harvest increased linearly with Cd exposure. Accordingly, the value observed in plants subjected to 1.0 mmol L<sup>-1</sup> Cd was almost three-fold higher than that of the control (Fig. 8a). At the end of the second growth period, plants grown with 100/0 showed the lowest NR activity, although it did not differ from that observed with the supply of 70/30 (Fig. 8b). At that time, the residual effect of Cd in the substrate also led to increased NR activity in diagnostic leaves (Fig. 8c).



**Fig. 8.** NR activity in diagnostic leaves (a-c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05).

Neither NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios nor Cd rates had significant effects on the GS activity in shoots at the first harvest, which had a mean of 49.2  $\pm$  5.4 mg  $\gamma$ -glutamyl hydroxamate g<sup>-1</sup> h<sup>-1</sup>. At the second harvest, plants supplied with 70/30 had this activity increased by more than two-fold after exposure to 0.5 and 1.0 mmol L<sup>-1</sup> Cd, while remaining unchanged with the supply of 100/0 and 50/50 (Fig. 9a). On the other hand, plants grown with 70/30 had the lowest GS activity in roots (Fig. 9b). The activity of this enzyme in roots was also affected by the presence of Cd in the nutrient solution, reducing 55.6% at 1.0 mmol L<sup>-1</sup> Cd compared with control plants (Fig. 9c).



**Fig. 9.** GS activity in shoots (a) and roots (b, c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **a**, lower-and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

# 2.3.4. $NO_3^-$ , $NH_4^+$ and total N concentrations

Shoot  $NO_3^-$  concentration at the first harvest changed with  $NO_3^-/NH_4^+$  ratios (Fig. 10a). Plants supplied with 70/30 and 50/50 showed the lowest and highest values, respectively, although both concentrations did not differ from that of plants grown exclusively with  $NO_3^-$ . At the second harvest, the same pattern was observed where no Cd was applied. However, the shoot  $NO_3^-$  concentration of plants supplied with combinations of  $NO_3^-$  and  $NH_4^+$  increased

substantially at 0.5 and 1.0 mmol L<sup>-1</sup> Cd (Fig. 10b). There was no significant difference for root  $NO_3^-$  concentration, which had a mean of  $3.9 \pm 1.5 \text{ mg kg}^{-1}$ .



**Fig. 10.** Shoot NO<sub>3</sub><sup>-</sup> concentration (a, b) of *Panicum maximum* cv. Tanzania as influenced by NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **b**, lower- and uppercase letters indicate significant differences within NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios and Cd rates, respectively.

The shoot  $NH_4^+$  concentration of plants supplied with 50/50 at the first harvest was 39.2 and 47.8% higher than those obtained with 100/0 and 70/30, respectively (Fig. 11a). The interaction  $NO_3^-/NH_4^+$  ratios × Cd rates was significant only at the second harvest, when the availability of Cd potentiated shoot  $NH_4^+$  concentration in plants supplied with 70/30 and 50/50, especially at 1.0 mmol  $L^{-1}$  Cd (Fig. 11b). Similar to what was observed for shoots at the first harvest, root  $NH_4^+$  concentration with the supply of 50/50 was greater than with any other ratio (Fig. 11c). Furthermore, regardless of the rate tested, Cd exposure resulted in increased concentration of this ion in roots (Fig. 11d).

Regarding total N, at the first harvest, plants supplied with 100/0 and 50/50 had essentially the same concentration of this nutrient in shoots, which was 17.7% higher than that of plants grown with 70/30 (Fig. 12a). At the second harvest, the supply of 70/30 also led to the lowest values at 0.0 and 0.5 mmol  $L^{-1}$  Cd. However, at 1.0 mmol  $L^{-1}$  Cd, shoot total N concentration was similar for all NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios (Fig. 12b). The lowest root total N concentration was observed in plants supplied with 70/30, although it did not differ from that obtained with 50/50 (Fig. 12c). The addition of Cd to the nutrient solution also played a role on root total N concentration. In comparison with control plants, this concentration increased 22.0 and 21.6% after application of 0.5 and 1.0 mmol  $L^{-1}$  Cd, respectively (Fig. 12d).



**Fig. 11.** Shoot (a, b) and root (c, d)  $NH_{4^+}$  concentration of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_{4^+}$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_{4^+}$  ratios and Cd rates, respectively.



**Fig. 12.** Shoot (a, b) and root (c, d) total N concentration of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

#### 2.3.5. Cd concentration and accumulation

At both harvests, shoot Cd concentration increased in a dose-dependent manner, which was found to be regulated by the  $NO_3^-/NH_4^+$  ratios. Five days after adding 1.0 mmol L<sup>-1</sup> Cd to the nutrient solution (first harvest), plants grown with both  $NO_3^-$  and  $NH_4^+$  had a shoot Cd concentration about three-fold higher than that observed with the exclusive use of  $NO_3^-$  (Fig. 13a). This effect was even more pronounced at the second harvest, when the supply of 50/50 compared with 100/0 led to over eight-fold more Cd in shoots (Fig. 13b). For roots,  $NO_3^-/NH_4^+$  ratios and Cd rates were analyzed independently. This indicated that the root Cd concentration with 50/50 was lower than that recorded for 100/0 and 70/30 (Fig. 13c), and that overall it increased proportionally to the Cd rates (Fig. 13d).



**Fig. 13.** Shoot (a, b) and root (c, d) Cd concentration of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **a** and **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

At the end of the first growth period, although shoots of plants supplied with 70/30 and 50/50 and subjected to 1.0 mmol L<sup>-1</sup> Cd showed similar Cd concentrations, the accumulation of this metal was 65.9% higher in the former (Fig. 14a). However, at the second

harvest, the amount of Cd accumulated using 50/50 was two- and almost eight-fold higher than with 70/30 and 100/0, respectively (Fig. 14b). The response pattern of root Cd accumulation was comparable to that obtained for root Cd concentration, for both  $NO_3^-/NH_4^+$  ratios (Fig. 14c) and Cd rates (Fig. 14d), except for the fact that plants supplied with 100/0 exhibited intermediate results.



**Fig. 14.** Shoot (a, b) and root (c, d) Cd accumulation of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **a** and **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

# 2.4. Discussion

Exposure to Cd can lead to biochemical, physiological and even morphological changes in plants [18, 30]. Leaf rolling, chlorosis and stunted growth, with a consequent decrease in the production of photosynthetically active leaves, are among the most common visual symptoms of Cd stress [31, 32]. In general, these symptoms are characteristic of high phytotoxicity [33]. The visual diagnosis of tanzania guinea grass under Cd rates suggested that while plants supplied with  $NO_3^-$  and  $NH_4^+$  at a 70/30 ratio were highly affected, those grown exclusively with  $NO_3^$ somehow limited the concentration of this metal in their tissues. The chlorosis observed even after a short period of exposure to Cd could be attributed to the decrease in leaf chlorophyll content, determined indirectly by the RCI. The chlorophyll *a* and *b* contents of *Zea mays* seedlings showed to be highly sensitive to Cd in the nutrient solution, decreasing considerably at 1.7  $\mu$ mol L<sup>-1</sup> Cd [33]. In *Medicago sativa* plants, Hattab et al. [19] observed that 12  $\mu$ mol L<sup>-1</sup> Cd altered mainly the synthesis of chlorophyll *b*, leading to a reduction in the total chlorophyll content. After exposure to much higher Cd rates than those, it was observed that, by and large, the availability of NH<sub>4</sub><sup>+</sup> benefited the leaf chlorophyll content of tanzania guinea grass.

High numbers of tillers and leaves, and consequently shoot dry weight, in plants supplied with 70/30 had already been reported for *P. maximum* cv. Aruana [13]. Also, under similar experimental conditions, Rabêlo et al. [8] found that 2.0 mmol L<sup>-1</sup> Cd basically inhibited the production of new tillers and leaves in tanzania guinea grass. However, in the present study, the fact that at the second harvest plants grown with 100/0 maintained normal tillering at 1.0 mmol L<sup>-1</sup> Cd reinforced the assumption of reduced Cd uptake with this NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratio. Moreover, based on their increased numbers of tillers and leaves at 0.5 mmol L<sup>-1</sup> Cd, plants supplied with 50/50 seemed to experience eustress when exposed to a low Cd rate.

Whereas at the end of the first growth period leaf area was proportional to the numbers of tillers and leaves, these parameters indicated that, in comparison with other  $NO_3^-/NH_4^+$  ratios, plants supplied with 50/50 had smaller leaves at the second harvest. This was observed in the control and became even more evident at 0.5 mmol L<sup>-1</sup> Cd. Besides a negative effect, reduced leaf size could also be an important strategy to cope with Cd stress [34].

Regarding root length and surface area, plants grown with 100/0 and 50/50 were the most and least affected by Cd exposure, respectively, indicating that the exclusive use of NO<sub>3</sub><sup>-</sup> might favor Cd accumulation in roots, while the supply of 50/50 leads to either reduced uptake or increased transport of this metal to shoots [8]. Taking into account that plants grown with 50/50 had visual symptoms of Cd toxicity, increased transport is the most likely explanation for this finding. In any case, the reduced root growth observed in plants exposed to Cd might be associated to a disintegration of the rhizodermis and cortical layers, as observed in *P. maximum* cv. Massai [35].

The shoot dry weight at the second harvest translated the stunted growth caused by Cd toxicity into numbers. Thus, it revealed that, at 0.5 mmol  $L^{-1}$  Cd, although this metal did not meaningfully alter the numbers of tillers and leaves, their relative growth was quite reduced. Fujimaki et al. [36] used imaging data to study Cd movement in intact *Oryza sativa* plants and observed that this metal accumulated mainly in the culm base. Therefore, when the shoots were

cut at 4 cm from the substrate surface (first harvest) and allowed to regrow, the Cd present in the culm base was probably the main factor restraining the development of new tissues. Also, it is possible to infer that 1.0 mmol  $L^{-1}$  Cd led to low specific root length in plants supplied with 50/50, because even though they showed the lowest root length, their root dry weight was similar to those observed with 100/0 and 70/30.

Previous works have suggested that Cd affects  $NO_3^-$  assimilation by inhibiting the NR and GS activities [20, 37, 38]. Nasraoui-Hajaji et al. [39] studied the effect of this metal on the N metabolism of *Solanum lycopersicon* seedlings and found that, regardless of the  $NO_3^-/NH_4^+$  ratio used (100/0, 90/10, 60/40, 10/80 or 0/100), Cd toxicity reduced the NR activity and  $NO_3^-$  concentration. Here, in contrast, it was found that the activity of this enzyme increased considerably in the presence of Cd. Moreover, at the second harvest, plants supplied with 70/30 and 50/50 had the highest NR activity. Therefore, these results pointed towards a high  $NO_3^-$  concentration in plants under Cd toxicity, especially when supplied with combinations of  $NO_3^-$  and  $NH_4^+$ .

After the reduction of  $NO_3^-$  to  $NH_4^+$ , the enzyme GS catalyzes the formation of glutamine from  $NH_4^+$  and glutamate, through the GS/GOGAT pathway [14]. Thus, an accumulation of  $NH_4^+$  in shoots might have resulted in the increased GS activity observed at the second harvest in plants supplied with 70/30 and subjected to Cd. Similarly, the reduced GS activity in roots grown with 70/30 and 50/50, or in those exposed to the metal, could be simply a consequence of rapid  $NH_4^+$  assimilation. In order to validate the results of both enzymes, the  $NO_3^-$  and  $NH_4^+$  concentrations in shoots and roots were also determined.

As suggested by the NR activity, during the regrowth, Cd exposure led to increased shoot  $NO_3^-$  concentration in plants supplied with 70/30 and 50/50. On the other hand, the concentration of this N form in shoots was maintained at normal levels with the supply of 100/0. Zhang et al. [15] demonstrated that Cd stress induced  $NO_3^-$  allocation to roots in *Arabidopsis thaliana*. This is a defense mechanism in which plants increase their tolerance to environmental stressors by enhancing  $NO_3^-$  assimilation in roots. Although the uptake of this ion plays a key role on biochemical mechanisms of adaptation to heavy metals [18], a high  $NO_3^-$  assimilation in shoots could compete for the energy produced during the photosynthetic electron transport [15].

The high shoot  $NH_4^+$  concentration observed at the first harvest in plants supplied with 50/50 could be mainly attributed to the increased availability of this N form in the nutrient solution [40]. However, under the residual effect of Cd, the accumulation of  $NH_4^+$  in shoots occurred not only in plants supplied with 70/30, but also with 50/50. Interestingly, root  $NH_4^+$  concentration also increased in plants grown with this latter ratio, or exposed to Cd. Thus, the

supply of 50/50 seems to disturb the overall GS activity in tanzania guinea grass. In roots of plants subjected to Cd, this is more likely a deleterious effect of the metal on this enzyme [38].

The low total N concentration observed in plants supplied with 70/30 are in accordance with the findings of Santos et al. [13], in which this same  $NO_3^-/NH_4^+$  ratio led to high N use efficiency. Here, it is demonstrated that the addition of Cd to the nutrient solution results in increased total N concentration in both shoots and roots. On the other hand, some reports indicate that Cd toxicity reduces the uptake of this nutrient [22]. More recently, Ma et al. [41] found that the total N uptake of *Brassica chinensis* seedlings was also negatively affected by Cd stress. However, this result was due precisely to reduced shoot and root biomass production. This was confirmed with the use of <sup>15</sup>N, whose abundance in plants exposed to Cd showed that the NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> concentrations actually increased.

Given that the simultaneous supply of  $NO_3^-$  and  $NH_4^+$  led to similar shoot Cd concentrations at the first harvest, the high shoot Cd accumulation with the use of 70/30 at that time can only be attributed to increased biomass production. Nevertheless, regardless of the  $NO_3^-/NH_4^+$  ratio used, plants exposed to Cd showed basically the same shoot dry weight at the second harvest. Even so, at the highest Cd rate, those grown with 50/50 proved to be way more effective in the uptake and accumulation of this metal. These results refute in part the initial hypothesis in this study, since the high Cd phytoextraction by tanzania guinea grass supplied with 50/50 was achieved through enhanced Cd uptake rather than increased plant growth.

Cheng et al. [6] assessed the influence of  $NO_3^-$  and  $NH_4^+$  on the phytoextraction efficiency of two hyperaccumulators (*Carpobrotus rossii* and *S. nigrum*) and observed that the supply of the latter N form increased Cd uptake, translocation and accumulation. In fact, in comparison with plants supplied with  $NO_3^-$ , those grown with  $NH_4^+$  accumulated 30% more Cd in their shoots. For both plant species, this effect was attributed to increased translocation. However, in contrast to their results, root Cd concentration with the supply of 100/0 was actually high. Therefore, it suggests that plants supplied exclusively with  $NO_3^-$  persist in highly contaminated environments by limiting Cd transport to shoots.

# 2.5. Conclusion

The development of tanzania guinea grass revealed that Cd uptake and accumulation are strongly related to the form of N available. Although it increased plant total N concentration, Cd exposure reduced the chlorophyll content and negatively affected shoot and root growth. In addition, it altered N metabolism and induced the accumulation of  $NO_3^-$  and  $NH_4^+$  mainly in shoots. It is concluded that the exclusive use of  $NO_3^-$  mitigates Cd toxicity by favoring the

accumulation of this metal in roots and maintaining normal N metabolism in the plant. Conversely, despite disturbing the GS activity and changing leaf and root morphology under Cd toxicity, the supply of  $NO_3^-$  and  $NH_4^+$  at a 50/50 ratio increases the uptake, transport and accumulation of this metal. Thus, high Cd phytoextraction by tanzania guinea grass grown with both these N forms is achieved through enhanced Cd uptake rather than increased plant growth.

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# 3. PARTIAL REPLACEMENT OF NITRATE BY AMMONIUM INCREASES PHOTOSYNTHESIS AND REDUCES OXIDATIVE STRESS IN TANZANIA GUINEA GRASS EXPOSED TO CADMIUM

#### ABSTRACT

In order to grow and effectively uptake and accumulate cadmium (Cd), plants used for phytoextraction have to cope with toxicity, which may be influenced by the supply of nitrate  $(NO_3^-)$  and ammonium  $(NH_4^+)$ . Thus, the effect of these nitrogen forms on the photosynthetic and antioxidant enzyme activities of P. maximum cv. Tanzania (tanzania guinea grass) under Cd stress was evaluated. Plants were grown in nutrient solution under greenhouse conditions and subjected to a 3  $\times$ 3 factorial experiment. They were supplied with three  $NO_3^-/NH_4^+$  ratios (100/0, 70/30 and 50/50) and exposed to three Cd rates (0.0, 0.5 and 1.0 mmol L<sup>-1</sup>), being arranged in a randomized complete block design with three replications. Gas exchange parameters, oxidative stress indicators, proline and protein concentrations, and antioxidant enzyme activities were studied. Exposure to Cd reduced photosynthesis by causing stomatal closure and impairing electron transport. However, the simultaneous supply of  $NO_3^-$  and  $NH_4^+$ , particularly at a 50/50 ratio, restored gas exchange and improved the function of photosystem II, increasing the photosynthetic capacity of the grass. Plants grown with 50/50 showed reduced lipid peroxidation along with increased synthesis of proline and protein. Moreover, this  $NO_3^{-}/NH_4^{+}$  ratio increased the tolerance of tanzania guinea grass to Cd by inducing high superoxide dismutase and glutathione reductase activities in shoots and roots, respectively, maintaining cellular homeostasis and reducing oxidative stress. The negative effects of Cd on photosynthesis and on the balance between oxidants and antioxidants in tanzania guinea grass are attenuated by the partial replacement of  $NO_3^-$  by  $NH_4^+$  in the nutrient solution.

Keywords: Antioxidant enzymes; Cadmium toxicity; Nitrogen fertilization; *Panicum maximum*; Photosynthetic activity; Phytoextraction

# 3.1. Introduction

Cadmium (Cd) is a non-essential element highly toxic to plants and animals, and that poses a serious threat to human health because it can be easily taken up by plants and enter the food chain [1-3]. The accumulation of this heavy metal in the environment has resulted mainly from anthropogenic actions such as mining, burning of fossil fuels, inappropriate disposal of contaminated waste and the indiscriminate use of pesticides and phosphate fertilizers [4, 5]. A major concern worldwide is the presence of high Cd concentrations in the soil, causing phytotoxicity and limiting agricultural production [6, 7].

Plants under Cd stress undergo several physiological and biochemical alterations [8, 9]. Their photosynthetic activity can be drastically reduced due to the inhibition of several processes, including electron transport, synthesis of chlorophyll a and b and stomatal conductance [10, 11]. By reducing gas exchange, Cd can also alter plant-water relations and, as a consequence, affect nutrient uptake and homeostasis [9, 12]. At the cellular level, this metal promotes the accumulation of reactive oxygen species, inducing oxidative stress and causing irreversible damages to biomolecules such as lipids, proteins and nucleic acids [13, 14]. In addition, Cd can inactivate some enzymes by replacing Zn in their active sites [13, 15], which may affect the function of important antioxidants.

Phytoextraction has been widely used as a low-cost and environmentally friendly method for remediation of Cd-contaminated soils [1, 16]. It consists of growing plants that are able to uptake and accumulate heavy metals and other soil contaminants in their biomass, which can then be harvested and removed from the affected sites [17]. In this sense, there is a need for agronomic strategies that enable plants to thrive in such environments. Among these strategies, proper nutrient management can improve the tolerance of plants to heavy metals, thus increasing phytoextraction efficiency [4, 18].

Plant growth and development depend on an adequate supply of nitrogen (N) [19], which is also known to influence the availability and uptake of soil contaminants [18, 20, 21]. Therefore, increasing attention has been paid to the role of N forms in plants exposed to toxic elements [4, 17, 22]. Nitrate ( $NO_3^-$ ) and ammonium ( $NH_4^+$ ) are the main inorganic forms of this nutrient absorbed by plants [19]. Nevertheless, the margin between the beneficial and toxic effects of the latter is rather narrow [23]. The simultaneous supply of these ions, on the other hand, enhances plant growth by leading to increased N use efficiency, as observed for *Panicum maximum* cv. Aruana grown with  $NO_3^-/NH_4^+$  ratios of 70/30 and 55/45 [24].

Hamlin and Barker [17] studied the effect of  $NO_3^-/NH_4^+$  ratios on *Brassica juncea* under Zn toxicity, and reported that plants supplied with 90/10 showed increased accumulation of the toxic element. Later, Souza Jr. et al. [22] reported that 70/30 was the most effective ratio of these ions for Cu phytoextraction by *P. maximum* cv. Tanzania (tanzania guinea grass). Regarding Cd, Nasraoui-Hajaji et al. [25] concluded that  $NO_3^-/NH_4^+$  ratios also affected the accumulation of this metal in tomato (*Solanum lycopersicum*) plants. However, there is no information on the influence of N supply, particularly in the form of  $NO_3^-/NH_4^+$  ratios, on the tolerance of tanzania guinea grass to Cd toxicity. Silva et al. [26] assessed the response of five forage grasses to increasing Cd rates in the soil and observed that tanzania guinea grass was one of the most tolerant to this metal. In fact, a recent study demonstrated the potential of this grass for Cd phytoextraction [27].

In order to grow and effectively uptake and accumulate Cd, plants used for phytoextraction have to cope with toxicity. In this study, the effect of  $NO_3^-/NH_4^+$  ratios on the photosynthetic and antioxidant enzyme activities of tanzania guinea grass under Cd stress was evaluated. The main hypothesis was that the simultaneous supply of  $NO_3^-$  and  $NH_4^+$  improves the tolerance of the grass to this metal, increasing photosynthesis and attenuating oxidative stress.

# 3.2. Materials and Methods

## 3.2.1. Plant culture and experimental design

A greenhouse study was conducted during the summer season, with tanzania guinea grass grown in 3.6 L plastic pots, containing ground quartz and nutrient solution (see Table 1 in section 2.2.1) [28]. A  $3 \times 3$  factorial arrangement was used, with three NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios (100/0, 70/30 and 50/50) and three Cd rates (0.0, 0.5 and 1.0 mmol L<sup>-1</sup>) tested, totaling nine combinations laid out in a randomized complete block design with three replications.

Seedlings were germinated for two weeks in sand moistened with deionized water, and 15 of them were transplanted to each pot. They were provided with 1/5 strength nutrient solution for seven days, after which it was replaced by a full strength solution and plants were thinned to five per pot. The nutrient solution was renewed every two weeks and had a N concentration of 15 mmol L<sup>-1</sup>. Aeration was performed by draining and refilling the pots three to six times a day, depending on the growth stage, and the volume lost through evapotranspiration was replaced daily with deionized water.

Four weeks after transplanting, the plants were subjected to the Cd rates for four days. Afterwards, the shoots were harvested and the contaminated nutrient solution replaced. Then, the plants were allowed to regrow under the residual effect of the metal. Overall, the study was conducted for two growth periods (of 32 days after transplanting and 26 days after the first harvest), taking into account the development of the grass in response to Cd toxicity. At the end of both periods, the harvested material was cryogenically ground and stored in a freezer at -80 °C for further biochemical analyses.

## 3.2.2. Gas exchange parameters

One day prior to each harvest, the plants were transferred to a growth chamber where gas exchange parameters were measured under controlled conditions of light, temperature and humidity. A GFS-3000 portable gas exchange system (Heinz Walz GmbH, Effeltrich, Germany) was used and readings were performed in the middle third of two diagnostic leaves (newly expanded leaves) of each experimental unit. Flow rate, CO<sub>2</sub> concentration, H<sub>2</sub>O concentration, light and the cuvette temperature were set at 750  $\mu$ mol s<sup>-1</sup>, 380 mg L<sup>-1</sup>, 18,000 mg L<sup>-1</sup>, 1,500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> and 27 °C, respectively, and five parameters were evaluated: stomatal conductance to water, transpiration rate, net photosynthetic rate, electron transport rate and quantum yield of photosystem II (PSII).

#### 3.2.3. Oxidative stress indicators

Hydrogen peroxide  $(H_2O_2)$  and malondialdehyde (MDA) were determined spectrophotometrically and used to assess oxidative stress. Thus, 0.2 g samples with 0.01 g PVPP were macerated in 2 mL of 1 g L<sup>-1</sup> TCA and centrifuged at 10,000 rpm for 5 min at 4 °C. The H<sub>2</sub>O<sub>2</sub> concentration in the supernatant was determined at 390 nm after a 0.2 mL aliquot was added to tubes containing 0.2 mL of 100 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.5) and 0.8 mL of 1 mol L<sup>-1</sup> KI [29]. As for MDA, 250 µL of the supernatant was homogenized in 1 mL of a solution of 200 g L<sup>-1</sup> TCA and 5 g L<sup>-1</sup> TBA and placed in a dry bath at 95 °C for 30 min. The concentration was calculated based on readings at 535 and 600 nm, taking into account an extinction coefficient of 155 mmol<sup>-1</sup> cm<sup>-1</sup>, as described by Heath and Packer [30].

# 3.2.4. Proline concentration

The concentration of free proline was calculated based on a standard curve, as proposed by Bates et al. [31]. Therefore, 0.3 g samples were homogenized in 10 mL of 30 g  $L^{-1}$ sulfosalicylic acid and centrifuged at 10,000 rpm for 20 min at 15 °C. Then, 1 mL of the supernatant was transferred to test tubes, along with 1 mL of acid ninhydrin (1.25 g of ninhydrin, 30 mL of glacial acetic acid and 20 mL of 6 mol  $L^{-1}$  phosphoric acid) and 1 mL of glacial acetic acid and placed in a water bath at 100 °C for 1 h. Next, they were mixed with 2 mL of toluene and the concentration of the amino acid was determined colorimetrically at 520 nm.

## 3.2.5. Protein assay

For protein extraction, 1 g samples were macerated with 0.05 g PVPP in 3 mL of extraction buffer (100 mmol L<sup>-1</sup> potassium phosphate, 3 mmol L<sup>-1</sup> DTT and 1 mmol L<sup>-1</sup> EDTA; pH 7.5) and centrifuged at 10,000 rpm for 30 min at 4 °C. Afterwards, a 20  $\mu$ L aliquot of the supernatant was used for protein assay, while the remaining volume (enzyme extract) was stored at -80 °C and used in the determination of antioxidant enzyme activities. The soluble protein

concentration was determined based on a standard curve of bovine serum albumin, as described by Bradford [32].

## 3.2.6. Antioxidant enzyme activities

#### 3.2.6.1. Superoxide dismutase (SOD, EC 1.15.1.1)

The SOD activity was estimated by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions, as described by Azevedo et al. [33]. Bovine SOD was used as a positive control and equal amounts of protein were loaded in each lane (i.e. 60 and 14 mg for shoots and roots, respectively). After running, each gel was then incubated with 15 mL of 130  $\mu$ mol L<sup>-1</sup> riboflavin and 35 mL of a solution of 100 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.8), 350  $\mu$ mol L<sup>-1</sup> NBT, 1 mmol L<sup>-1</sup> EDTA and 30 mmol L<sup>-1</sup> TEMED for 30 min, after which they were exposed to light. The reaction was stopped 10 min later by immersing the gels in 70 mL L<sup>-1</sup> acetic acid. Subsequently, they were scanned and the images analyzed using the ImageJ software (version 1.52) [34].

# 3.2.6.2. Catalase (CAT, EC 1.11.1.6)

After mixing 25  $\mu$ L of the enzyme extract with 1 mL of 100 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.5) and 2.5  $\mu$ L of 9.8 mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, the CAT activity was determined spectrophotometrically at 240 nm based on the decomposition of H<sub>2</sub>O<sub>2</sub> at 25 °C for 1 min [33].

# 3.2.6.3. Ascorbate peroxidase (APX, EC 1.11.1.11)

The oxidation of ascorbate was monitored at 30 °C for 1 min in order to calculate the APX activity [35]. Thus, 40  $\mu$ L of the enzyme extract were mixed with 100  $\mu$ L of 1 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> and 860  $\mu$ L of 80 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.0) containing 5 mmol L<sup>-1</sup> ascorbic acid and 1 mmol L<sup>-1</sup> EDTA, and absorbance values were recorded at 290 nm.

# 3.2.6.4. Guaiacol peroxidase (GPX, EC 1.11.1.7)

A 2  $\mu$ L aliquot of the enzyme extract was mixed with 50  $\mu$ L of 5 mL L<sup>-1</sup> guaiacol, 50  $\mu$ L of 294 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> and 750  $\mu$ L of 200 mmol L<sup>-1</sup> sodium phosphate buffer (pH 5.0) containing 110 mmol L<sup>-1</sup> citric acid, and placed in a water bath a 30 °C for 15 min. Next, the reaction mixture received 50  $\mu$ L of 20 g L<sup>-1</sup> sodium metabisulphite and absorbance values were recorded at 450 nm [36].

# 3.2.6.5. Glutathione reductase (GR, EC 1.6.4.2)

The GR activity was determined by monitoring the reduction of oxidized glutathione at 30 °C for 1 min [33]. The analysis was performed using 10 and 50  $\mu$ L of the enzyme extract of shoots and roots, respectively. They were added to 1 mL of 100 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.5), along with 500  $\mu$ L of 1 mmol L<sup>-1</sup> DTNB, 100  $\mu$ L of 1 mmol L<sup>-1</sup> GSSG and 100  $\mu$ L of 100  $\mu$ mol L<sup>-1</sup> NADPH, and the activity was detected at a wavelength of 412 nm.

## 3.2.7. Statistical analysis

The data were analyzed by two-way analysis of variance, followed by Tukey's test at the 5% level, with the Assistat software (version 7.7) [37]. Significant interaction effects were studied by comparing  $NO_3^-/NH_4^+$  ratios within each Cd rate and Cd rates within each  $NO_3^-/NH_4^+$  ratio. Alternatively, each factor was analyzed independently where no significant interaction was observed.

# 3.3. Results

## 3.3.1. Photosynthetic activity

There were significant changes in all gas exchange parameters of tanzania guinea grass after exposure to the Cd rates and their residual effect, most of which were found to be regulated by the  $NO_3^-/NH_4^+$  ratios. Control plants (no Cd added) grown with 100/0 and 70/30 had the highest stomatal conductance to water at the first harvest (Fig. 15a). Nevertheless, the supply of these  $NO_3^-/NH_4^+$  ratios was also associated to the lowest values at 0.5 mmol L<sup>-1</sup> Cd. Despite the negative effect of Cd at the first harvest, this metal induced higher stomatal conductance to water at the end of the second growth period, except for plants supplied with 100/0 (Fig. 15b).

At both harvests, transpiration rate was strongly related to stomatal conductance to water. As a result, four days of exposure to Cd caused a decrease in this parameter, and at 1.0 mmol  $L^{-1}$  Cd all plants were equally affected (Fig. 16a). In relative terms, plants supplied with 50/50 were the least sensitive to 0.5 mmol  $L^{-1}$  Cd, considering that they transpired only 15.4% less than those of the control. The residual effect of Cd also led to increased transpiration in plants grown with and 70/30 and 50/50 (Fig. 16b).



Fig. 15. Stomatal conductance to water in diagnostic leaves (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). Lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.



**Fig. 16.** Transpiration rate in diagnostic leaves (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). Lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

The response pattern of the net photosynthetic rate was very similar to those found for the two previous parameters. At the first harvest, the highest value was observed in control plants supplied with 70/30 (Fig. 17a). After application of 0.5 mmol L<sup>-1</sup> Cd, whilst those grown with 70/30 and 50/50 had similar results, only the latter were able to maintain the same levels observed in the control. Conversely, at the second harvest, plants supplied with combinations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> and exposed to Cd actually increased their net photosynthetic rate, which was overall two-fold higher than that observed with the exclusive use of NO<sub>3</sub><sup>-</sup> (Fig. 17b).



**Fig. 17.** Net photosynthetic rate in diagnostic leaves (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). Lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

The electron transport rate at the first harvest decreased with the increase in the Cd rate, with no significant effect of the  $NO_3^-/NH_4^+$  ratios (Fig. 18a). Interestingly, at the second harvest, only plants supplied with 50/50 had this parameter altered by the presence of Cd in the nutrient solution (Fig. 18b). In comparison with control plants, their electron transport rate increased 88.9% after application of 0.5 mmol L<sup>-1</sup> Cd. At the highest Cd rate, this value was limited to 44.0%, and combinations of  $NO_3^-$  and  $NH_4^+$  led to similar results.



**Fig. 18.** Electron transport rate in diagnostic leaves (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

The quantum yield of PSII at both harvests showed the exact same pattern found for the electron transport rate. Thus, compared with the control, a 32.6% decrease was observed four days after exposure to 1.0 mmol  $L^{-1}$  Cd (Fig. 19a). At the second harvest, plants supplied

with 50/50 and treated with 0.5 mmol  $L^{-1}$  Cd had the highest quantum yield of PSII (Fig. 19b). Moreover, at 1.0 mmol  $L^{-1}$  Cd, the value observed in plants grown exclusively with NO<sub>3</sub><sup>-</sup> was 29.2 and 28.7% lower than in those supplied with 70/30 and 50/50, respectively.



**Fig. 19.** Quantum yield of PSII in diagnostic leaves (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

# 3.3.2. H<sub>2</sub>O<sub>2</sub>, MDA and proline concentration

At the first harvest, the supply of 50/50 led to the highest shoot  $H_2O_2$  concentration in the control plants (Fig. 20a). However, the concentration of the peroxide in shoots of plants grown with 100/0 and 70/30 increased after Cd exposure, becoming virtually the same for all  $NO_3^-/NH_4^+$  ratios. At the end of the second growth period, only Cd rates had a significant effect on shoot and root  $H_2O_2$  concentrations. In this case, in comparison with control plants, those subjected to 0.5 and 1.0 mmol  $L^{-1}$  Cd had 48.5 and 52.6% more  $H_2O_2$  in their shoots, respectively (Fig. 20b). Conversely, the root  $H_2O_2$  concentration of plants exposed to Cd was about half of that observed where this metal was not applied (Fig. 20c).

Regardless of the form of N available to the plants, the addition of Cd to the nutrient solution increased the shoot MDA concentration. The supply of 50/50, even though similar to 70/30, also led to the highest values in the control plants at the first harvest (Fig. 21a). Nevertheless, unlike for H<sub>2</sub>O<sub>2</sub>, plants grown with this latter  $NO_3^-/NH_4^+$  ratio showed a higher shoot MDA concentration after Cd exposure. At the second harvest, the residual effect of Cd also increased this variable by up to 49.7% compared with the control, as observed at 1.0 mmol  $L^{-1}$  Cd (Fig. 21b). No detectable MDA levels were found in roots.



Fig. 20. Shoot (a, b) and root (c)  $H_2O_2$  concentration of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **a**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.



**Fig. 21.** Shoot MDA concentration (a, b) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **a**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

The  $NO_3^-/NH_4^+$  ratios and Cd rates had independent effects on the concentration of free proline. At the first harvest, the concentration of this amino acid in shoots of plants supplied with 50/50 was 140.8 and 56.7% higher than those recorded for 100/0 and 70/30, respectively (Fig. 22a). Furthermore, it increased proportionally to the availability of Cd to the plants (Fig.

22b). Like at the first growth period, the highest shoot proline concentration at the second harvest was observed in plants supplied with 50/50, although it did not differ from that of plants grown with 70/30 (Fig. 22b). None of the factors tested led to significant differences in root proline concentration, which had a mean of  $0.6 \pm 0.1 \,\mu\text{mol g}^{-1}$ .



**Fig. 22.** Shoot proline concentration (a-c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05).

# 3.3.3. Protein concentration and antioxidant enzyme activities

Four days after Cd exposure, there were no significant differences in shoot soluble protein concentration (mean =  $1.6 \pm 0.1 \text{ mg mL}^{-1}$ ). At the end of the second growth period, it was also comparable in control plants, but increased considerably in the presence of the metal, especially in plants supplied with combinations of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> (Fig. 23a). For roots, the soluble protein concentration was influenced by both NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios and Cd rates, albeit without significant interaction. Accordingly, the lowest values were recorded in plants grown with 70/30 (Fig. 23b) and in those subjected to 1.0 mmol L<sup>-1</sup> Cd (Fig. 23c).

In view of the differences in the soluble protein concentration of shoots and roots, the SOD activity was more evident in the former (Fig. 24). At the first harvest, the total activity of this enzyme in shoots of plants supplied with 100/0 and 70/30 increased after Cd exposure,

whereas it remained almost unchanged (with high values) in those grown with 50/50 (Fig. 24a). At the second harvest, the supply of 50/50 led to the highest total SOD activity in shoots, particularly in plants subjected to 0.5 mmol  $L^{-1}$  Cd (Fig. 24b). At the same Cd rate, a noticeable increase was also observed for plants grown with 70/30. On the other hand, the total SOD activity in roots decreased with the increase in the availability of Cd (Fig. 24c).



Fig. 23. Shoot (a) and root (b, c) soluble protein concentration of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **a**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

For the CAT activity, there were significant effects only on shoots at the second harvest. At the end of the first growth period, the activity of this enzyme in shoots was about  $38.8 \pm 3.7$  µmol min<sup>-1</sup> mg protein<sup>-1</sup>. The results obtained for control plants at the second harvest did not differ between NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios (Fig. 25). However, while the CAT activity in shoots of plants supplied with both N forms decreased substantially after exposure to 0.5 mmol L<sup>-1</sup> Cd, in those grown exclusively with NO<sub>3</sub><sup>-</sup> it was only affected at 1.0 mmol L<sup>-1</sup> Cd. In roots, the activity of this enzyme was highly variable and had a mean of  $61.6 \pm 18.0 \,\mu\text{mol}$  min<sup>-1</sup> mg protein<sup>-1</sup>.



**Fig. 24.** Non-denaturing PAGE showing the SOD activity in shoots (a – first harvest, b – second harvest), and roots (c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates.



**Fig. 25.** CAT activity in shoots of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). Lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

High variability was also observed in the APX data. As a consequence, the activity of this enzyme was not influenced by either  $NO_3^-/NH_4^+$  ratios or Cd rates, and the means, expressed as nmol min<sup>-1</sup> mg protein<sup>-1</sup>, were as follows: shoots at the first harvest = 123.9 ± 37.1; shoots at the second harvest = 59.9 ± 30.7; and roots = 205.4 ± 119.4.

At the first harvest, the GPX activity in shoots changed as a function of the Cd rates, increasing by up to 51.0% after application of 1.0 mmol L<sup>-1</sup> Cd (Fig. 26a). At the second harvest, each  $NO_3^-/NH_4^+$  ratio resulted in a different response to the residual effect of the metal. The GPX activity in shoots of plants exposed to Cd and supplied with 100/0, 70/30 and 50/50 was increased, maintained and decreased compared with the control plants, respectively (Fig. 26b). For roots, there was a significant effect of  $NO_3^-/NH_4^+$  ratios, and this enzyme proved to be more active with the supply of 70/30 (Fig. 26c).



**Fig. 26.** GPX activity in shoots (a, b) and roots (c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **b**, lower- and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

The GR activity in shoots at the first harvest was not influenced by any of the  $NO_3^-/NH_4^+$  ratio × Cd rate combinations, and had a mean of 0.8 ± 0.1 µmol min<sup>-1</sup> mg protein<sup>-1</sup>. At the end of the regrowth period, there were independent effects of  $NO_3^-/NH_4^+$  ratios and Cd rates on the activity of this enzyme in shoots. Regarding the former factor, the highest and lowest values were recorded in plants supplied with 100/0 and 50/50, respectively (Fig. 27a). As for the metal, compared with the control, the GR activity in shoots reduced 59.6% after exposure to 1.0 mmol L<sup>-1</sup> Cd (Fig. 27b). Also in comparison with control plants, those





**Fig. 27.** GR activity in shoots (a, b) and roots (c) of *Panicum maximum* cv. Tanzania as influenced by  $NO_3^-/NH_4^+$  ratios and Cd rates. Values are expressed as mean  $\pm$  SD (n = 3) and compared by Tukey's test (P < 0.05). In **c**, lower-and uppercase letters indicate significant differences within  $NO_3^-/NH_4^+$  ratios and Cd rates, respectively.

# 3.4. Discussion

One of the main effects of Cd on photosynthesis is related to changes in stomatal regulation, which directly influences the water balance in the plant [2]. In all NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratio × Cd rate combinations, stomatal conductance was probably the major factor regulating the transpiration rate of tanzania guinea grass. A short period of exposure to Cd induced stomatal closure and reduced water loss through the leaves, proving the deleterious effect of this metal on plant-water relations [16]. Plants supplied with NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> at a 50/50 ratio were less prone to this problem and, along with those grown with 70/30, even showed increased stomatal conductance at the end of the second growth period. This indicated a beneficial effect because, despite increasing transpiration, plants have to open their stomata in order to obtain CO<sub>2</sub> [19].

The decrease in net photosynthesis at the first harvest can be attributed to the reduced stomatal conductance observed at that time [11]. Moreover, as previously suggested, plants

supplied with combinations of  $NO_3^-$  and  $NH_4^+$  in fact benefited from the residual effect of Cd. There are conflicting reports on the influence of N forms on the net photosynthetic rate of plants under Cd stress. Hassan et al. [38] found that, compared to  $NO_3^-$ , the supply of  $NH_4^+$  attenuated the negative effect of Cd on this parameter in rice (*Oryza sativa*) grown in nutrient solution. The opposite was observed for another rice cultivar in a pot experiment [2]. Nevertheless, it is important to stress that in the latter study  $NO_3^-$  and  $NH_4^+$  were applied to the soil as  $Ca(NO_3)_2$  and  $(NH_4)_2SO_4$ , respectively, with no mention of the availability or influence of other plant nutrients.

The direction (positive or negative) of the effect of Cd on physiological processes is dose-dependent [7]. At high rates, this heavy metal can negatively affect photosynthesis by inhibiting the function of the oxygen-evolving complex, impairing electron transport and reducing the quantum yield of PSII [10]. During the second growth period, when the highly contaminated nutrient solution was replaced, the effect of Cd on electron transport rate and quantum yield of PSII was restricted to plants supplied with 50/50. However, in this case, there were increases in these parameters, which suggest a eustress state that also increased the photosynthetic capacity of the plants. This could be associated with the upregulation of antioxidant enzymes and their protective role in photosynthesis [7].

In biochemical terms, Cd toxicity alters the redox state of the cell and leads to the accumulation of reactive oxygen species such as  $O_2^-$  and  $H_2O_2$ , ultimately causing oxidative stress [8]. Although this metal does not participate in Fenton and Haber-Weiss reactions [6, 39], it can augment the production of  $H_2O_2$  by indirectly activating plasma membrane NADPH oxidases [6]. Four days after Cd exposure, there was no significant difference in the shoot  $H_2O_2$  concentration of plants supplied with 50/50, which, curiously, were the only ones to show increased accumulation of this peroxide where no Cd was applied. Furthermore, application of this metal actually decreased the root  $H_2O_2$  concentration. In contrast, Schützendübel et al. [40] reported that, a few hours after exposure, Cd inhibited the activity of antioxidant enzymes and increased the root  $H_2O_2$  concentration of *Pinus sylvestris*.

The MDA results showed that Cd triggered increased lipid peroxidation specifically in shoots of tanzania guinea grass, as previously evidenced by Rabêlo et al. [27]. Like for H<sub>2</sub>O<sub>2</sub>, the supply of NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> at a 50/50 ratio led to the highest shoot MDA concentration in the control plants at the first harvest. Nevertheless, even though at that time plants subjected to 1.0 mmol L<sup>-1</sup> Cd had essentially the same shoot H<sub>2</sub>O<sub>2</sub> concentration, compared with the other NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratios, those supplied with 70/30 had higher MDA concentrations. Jalloh et al. [41] reported that, regardless of the presence of Cd in the soil, rice plants showed high leaf MDA

concentration when fertilized with  $NH_4^+$  in lieu of  $NO_3^-$ . Thus, increased oxidative stress could lead to impaired growth of plants supplied with 70/30 in highly contaminated soils. Interestingly, the supply of 50/50 does not appear to result in the same problem.

A likely explanation for plants supplied with 50/50 being more tolerant to oxidative stress is their ability to concentrate proline. The shoot proline concentration observed a few days after Cd application was about two-fold higher than at the second harvest, and in both cases plants supplied with 50/50 naturally showed the highest concentrations. Proline confers tolerance to a wide range of heavy metals, and increased synthesis of this amino acid under Cd toxicity has been reported for rice [14], maize (*Zea mays*) [42] and tropical forage grasses [1]. As a result, recent studies have even considered the application of exogenous proline to mitigate Cd stress [43], reducing lipid peroxidation and increasing the SOD and CAT activities [44].

Lagriffoul et al. [10] observed that the leaf soluble protein concentration of maize seedlings increased after treatment with 25  $\mu$ mol L<sup>-1</sup> Cd. Similarly, tanzania guinea grass exposed to Cd rates much higher than this showed increased shoot soluble protein concentration, especially when supplied with 70/30 and 50/50; however, this benefit was limited to shoots. Though Cd exposure combined with low NH<sub>4</sub><sup>+</sup> levels can result in increased soluble protein concentration, the protein synthesis of *Matricaria chamomilla* plants supplied exclusively with NH<sub>4</sub><sup>+</sup> was considerably affected [23]. This was attributed to the toxic effect of NH<sub>4</sub><sup>+</sup> and emphasizes the importance of an adequate balance between the supply of this ion with that of NO<sub>3</sub><sup>-</sup> [24].

SOD is responsible for catalyzing the dismutation of  $O_2^-$  into  $O_2$  and  $H_2O_2$  [7, 45]. Therefore, by comparing the activity of this enzyme with the  $H_2O_2$  concentration in shoots and roots, it can be inferred that  $H_2O_2$  derived mainly from the dismutation of  $O_2^-$ , rather than any other endogenous source of reactive oxygen species. The increased SOD activity observed at both harvests in shoots of plants supplied with 50/50, even in the absence of Cd, suggested a permanent stress condition [15]. This explains why control plants grown with this  $NO_3^-/NH_4^+$  ratio also showed increased shoot  $H_2O_2$  concentration at the first harvest. Besides toxic to the cell,  $H_2O_2$  acts as a signaling molecule, and high concentrations of this peroxide are necessary for a fast and effective activation of the antioxidant system [40]. Consequently, the supply of 50/50 may lead to increased tolerance to Cd and other environmental stressors.

Increased SOD activity per se does not explain the accumulation of  $H_2O_2$  in the plant. In a properly functioning system, excessive  $H_2O_2$  is decomposed to  $H_2O$  by CAT, APX and GPX [46]. Thus, the activities of these enzymes were determined and showed that, regardless of the form of N supplied to the plants, Cd toxicity reduced the CAT activity in shoots. This effect was more pronounced in plants grown with combinations of  $NO_3^-$  and  $NH_4^+$ , especially at a 70/30 ratio. Although some authors have reported the occurrence of increased CAT activity in plants subjected to Cd [11, 13], the response of the main components of the antioxidant system vary greatly based on the species, Cd rate and exposure time [40, 47]. Furthermore, there are reports on the accumulation of H<sub>2</sub>O<sub>2</sub> as a result of reduced CAT activity [47].

GPX also plays an important role in cellular detoxification under Cd stress [13]. At the end of the second growth period, the higher the availability of  $NH_4^+$  in the nutrient solution, the higher the GPX activity in shoots of control plants. In contrast, in the presence of Cd, the activity of this enzyme in plants supplied with 50/50 decreased in relation to the control plants, while in those grown with 100/0 it increased. The results indicate that, separately,  $NH_4^+$  and Cd increase the GPX activity in shoots. However, their synergistic effect inhibits this enzyme, as previously suggested by Kováčik and Hedbavny [23]. In roots, the GPX activity was stimulated by the supply of  $NO_3^-$  and  $NH_4^+$  at a 70/30 ratio.

Both NH<sub>4</sub><sup>+</sup> and Cd had a negative effect on the GR activity in shoots. This enzyme catalyzes the reduction of glutathione, which, in its reduced form, acts directly as an antioxidant and indirectly by activating the transcription of an array of stress genes [45]. The reduced glutathione is also a precursor of phytochelatins, and these peptides are involved in the chelation of heavy metals in the cytosol and their sequestration in the vacuole [7, 27]. The decrease in the GR activity in shoots with the increase in the availability of NH<sub>4</sub><sup>+</sup> might be associated with the accumulation of Cd in theses tissues, limiting the synthesis of glutamate [21]. Nevertheless, under Cd stress, plants supplied with 50/50 were able to maintain increased GR activity in roots, regardless of the Cd rate they were exposed to.

# 3.5. Conclusion

The negative effects of Cd on photosynthesis and on the balance between oxidants and antioxidants in tanzania guinea grass are attenuated by the partial replacement of  $NO_3^-$  by  $NH_4^+$  in the nutrient solution. This metal reduced photosynthesis by causing stomatal closure and impairing electron transport. However, the simultaneous supply of  $NO_3^-$  and  $NH_4^+$ , particularly at a 50/50 ratio, led to a eustress state that restored gas exchange and improved the function of PSII, increasing the photosynthetic capacity of the grass. Plants grown with 50/50 showed reduced lipid peroxidation along with increased synthesis of proline and protein. Moreover, despite inhibiting the GPX activity in the presence of Cd, this  $NO_3^-/NH_4^+$  ratio increased the tolerance of tanzania guinea grass to the metal by inducing high SOD and GR activities in shoots and roots, respectively, maintaining cellular homeostasis and reducing oxidative stress.

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